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Detection of *Clavibacter michiganensis* subsp. *sepedonicus*, the causal agent of potato ring rot, in the breeding and propagation materials of the three-stage control process

Iveta Pánková*, V. Krejzar

Crop Research Institute, vvi, Drnovská 507, 161 06 Prague 6, Czech Republic.

Abstract

To maintain *Clavibacter michiganensis* subsp. *sepedonicus*-free (*Cms*-free) potato genetic resources, a three-stage process of checking limited lots of initial and new breeding potato materials was designed for small breeders in terms of costs, personnel and facilities. For an intensified evaluation of the presence of *Cms* in different vegetative stages within one growing season a mixture of 10 randomly selected artificially infected and healthy tubers of individual cultivars and breeding materials were prepared. In the first stage, a visual assessment of the vascular vessels of mother tubers was performed and at least 20% of each material was deemed positive, indicating that it should be discarded. In the second stage, one half of mother tubers were grown in the greenhouse and the other in a netting house. Plants in bloom and daughter tubers were evaluated. Within the second stage 1.4%–28% of each individual potato material could be excluded based on double-antibody sandwich enzyme-linked immunosorbent assays and SYBR Green real-time PCR assays, including a melting point analysis using PSA 1/R and *CelA* F/R primer sets and biological tests on aubergines. In the third stage, *in vitro* plants transferred from potato resources that tested negative in the previous stages were evaluated using the methods used in the second stage. The specificity of the antibodies and the two primer sets, PSA1/R and *CelA* F/R, were evaluated with 100% success.

Key words: bacterial ring rot of potato, breeding material, cultivars, three-stage control process.

* **Corresponding author:** Iveta Pánková,
E-mail: pankovai@vurv.cz

Introduction

Bacterial ring rot, caused by the gram-positive bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (Spieckermann & Kotthoff, 1914) Davis et al. 1984 (*Cms*), is included in the list of quarantined harmful organisms that require special measures to either restrict or prevent its introduction and spread in potato-production regions (Act no. 245/2011 Coll.; Decree 382/2011 Coll.). A zero-tolerance policy for the disease was implemented by the European and Mediterranean Plant Protection Organization (EPPO; Bulletin OEPP/EPPO Bulletin, 2011) (Gutbrod, 1987). During the first years after introducing the eradication program, a significant reduction in the incidence of the causal agent of bacterial ring rot was recorded (Kůdela, 2007), but despite the high cost of testing seed tubers, the pathogen was not eradicated in central Europe. The attention of legislators and researchers was still focused on minimizing the possible horizontal spread of *Cms* through technological measures and sanitation. In the temperate climatic zone of central Europe, *Cms* does not cause serious crop losses or significant symptoms on potato plants or tubers (ACP, 2003; Bishop & Slack, 1987). Infections in potato are symptomless because of the low population rates and can persist in certified seed stocks for many years without positive test results (Nelson, 1982). Positive findings occurred most often in the lowest levels of commercial seed potatoes, E, A and B. Even seed lots that were evaluated as healthy prior to harvesting were positive when sampled a few months later after shipping (personal communication). The combination of several years of proliferation and subsequent unsuitable conditions during the transport and storage of seed tubers can lead to the pathogen's multiplication, which was below the threshold limits of the main screening methods prior the harvesting. Attention should be focused on the vertical spread of the pathogen. Initial breeding materials that are from different sources are still not being checked properly or at all before the breeding process. Even new breeding materials have only been checked randomly. The first regular inspection of breeding materials is made before their transfer to tissue cultures. This one-time determination of the pathogen appears to be insufficient. Breeding farms are situated in the highlands (400–600 m above the sea), where it is not warm enough (average temperature during the growing season 10–13°C) for *Cms* to multiply over the threshold level of the screening methods. *Cms* detection can be performed using immunochemical techniques, immunofluorescence assays (Roozen & Van Vurde, 1991; De Boer & Copeman, 1980), enzyme-linked immunosorbent assays (ELISA; De Boer et al., 1988) or fluorescent *in situ* DNA hybridization (Li et al., 1997). All of the methods have similar detection limits of 10^3–10^4 colony forming units (cfu) mL^{-1}, at an acceptable level > 80%. In an effort to increase the sensitivity of these methods to approximately 10^3 cfu mL^{-1} *Cms*, extraction buffers containing lysozyme, *Cms* semi-selective nutrient media for incubation, and magnetic beads for trapping *Cms* antigens (Poussiere et al., 2002) were used. The slow-growing pathogen is often overgrown by other rapidly growing

saprophytic microorganisms during these enrichment steps. Thus, the polymerase chain reaction (PCR) techniques are easier, and the sensitivity is higher than that of immunodetection techniques (detection limit 10^3 cfu mL^{-1}; at an acceptable level > 80%–90%). The most widespread PCR methods for detecting *Cms* use primers and protocols based on Pastrik (2000) and Mills et al. (1997). TaqMan PCR (Batch et al., 2003; Schaad et al., 1999), BIO-PCR (Cho et al., 2015; Schaad et al., 1999) and the AmpliDet RNA assay (Van Beckhoven et al., 2002) were also used. Some primers developed for a classical PCR format have been successfully applied in a real-time format (Smith et al., 2008; Bach et al., 2003; Beckhoven et al., 2002; Schaad et al., 1999). Positive results obtained by any method must be confirmed by a positive biological test on aubergines and the subsequent re-isolation of the pathogen (Bulletin OEPP/EPPO Bulletin, 2011). The detection limit for most *Cms* strains in biological tests with a high reproducibility is 10^2–10^3 cfu mL^{-1}(Brown et al., 2002), while at a concentration of 10^2 cfu mL^{-1} aubergines are usually symptomless (Pankova & Krejzar, 2015). The objective of this study was to improve an inspection procedure that would eliminate the risk of latent infections occurring in breeding and propagation potato materials due to insufficient controls. In this study, to maintain *Cms*-free potato genetic resources, a three-stage process of checking breeding and propagation inputs was designed to meet the labour and financial limits of small breeders. For this study artificially infected tubers were grown, mixed with the same number of "healthy" seed tubers, and then 10 randomly selected tubers were assessed for the presence of the pathogen in their different vegetative stages within one growing season. The detection of *Cms* was based on a double-antibody sandwich (DAS) ELISA and SYBR Green real-time PCR assays, including a melting point analysis using PSA 1/R (Pastrik, 2000) and *CelA* F/R (Gudmestad et al., 2009) primer sets and biological tests on aubergines.

Materials and methods

Bacterial strains and culture conditions: *Cms* strains NCPPB 3467 (Collection of Plant Pathogenic Bacteria, United Kingdom) and CPPB 97 (Collection of Plant Pathogenic Bacteria, Czech Republic) were cultured on medium C (1 L distilled water, 5 g peptone, 3 g casein hydrolysate, 3 g yeast extract, 2 g maltose, 1 g lactose, 18 g agar) and incubated at 22°C for 5 d. For tuber inoculation, a 1:1 mixture of the collection strains was prepared in a blender using pieces of nutrient medium C with actively growing *Cms* colonies and sterile water, and the concentration was set on 10^8 cfu mL^{-1}, which corresponded to an optical density (OD) of 0.1 at 560 nm.

Tuber inoculations and sample preparation: Ten *Cms*-free potato tubers (*Solanum tuberosum* L.) from 16 potato cultivars (pre-basic seed class) and 11 new materials at different breeding stages (Table 1) were incubated 1–3 weeks in the dark at 20°C prior to inoculation. Approximately 2 or 3 cm tall "eyes" were trimmed back to half their lengths with sterile scissors and

immersed in suspensions of individual *Cms* strains. The tubers were allowed to rest for 30 min to stabilise the *Cms* (Slack et al., 1996) and then were sown in 20-cm diameter pots filled with a mix (1:1) of field soil and special vegetable substrate (Rašelina Inc., Soběslav, Czech Republic). These pots were placed in a greenhouse at 20°C to 22°C during the growing period and watered daily until they withered. Daughter tubers were harvested after 10–12 weeks and mixed with the same number of "healthy" seed tubers from the same cultivar.

Table 1: Potato cultivars and new breeding materials used in this study.

Cultivar	Abbreviation	Source	Breeding material	Source
Alice	AL	Research Institute collection	B15/2	Private farm breeding collection
Borek	BR		C18/18	
Dicolora	DI		E8/1	
Ditta	DT		J47/2	
Dominátor	DO		J59/4	
Magda	MA		J61/7	
Monika	MO		J66/3	
Nancy	NA		J66/5	
Primarosa	PR		K20/5	
Rebel	RB		K70/3	
Red Anna	RE		M 12/1	
Suzan	SU			
Terka	TE			
Vendula	VE			
Verne	VR			
Vlasta	VL			

Three-stage control process: The experiment was carried out in a randomised block design with 10 randomly selected same-sized tubers of each potato material. In the first stage of the control process, selected tubers from each of the potato cultivars and breeding materials were stored for 4 weeks at 22°C in the dark. Then, the tubers were washed under running tap water, surface-disinfected with 75% ethyl alcohol and cut in two equal parts containing the stem and apical bud ends. The colour and appearance of xylem vessels were evaluated. In the second stage of the control process, a half of each tuber was sown and planted in the greenhouse in the same manner as described above. Each pot was on its own deep plate to avoid cross-contamination. The other half of each tuber was planted in a netting house trial. The tubers were planted 50 cm apart with individual cultivars on rows 30 cm apart. Potato plants were watered regularly. Samples of 1 g of vascular bundles were taken independently from the base, middle and top parts of the main stem of each blooming plant. Each sample was macerated in 1.5 mL of sample buffer (20 g polyvinylpyrrolidone K10-K40, 2 g bovine serum albumin, 1 L distilled

water, pH = 7.4) and incubated on an orbital shaker overnight at room temperature. Plant extracts were analysed using DAS ELISA tests and SYBR Green real-time PCR assays, including melting point analyses. After senescence, daughter tubers from each half of the mother tuber were harvested. After 4 weeks of incubation at 22°C, tubers were cut in two pieces, and the vascular ring vessels at the stem ends of five tubers were removed and macerated in 1.5 mL of sample buffer. Then, they were processed and analysed in the same manner as the potato plants. After incubation, 10 µL samples from tuber tissues were streaked on C medium to test for the presence of *Cms* using DAS ELISA and real-time PCR assays. In the third stage of the control process, several lateral sprouting "eyes" of daughter tuber samples that had tested negative for the presence of *Cms* in the first and second stages were used for the establishment of *in vitro* tissue cultures (George & Klerk, 2008; Chawla, 2002). The most vital 2-3 clones from the *in vitro* cultures of each daughter tuber sample were stored. Micro-plants from nuclear stocks were multiplied and inspected 2 months later. Altogether, 40 *in vitro* plants from each clone were transferred at 10 cm lengths to the soil substrate (Rašelina Inc., Soběslav, Czech Republic). Four plants per pot (10-cm in diameter) were planted in a quarantined greenhouse at 20°C to 22°C. Four weeks later, the plants from each pot were cut, washed under running tap water and surface-disinfected with 75% ethyl alcohol. Approximately 1 g of defoliated stems were macerated in 1.5 mL of sample buffer, treated and analysed as the potato plant extracts described above.

DAS ELISA test: To determine the sensitivity of polyclonal antibodies, serial 10-fold dilutions of *Cms* strains NCPPB 3467 and CPPB 97 from 10^8 cfu mL^{-1} (OD_{560nm} = 0.1) to 10^1 cfu mL^{-1} in sample buffer were prepared. For the specificity evaluation, different types of saprophytic bacterial colonies were isolated from 10 µL samples of potato plants and tuber extracts streaked on medium C plates and incubated for 2–4 days at 22°C. Bacterial strains were identified using Biolog GEN III (Biolog Inc., Hayward, CA, USA) and the Gas Chromatography-Analysis of Fatty Acid Methyl Esters (GC-FAME) method (MIDI, Microbial ID, Inc., Newark, DE, USA) with a Sim Index ≥ 0.5 (Table 2). The concentrations of bacterial suspensions were set at OD_{560nm} = 0.1, and 1:1 dilutions in sample buffer were prepared and processed in the same way as plant extracts and controls. Suspensions of 10^8 cfu mL^{-1} and 1:1 dilutions of *Cms* collection strains in sample buffer were used as positive controls. The sample buffer and extracts from healthy potato plants or tuber tissues were used as negative controls. All of the tested plants and tuber extracts were used both in a concentrated form and diluted 1:1 in sample buffer. All of the samples and controls were reproduced in duplicate in one procedure. The DAS ELISA was performed in a 200-µL reaction in Nunc-Immuno™ MicroWell™ 96-well solid microplates (Nunc Systems Pvt Ltd, Hyderabad, India). The assay, setting of the threshold level and the final evaluation of absorbance values using a spectrophotometer at 405 nm (A_{405nm}), were carried out using polyclonal antibodies according to the

manufacturer's instructions (Loewe Biochemica GmbH, Sauerlach, Germany).

DNA extraction and real-time PCR conditions: The total genomic DNA from potato plants and tuber extracts, positive and negative controls, saprophytic bacteria (Table 2), and serial 10-fold dilutions of *Cms* strains NCPPB 3467 and CPPB 97 from 10^8 cfu mL^{-1} (OD_{560nm} = 0.1) to 10^1 cfu mL^{-1} was prepared using a DNAeasy Plant Minikit (Qiagen, Hilden, Germany) or GeneAll Plant SV Mini kit (GeneAll, Seoul, Korea) according to the manufacturer's instructions. The real-time PCR amplification was performed using a Rotor-Gene Q 5plex HRM (Qiagen, Hilden, Germany) with the dye SYBR Green (detection wavelength 510 nm) with the following cycling conditions: initial denaturation for 30 s at 95°C; then 40 cycles of 5 s at 95°C, and 30 s at 60°C. This was followed by a melting curve from 57°C to 95°C, at 1.0°C increments, for the melting point analysis. The SYBR Green real-time PCR assay was performed in a 25-µL reaction. The real-time PCR amplifications were carried out using a mixture of the two primer sets, given in Pastrik (2000), PSA-1 (5'-CTCC TTGTGGGGTGGGAAAA-3') /PSA-R (5'-TACTGAGATGTTTCACTTCCCC-3'), and in Gudmestad et al. (2009), *CelA*-F (5'-TCTCTCAGTCATTGTAA GATGA T-3')/*CelA*-R (5'-ATTCGACCGCT CTCAAA-3'), at final concentrations of 0.2 and 0.5 µM per reaction, respectively. A Rotor-Gene SYBR® Green PCR Kit (Qiagen) was used, with 3 µL of template DNA, according to the manufacturer's instructions. All of the reactions were duplicated. The data analysis included the determination of the cycle thresholds (c_ts) and the melting temperatures (t_ms) of the PCR products. To determine the sensitivity of the two primer sets, template DNA from the 10-fold dilutions of *Cms* collection strains were used. To determine the specificity of the two primer sets, both concentrated and diluted DNA samples from saprophytic bacteria (Table 2) were amplified. DNA samples from potato and controls were used in either concentrated or diluted form.

Bioassay: Aubergines (*Solanum melongena L.*) variety Black Beauty was inoculated through the stem at the two-leaf stage. Concentrated potato plant and tuber tissue extracts, along with controls, saprophytic bacterial suspensions, and serial 10-fold dilutions of *Cms* collection strains were injected by syringe into four plants per sample. The inoculations, plant incubation conditions and the evaluation of leaves wilting symptoms were conducted according to the OEPP/EPPO Bulletin (2006).

Results

Sensitivity and specificity of DAS ELISA, SYBR Green real-time PCR and the bioassay: A positive absorbance threshold for the DAS ELISA assay was set according to the manufacturer's instructions to $A_{405nm} \geq 0.4$. These values were reached for the *Cms* concentration of $\geq 10^4$ cfu mL^{-1}. At a concentration of 10^3 cfu mL^{-1}, the absorbance values differed in replicates (A_{405nm} = 0.22 and 0.36), and at concentrations lower than 10^3cfu mL^{-1}, the absorbance was the

same as that of the negative controls ($A_{405nm} \leq 0.2$). All of the 35 saprophytic bacteria isolated from potato plants and tuber extracts produced negative results, absorbance values ranged between 0.1 (blank) and 0.2 (negative and commercial negative controls) for 10^8 cfu mL^{-1} concentrations and 1:1 dilutions (Table 2). For the real-time PCR assays, the positive c_t values were set as $c_t \leq 25$ and ≤ 30 or less for the PSA 1/R and C*elA* F/R *Cms* primer sets, respectively, to determine the presence of *Cms* at 10^3–10^4 cfu mL^{-1} (2.0–2.5 ng μL^{-1} of DNA). At a *Cms* concentration $\geq 10^3$ cfu mL^{-1}, the PCR products' $t_m = 85.5 \pm 0.1$°C for the PSA primer set and 86.5 ± 0.1°C for the C*elA* primer set, respectively. At lower *Cms* concentrations ($\leq 10^3$ cfu mL^{-1}), c_t values often differed between replicates, and the t_m values of the PCR products became lower, the melting curves for *Cms* concentrations $\leq 10^2$ cfu mL^{-1} became similar to those of the negative controls. When using *Cms*-specific primers, the c_t values for the saprophytic bacteria ranged from 27.5 (*Pseudomonas corrugata* at 1:1 dilution) to 37.3 (*Ochrobactrum grignonense* at 10^8 cfu mL^{-1}) for the PSA primer set, and from 28.1 (*Rhizobium rhizogenens* at 10^8 cfu mL^{-1}) to 37.1 (*O. grignonense* at 10^8 cfu mL^{-1}) for the C*elA* primer set (Table 2). According to the melting point analysis of the PCR products, the main peaks had significantly different positions at 58°C–78°C. The threshold for biological tests on aubergine plants was set at a *Cms* concentration of 10^3cfu mL^{-1} (Figure 1). At a *Cms* concentration of 10^2 cfu mL^{-1}, wilting symptoms and the ability to re-isolate the pathogen differed between replicates. At a 10^1 cfu mL^{-1} dilution, no wilting symptoms were observed and re-isolation was completely unsuccessful. None of the saprophytic bacteria, when injected, caused wilting symptoms in aubergines (Table 2).

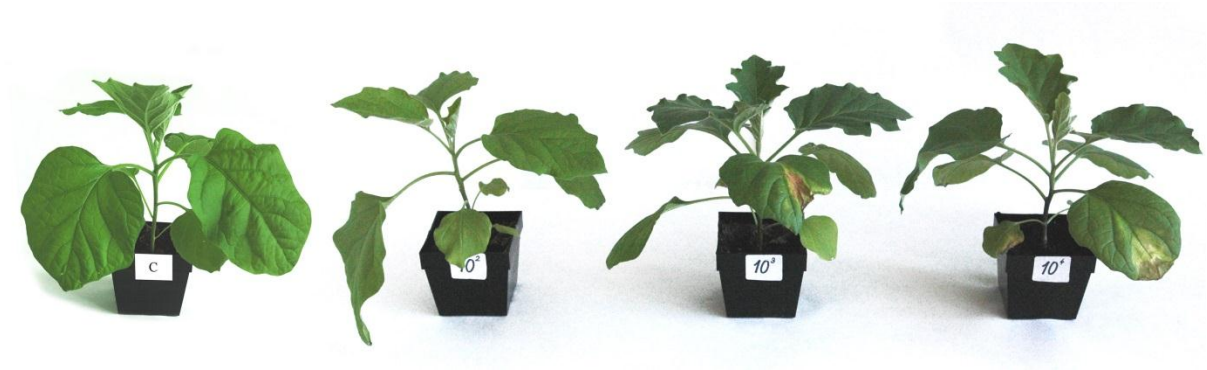

Figure 1: The threshold for biological tests on aubergines (*Solanum melongena L.*) variety Black Beauty was set at a concentration of 10^3 cfu mL^{-1} for *Clavibacter michiganensis* subsp. *sepedonicus*. Symptoms of wilting were evaluated 6 weeks after stem inoculations. Healthy non-inoculated control (c), and the *Cms* concentrations of 10^2, 10^3, and 10^4 cfu mL^{-1} are shown.

Table 2: Specificity of DAS ELISA antibodies and PCR primer sets to saprophytic bacteria.

Species[A] concentration/dilution	DAS ELISA (A_{405nm}) 10^8 cfu mL^{-1}	DAS ELISA (A_{405nm}) 1:1	PSA1/R (ct) 10^8 cfu mL^{-1}	PSA1/R (c_t) 1:1	CelA F/R (c_t) 10^8 cfu mL^{-1}	CelA F/R (c_t) 1:1
Acinetobacter ursingii	0.1	0.2	29.6	28.0	29.1	29.8
Aeromonas veronii	0.2	0.1	32.8	32.5	34.1	30.3
Arcanobacterium phocae	0.1	0.1	28.9	28.2	30.5	31.9
Bacillus atrophaeus	0.2	0.2	36.8	33.2	32.8	32.5
Bacillusc ecembensis	0.2	0.2	35.3	29.4	28.2	34.5
Bacillus halodurans	0.2	0.1	28.5	33.2	29.9	35.0
Bacillus indicus	0.2	0.1	34.4	27.8	28.0	32.3
Bacillus subtilis	0.2	0.2	27.9	28.5	28.5	30.6
Brevibacterium epidermis	0.1	0.1	31.5	28.5	30.4	35.0
Cellulomonas cellasea	0.2	0.1	29.9	27.9	28.8	32.2
Corynebacterium lipophiloflorum	0.2	0.1	27.6	28.1	28.1	31.7
Curtobacterium flaccumfaciens	0.2	0.1	30.7	29.7	28.3	36.1
Enterobacter aerogenes	0.1	0.1	35.3	32.1	30.5	31.2
Erwinia americana	0.1	0.1	32.3	32.0	33.0	32.0
Flavobacterium columnare	0.1	0.1	29.5	28.0	28.2	32.2
Klebsiella sp.	0.1	0.1	33.0	31.9	29.9	31.4
Microbacterium flavescens	0.2	0.2	28.2	27.8	28.7	36.1
Microbacterium lacticum	0.2	0.2	35.8	30.7	29.1	34.9
Mycobacterium confluentis	0.2	0.2	34.0	29.7	33.0	32.6
Ochrobactrum grignonense	0.2	0.2	37.3	35.6	35.3	37.1
Paenibacillus polymyxa	0.2	0.2	29.3	30.4	31.6	33.4
Paenibacillus soli	0.2	0.2	32.3	31.0	30.8	32.0
Pantoea agglomerans	0.1	0.1	30.1	27.7	29.5	30.4
Pseudomonas corrugata	0.1	0.1	28.1	27.5	29.8	30.1
Pseudomonas fluorescens	0.1	0.1	31.1	29.9	29.4	30.0
Pseudomonas marginalis	0.1	0.1	29.1	27.6	29.4	31.5
Pseudomonas syringae	0.1	0.1	30.3	31.2	28.9	29.9
Rhizobium rhizogenens	0.1	0.1	31.4	28.7	28.1	29.1
Rhodococcus sp.	0.2	0.2	30.5	30.9	30.1	29.0
Roseomonas fauriae	0.2	0.1	30.7	31.7	29.5	32.3
Sporosarcina sp.	0.2	0.1	30.6	27.8	29.5	30.2
Stenotrophomonas maltophilia	0.1	0.1	27.6	27.8	28.5	28.5
Virgibacillus halophilus	0.2	0.1	29.4	31.6	29.1	30.1
Virgibacillus sediminis	0.2	0.1	31.8	31.2	32.4	30.3
Clavibacter mich. subsp. sepedonicus NCPPB 3467	2.6	2.4	16.3	17.2	18.2	18.7
Clavibacter mich. subsp. sepedonicus CCPB 97	2.8	2.4	15.7	16.9	16.9	17.7
Deionized water	0.1	0.1				
Healthy potato tissue extract	0.1	0.1	32.0	31.8	33.6	33.6

The positive threshold for the DAS ELISA assay was set at $A_{405nm} \geq 0.4$. For the real-time PCR assay, the threshold was set at $c_t = 25$ for the PSA1/R primer set and $c_t = 30$ for the C*elA*F/R *Cms* primer set. [a] Bacterial species were determined when the SIM index ≥ 0.5 in the Biolog GENIII and GC-FAME systems.

Three-stage control process: In 2013–2014, 16 potato cultivars, and in 2014–2015, 16 potato cultivars and 11 breeding materials were evaluated using a three-stage process (Table 1). In the first stage, the presence of *Cms* in vascular vessels of mother tubers was evaluated by visual assessment, producing the highest number of positives within the three-stage evaluation of potato materials (Figures 2 and 3). In 2014, 32.2% of tubers in each potato cultivar appeared positive, while in 2015, 20% of each cultivar, and 25.5% of the breeding materials, appeared positive. The Spearman's rank coefficient $\rho = 0.30$ ($p = 0.05$) indicated moderate interactions between the annual results of the individual cultivars within the first stage of the control process.

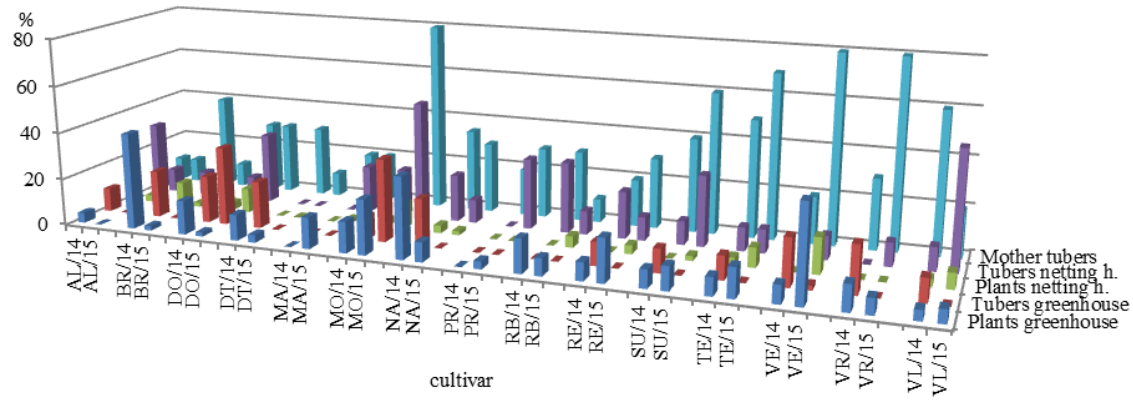

Figure 2: Comparison of the percentage of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) positive results for individual potato cultivars in the first and second control stages in vegetative seasons 2014 and 2015. In the second stage, samples of plant and tuber tissues were determined to be *Cms* positive when the DAS ELISA had $A_{405nm} \geq 0.4$ or when the real-time PCR with primer set PSA1/R had a $c_t \leq 25$ and $t_m = 85.5 \pm 0.1$°C, primer set C*elA*F/R had a $c_t \leq 30$ and $t_m = 86.5 \pm 0.1$°C, the leaves of inoculated aubergines wilted within 6 weeks and the pathogen was re-isolated. Each bar represents the percentage of positive samples for each potato cultivar.

In the second control stage, the presence of *Cms* in vascular vessels of potato plants grown from mother and daughter tubers was evaluated using three methods. The samples were determined to be positive when they had an absorbance value $A_{405nm} \geq 0.4$ in the DAS ELISA assay or the real-time PCR assays with the PSA 1/R primer set had a $c_t \leq 25$ and a main peak at the $t_m = 85.5 \pm 0.1$°C, the *CelA* F/R primer set had a $c_t \leq 30$ and $t_m = 86.5 \pm 0.1$°C. The positive result was confirmed by the development of wilting symptoms on aubergine plants within 6 weeks after inoculation and the re-isolation of *Cms*. In 2014, the highest percentage of *Cms* positive samples per cultivar was 12.5% of daughter tubers harvested from the greenhouse, followed by 12.1% of plants grown in the greenhouse, 11.9% of daughter tubers harvested from the netting house and 1.9% of the plants grown in the netting house (Figure 2). In 2015, the detection rates in the second control stage were, in decreasing order, 12.7% in the daughter

tubers of each cultivar harvested from the greenhouse, 11.8% of potato plants grown in the greenhouse, 6.0% in plants grown in the netting house and 4.0% in daughter tubers harvested from the netting house (Figure 2). For breeding materials, *Cms* was found in 21.2% of greenhouse plants, 16.2% of greenhouse tubers, 7.7% of netting house plants and 5.1% of netting house tubers (Figure 3). In 2015, the order of the infected potato cultivars and breeding materials differed slightly, but in total the *Cms* positive percentages were much higher in the new breeding materials. The highest positive detection rate of 21.2 % per cultivar was detected in plant extracts of breeding materials grown in the greenhouse. The Spearman's rank coefficient $\rho = 0.32$ ($p = 0.05$), indicating that moderate interactions among the individual cultivars' annual results at the second control stage (Figure 2). Generally, over the two years, no significant symptoms of wilting on potato plants were observed in the greenhouse and netting house. More *Cms* positive samples were detected in the greenhouse: 15% per potato material for plant extracts and 13.8% for tuber extracts. In the netting house the average positive detection rate over the two years reached 5.2% for plants per potato material and 7.0% for tubers per potato material (Table 3).

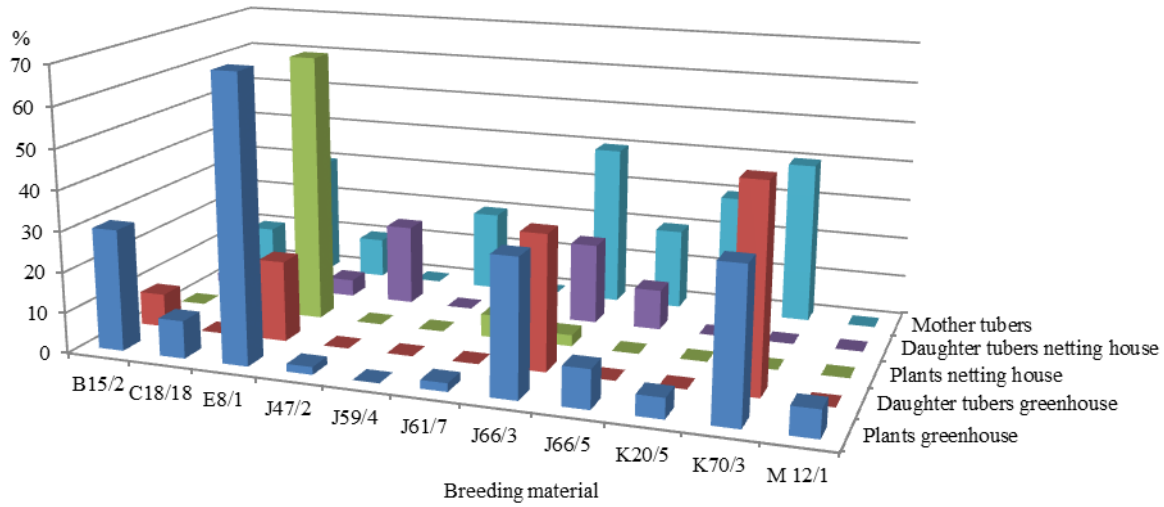

Figure 3: Evaluation of the *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) presence in the first and second control stages of potato breeding materials in the 2015 vegetative season. In the second stage, samples of plant and tuber tissues were determined to be *Cms* positive when the DAS ELISA had $A_{405nm} \geq 0.4$, or the real-time PCR with primer set PSA1/R had a $c_t \leq 25$ and $t_m = 85.5 \pm 0.1°C$, primer set C*el*AF/R had a $c_t \leq 30$ and $t_m = 86.5 \pm 0.1°C$, leaves of inoculated aubergines wilted within 6 weeks, and the pathogen was re-isolated. Each bar represents the percentage of positive samples for each potato cultivar.

The detection rates in the second control stage differed from those of the first stage regardless of the type of potato material (Figures 2 and 3). Only for three breeding materials (B 15/2, E 8/1 and K70/3) planted under greenhouse conditions was the percentage of positive samples greater than that of the visual

assessment (Figure 3). The Spearman's rank coefficient $\rho = 0.2$ ($p = 0.05$) indicated moderate interactions between the first and the second stage evaluations of the individual potato materials. The resulting detection rates of the assays used in the second control stage, in decreasing order, were the real-time PCR assay with the PSA1/R primer set, the real-time PCR assay with the *CelA* F/R primer set, the bioassay and the DAS ELISA. The last two were nearly the same. Results from the PCR primer sets differed for approximately 3% extracts over the two years. In the third control stage, the presence of *Cms* in vascular vessels of all of the potato materials transferred and subsequently grown from *in vitro* tissue cultures were evaluated using the same diagnostic methods. The assays were performed under the same conditions as in the second stage. All of the potato materials were determined to be negative for the presence of *Cms* in 2014 and 2015. Several values obtained from the real-time PCR analysis using the PSA1/R primer set reached the c_t and t_m detection threshold levels but were not confirmed by a positive bioassay. The resulting detection rates after two years using the three-stage control process were, in decreasing order, mother tubers, potato plants, daughter tubers, and *in vitro* plants. According to the results of the visual assessment, DAS ELISA, real-time PCR and bioassay, 38.2%, 15.5%, and 0% of potato materials were positive for the presence of the *Cms* pathogen in the first, second, and third control stages, respectively.

Table 3: Comparison of percentages of *Clavibacter michiganensis* subsp. *sepedonicus* positive results in the individual assessment steps of the three-stage control process.

Propagation and breeding potato materials	First control stage: visual assessment		Second control stage: DAS ELISA, real-time PCR and bioassay positive results								TSP[c]
			Greenhouse				Netting house				
	Mother tubers[a] (%)	TSP[b] (%)	Plants[a] (%)	TSP[b] (%)	Daughter tubers[a] (%)	TSP[b] (%)	Plants[a] (%)	TSP[b] (%)	Daughter tubers[a] (%)	TSP[b] (%)	
2015/16 potato cultivars	280.0	36.6	165.6	21.7	178.3	23.3	84.2	11.0	56.3	7.4	764.4
2015 average per cultivar	20.0	--	11.8	--	12.7	--	6.1	--	4.2	--	--
2015/11 breeding materials	280.0	33.7	232.8	28.0	178.3	21.4	84.2	10.2	56.3	6.8	831.6
2015 average per breeding material	25.5	--	21.2	--	16.2	--	7.7	--	5.1	--	--
2014/16 potato cultivars	515.0	44.2	192.1	13.5	200.0	18.1	29.8	1.4	190.0	22.8	1126.9
2014 average per cultivar	32.2	--	12.0	--	12.5	--	1.9	--	11.9	--	--
Two year ∑ of positive results	1075.0	--	590.5	--	556.6	--	198.2	--	302.6	--	--
Annual average of potato materials[d]	25.9	--	15.0	--	13.8	--	5.2	--	7.0	--	--

[a] Annual sum of *Cms* positive results in tubers and plants; [b] Percentage of individual assessment steps of the TSP; [c] Annual sum of *Cms* positive results in the TSP; [d] Annual average of *Cms* positive results in potato tubers and plants.

Discussion

Due to the persisting difficulties in maintaining zero tolerance for the causal agent of potato ring rot, attention was focused on vertical disease transmission. This has led to an effort to set or improve the control of initial breeding materials. A three-stage control process for potato materials was suggested and designed, taking into consideration the time, personnel and financial limitations of small breeding and propagation stations. Breeders are well acquainted with differences among potato cultivars, therefore, they are qualified to visually assess potato tubers. According to the percentage of potential *Cms* positives obtained by the visual assessment, in comparison with the results of the second stage analysis, reviewers tended to overestimate the presence of discoloured vessels in tubers. The detection rate of the visual assessments was also enhanced after one month incubation at a temperature that supported pathogen multiplication. The Spearman's rank coefficient for annual results indicated that individual potato cultivars responded differently to the artificial inoculation. The percentage of positives in the first stage was the highest within the three stage process, but an average of at least 20% of tubers of individual potato materials (Table 3) could be excluded during the first stage. In the second control stage of the presented trials, significant differences were shown between detection rates in potato plants and tubers (15% and 13.8%, respectively) in the greenhouse, in which they were grown under the optimal conditions for the growth and multiplication of *Cms* colonies, and rates in netting house (5.2 % and 7%, respectively) under nearly field conditions (Table 3). The planting of both parts of the mother tubers under *Cms* optimal conditions in the greenhouse should be more valuable for revealing the presence of the pathogen. However, the second stage of control was designed to use the equipment and space available at most breeding farms. Breeders usually have limited greenhouse space without the capability to continuously regulate the temperature and humidity, so the chance to increase the *Cms* concentrations above the detection methods thresholds is limited. The possibility of enhancing the determination rate was established by growing two parts of the mother tubers under different conditions, sampling blossoming plants and incubating daughter tubers at temperatures suitable to *Cms* growth (Pankova & Krejzar, 2015; Krejzar et al., 2007). The greatest differences between the detection rates in plants and daughter tuber extracts, and between greenhouses and netting houses, were observed for potato breeding materials (Figure 3). These expected divergences were caused by the diversity of the new breeding materials (size and number of stems, number of tubers, etc.). Thus, within these materials the importance of multiple testing increased. Moderate correlations between the results in 2014 and 2015 for individual cultivars at the second stage showed, as in the first stage that individual potato cultivars responded differently to the artificial inoculations. After the second stage, 1.4%–28% of individual potato materials in plants or daughter tubers (Table 3) could be excluded. The

Spearman's rank coefficient $\rho = 0.2$ ($p = 0.05$) indicated moderate interactions between the first and the second stage evaluations of individual potato materials, so visual assessments of the vascular vessels were valuable and an overestimation in visual assessment correlated with the determination rate in the second stage. These results indicated that most of the *Cms* positive tubers should be discarded already during the first stage of the control process. For breeders, the main determination method in the second stage of control, the DAS ELISA test, has some limitations (Lee et al., 2001), but its simplicity and reasonable cost allows it to be used in the large-scale indexing of commercial seed potatoes, as well as in the estimation of breeding materials almost everywhere. In this study, the DAS ELISA kit was tested with 100% success for the antibody specificity against local saprophytic bacteria isolated from potato plants and tubers. None of these bacterial strains reacted with antibodies even at high concentrations, such as 10^8 cfu mL^{-1} (Table 2). The negative results and those near the DAS ELISA threshold level (10^4 cfu mL^{-1}) should be confirmed using a PCR-based method in an approved laboratory. Real-time PCR with two different primer sets, plasmid-based *Cel*AF/R and genomic PSA1/R, were used to avoid false negative reactions due to excision of the plasmid, and a melting point analysis was used to exclude nonspecific amplifications. In this study, using a non-specific SYBR Green dye, no cross-reacting saprophytic bacteria were revealed (Beckhoven et al., 2002). At the detection limit for real-time PCR methods, 10^3cfu mL^{-1} *Cms* should be detected in important parts of symptomless potatoes tubers and plants, and can be confirmed with certainty using the bioassay. Nevertheless, in our trials a few real-time PCR threshold positive and aubergine negative samples were found (< 1% of all of the samples). Within the third stage of the inspection, among the 2–3 clones of individual potato cultivars and breeding materials, no positives were revealed in 2014 and 2015. As in the second stage, several samples measured in the real-time PCR analysis using PSA1/R primer set reached the c_t and t_m detection threshold levels but were not confirmed by a positive bioassay. Thus, in this study, all of the *Cms* positive samples must be revealed in the first and second control stages of the control process. A number of factors are associated with the lack of disease symptoms in potato plants and tubers, including the potato cultivar, *Cms* strain virulence and environmental conditions (Bishop & Slack, 1987; Manzer et al., 1987; Nelson & Kozub, 1983; Nelson, 1980). In this study, the ability to detect all of the *Cms* artificially inoculated tubers of potato cultivars and breeding materials during the three-stage control process was evaluated. This control process was designed for small breeders, which have limited funds, personnel and facilities, to evaluate limited lots of initial and new breeding potato materials within one growing season and simultaneously knowledge on how to reveal quarantined pathogens were used (Schuld et al. 1992; Slack, 1987). All of the positive samples were successfully revealed. Thus, the three-stage control process can be valuable and helpful to breeders in the effort to maintain a zero tolerance for the causal agent of bacterial ring rot.

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