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Categorisation of reactions and enumeration of bacteria in potato cultivars inoculated with the causal agent of bacterial ring rot

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Abstract: Variability in the responses of plants propagated from *in vitro* tissue cultures of 52 ware and industrial potato cultivars to different *Clavibacter michiganensis* subsp. *sepedonicus* inoculum size was tested during 2015–2017. Bacterial ring rot symptoms on plants and tubers xylem vessels were recorded for 8 weeks and the susceptibility index (*SI*) for individual cultivars was calculated. Based on foliage symptoms, potato cultivars were placed into three symptoms groups. The symptomless group had *SI*s ≤ 1, for the moderate symptom group *SI*s ranged from 1.01 to 2.99, and the severe symptom group had *SI*s ≥ 3.0. The pathogen concentrations in vascular vessels of all infected potato plants increased during the experiment regardless of the foliage symptom group.

Keywords: bacterial ring rot of potato; *Clavibacter michiganensis* subsp. *sepedonicus*; industrial potato cultivar; susceptibility index; tolerance/susceptibility; ware potato cultivar

Bacterial ring rot is caused by the Gram-positive bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (Spieckermann & Kotthoff 1914) Davis et al. 1984 (*Cms*), which is included in the list of quarantine harmful organisms (Act No. 245/2011 Coll.; Decree 382/2011 Coll.). A zero-tolerance policy for this quarantine bacterium was implemented by the European and Mediterranean Plant Protection Organization (Bulletin OEPP/EPPO Bulletin 2011) for the major part of Europe (Gutbrod 1987). In the temperate climatic zone of Central Europe, *Cms* still does not express symptoms in field or even cause serious crop losses. However, if it were to become established here, then the effect on the seed-potato industry would be substantial, especially for exports (Carrasco *et al.* 2010). Control of this organism requires vigilance from growers and screening measures for all potato sources (Dehnen-Schmutz *et al.* 2010). The occurrence of latent *Cms* infections may be more common than previously believed and may serve as inoculum for the horizontal infection of other seed lots. However, keeping planting materials free of *Cms* is very difficult because of the pathogen's behaviour. Even low population sizes can plug vessels and persist in seed stocks (Narayanasamy 2011). It is very difficult to detect pathogens, especially in small breeding material samples or materials that are transferred into tissue culture (Stead 1999; Smith & De Boer 2009). Recent studies have shown that only the presence of virulent and highly concentrated *Cms* can be manifested by difficulties during the transfer, slower growth or higher percentage of withering plants during initial propagation steps of infected *in vitro* cultures (Toyoda *et al.* 1989). Foliage symptoms in propagated materials and in the field (leaf necrosis and wilting) are dependent on many interactions between the pathogen and plants. For disease induction pathogens evolve strategies

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to evade or suppress plant defence responses and induce disease susceptibility with plasmid-encoded virulence and chromosomally encoded genes responsible for the successful colonisation of the host plant. Host answers include programmed cell death, cell wall-based defence, hormone signalling, and the expression of defence genes (Abrahamovitch & Martin 2004; Eichenlaub & Gartemann 2011). Quorum sensing and regulatory virulence have been extensively studied in Gram-negative plant pathogens, such as *Ralstonia solanacearum* (Liu *et al*. 2005), and for many of *Pseudomonas syringae* pathovars (Monier & Lindow 2003). Roots, vascular vessels or leaf-epiphytic colonization with high density populations of 10^5 or 10^7 colony forming units (CFU)/ml are considered the first steps before initiating disease symptoms. Gram-positive pathogens have received limited attention (Jahr *et al*. 2000; Gartemann *et al*. 2003). Several serine proteases, defined based on the known genome sequences, have allowed the genome-wide investigation of *Cms*–host interactions (Eichenlaub & Gartemann 2011). The potato cultivars' responses to pathogen attacks and the population density initiating ring rot symptoms differ (Bishop & Slack 1987a; De Boer & McCan 1990; Westra & Slack 1994). Biological testing on eggplants (*Solanum melongena* L.) with a highly reproducible wilting symptoms at *Cms* population density 10^3 CFU/ml is still essential for the virulence evaluation of *Cms* strains determined in potato seed (Bishop & Slack 1987b; Yasuhara & Alvarez 2015; Pánková & Krejzar 2016).

Over the last decade, no significant diversity in the virulence of *Cms* strains was found in Central Europe (unpublished project results). The unexpected occurrence of *Cms*-positive potato seeds over several years in fields under strict management measures against horizontal spread and without any visual disease symptoms on plants and tubers led to the verification of the susceptibility and symptoms expression in ware and industrial potato cultivar resource materials in the Czech Republic. Detailed knowledge of the resistance, tolerance or susceptibility of potato genotypes is needed for breeding programs. Tolerant cultivars that generally do not show symptoms are dangerous because of their tendency to serve as symptomless carriers of ring rot (Hooker 1984; De Boer & McCan 1990; Kriel *et al*. 1995). Breeding for bacterial disease resistance in potato is not the primary goal of breeders; resistance to late blight, some viruses and nematodes is a higher priority (Milczarek *et al*. 2017). The demand for resistant varieties has increased among organic farmers and starch potato growers recently (Plich *et al*. 2015). New potato cultivars for processing to French fries and chips, tubers with excellent skin suitable for washing and packing, showing high level of anthocyanins or high content of starch are most desirable. The resistance/susceptibility level for bacterial ring rot in these new potato cultivars is not known or investigated.

The objectives of this study were to determine the variation in susceptibility of *in vitro* cultures of currently most requested ware and industrial potato cultivars to *Cms* infection, and to determine the relationship between disease symptoms expression and inoculum concentration and bacterial population densities in host plants. We hypothesised that a detailed characterisation of symptoms and precise enumeration of bacterial population densities would allow categorisation of disease reactions that would aid in management of ring rot.

MATERIAL AND METHODS

Bacterial strains and culture conditions. *Cms* strains NCPPB 3467 (Collection of Plant Pathogenic Bacteria, UK) 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) at 22°C for 5 days. The cells were harvested from plates with a sterile loop and suspended in sterile water.

For plant inoculations a 1 : 1 mixture of the collection strains was prepared in sterile water, the concentration was set at 10^8 (OD_{560} = 0.1), and a dilution 10^6 CFU/ml was prepared. Collection strains at 10^5 CFU/ml were used as positive control. Extracts from healthy plants and tubers were used as negative control.

Inoculation of plants. During 3 years (2015–2017) the susceptibilities of 52 ware and industrial potato cultivars to *Cms* were evaluated. In total, 88 of the sterile viable 10-cm tall plants propagated from the *in vitro* tissue culture of each cultivar were removed from the solid growing medium (Murashige & Skoog 1962) resulting in slight injuries to the roots. Then, 40 plants were immersed in the 10^8 CFU/ml *Cms* suspension for 2 s, another 40 plants in the 10^6 CFU/ml *Cms* suspension, and 8 plants were used as negative controls in sterile water. After 10 min,

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four partly drained plants were planted in one 10-cm diameter pot containing a soil mixture (Garden Substrate B Universal; Rašelina, Soběslav, Czech Republic). Inoculated plants were grown in a quarantine greenhouse at 22/15°C day/night on a 14-h day/10-h night cycle and watered with 20 ml of water every other day for 8 weeks. Plants were not fertilized. After 5 weeks five plants and at the end of the experiment all living plants from each cultivar and both treatments were harvested, cut, and the presence of *Cms* in vascular tissues was determined. Daughter tubers of each cultivar were evaluated for ring rot symptoms and the *Cms* concentration in the vascular vessels was determined.

Susceptibility levels of potato cultivars. Infected plants (*N* –number of observed plants 4, 5, 6, and 8 weeks after inoculation) of each potato cultivar (*X*) were assessed four times (*n*), at 4, 5, 6, and 8 weeks after inoculation for severity of foliage symptoms (*F*). In comparison to control plants, the numbers of plants with small noticeable differences in growth and wilting symptoms on several leaves (*Fsd*), plants with great differences in growth and whole-plant wilting (*Fgd*), and dead plants (*Fd*) were counted. At the end of the experiment the number of daughter tubers (*T*) and tubers with ring rot symptoms (*Ts*) was counted. A mean susceptibility index (SI_X) for each potato cultivar at the end of experiment was calculated using the formula:

$$SI_X = 1/n \sum_{i=1}^{n} \frac{Fsd_i + 5\ Fgd_i + 10\ Fd_i}{N} + \frac{Ts}{T}$$

All measurements were processed in UNISTAT, Version 5.1 (Unistat Ltd., London, UK).

Determination of Cms in potato plants and daughter tubers. To determine the presence of *Cms* in potato plants 5 and 8 weeks after inoculation, and in daughter tubers at the end of the experiment, DAS ELISA and real-time polymerase chain reaction (PCR) tests were performed. Stems of inoculated and control plants of each cultivar were divided into approximately 1-g samples, macerated in 1.5 ml of sample buffer (1 l distilled water, 20 g polyvinylpyrrolidone K10–K40, 2 g bovine serum albumin, pH = 7.4). Xylem vessels of daughter tubers were excised and macerated in 1.5 ml of sample buffer. All samples were incubated overnight at room temperature with shaking at 150 rpm. The next day liquid extracts from samples were collected and used for *Cms* determination. Extracts from stems and daughter tubers from control plants treated with water were used as negative controls.

For the assessment of *Cms* concentrations in vascular tissues of inoculated plants and daughter tubers, 10-fold dilutions of *Cms* collection strain NCPPB 3467 from 10^8 (optical density OD_{560} = 0.1) to 10 CFU/ml in extracts from healthy plants and tubers were prepared and determined under the same conditions as potato samples.

All measurements were processed in UNISTAT, Version 5.1.

DAS ELISA test. The determination of *Cms* concentrations in the samples was performed in triplicates in 200-µl reactions in Nunc-Immuno™ MicroWell™ 96-well solid microplates (Nunc Systems, Telangana, India) using commercial polyclonal antibodies according to the manufacturer's instructions (Loewe Biochemica, Sauerlach, Germany). The evaluation according to absorbance values at wavelength 405 nm (A_{405}) was carried out according to earlier sensitivity and specificity validation (Pánková & Krejzar 2016). The standard curve for DAS ELISA was generated according to A_{405} for 10-fold dilutions of *Cms* collection strain NCPPB 3467 from 10^8 to 10 CFU/ml.

DNA extraction and real-time PCR conditions. The total genomic DNAs from all potato samples, as well as positive and negative controls (described previously), were prepared using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The real-time PCR method was performed using a previously published protocol for specific detection and optimal quantification of *Cms* in potato plants and tubers (Pánková & Krejzar 2016). The thermocycling conditions with the dye SYBR® Green (detection wavelength 510 nm) using a Rotor-Gene Q 5plex HRM (Qiagen, Germany) were the following: initial denaturation for 30 s at 95°C; then 40 cycles of 5 s at 95°C and 30 s at 60°C, followed by a melting curve from 57°C to 95°C with increments of 1.0°C. The SYBR® Green real-time PCR assay was performed in a 25-µl reaction. The real-time PCR amplifications were carried out using two primer sets, PSA-1 (5'-CTC CTT GTG GGG TGG GAA AA-3')/PSA-R (5'-TAC TGA GAT GTT TCA CTT CCC C-3') (Pastrik 2000), and *CelA*-F (5'-TCT CTC AGT CAT TGT AAG ATG AT-3')/*CelA*-R(5'-ATT CGA CCG CTC TCA AA-3') (Gudmestad *et al*. 2009) at final concentrations of 0.2 and 0.5 µM per reaction, respectively. A Rotor-Gene SYBR® Green PCR Kit (Qiagen, Germany) was used according to the manufacturer's instructions, with 3 µl of purified DNA from each sample. All of the DNA samples were used concentrated and

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diluted 1 : 1 in water (Sigma-Aldrich Inc., St. Louis, USA) and amplification reactions were performed in triplicate for each procedure. The data analysis included determining the cycle threshold (C_t) and the melting temperatures of the PCR using a melting point analysis, which was performed with Rotor-Gene Software (Qiagen, Germany). The standard curve for SYBR® Green real-time PCR methods was generated according to the C_t for total genomic DNAs from 10-fold dilutions of *Cms* collection strain NCPPB 3467 from 10^8 to 10 CFU/ml.

RESULTS

Symptoms of bacterial ring rot. Three groups of cultivars could be identified based on foliage and tuber symptoms expression (Table 1). A group of 10 cultivars (19.2%) had no foliage and tuber symptoms during the two months of observation. The susceptibility to water stress was slightly greater than that in the control plants (not shown). The numbers (2–5) and sizes of daughter tubers were similar in pots with infected and control plants depending on cultivar (Table 1). Within this group of cultivars no significant above-ground and tuber symptoms were observed regardless of *Cms* inoculum concentration (not shown).

The second group of 31 cultivars (59.6%) showed noticeable moderate foliage wilting symptoms in comparison to the control plants (Figures 1A and 1B). The *Cms* inoculum concentrations resulted in noticeable differences in foliage disease symptoms 4 weeks after inoculation. In this group, up to 13% of plants, which were predominantly infected with the higher inoculum concentration (up to 10%), died during the experiment. A significant susceptibility to water stress was observed 2–3 weeks after inoculation for all 31 cultivars (not shown). Differences in the numbers (0–5) and sizes of daughter tubers harvested from inoculated and control plants were observed (Table 1). A subset of the group, consisting of 17 cultivars, tended to grow more slowly (Figure 1A). Their leaves were smaller and lighter coloured, and after 2 months some of them were predominantly withered. Symptomatic daughter tubers were observed in individual pots (up to 30%) within the given cultivars (Table 1). The second subset of 14 cultivars developed significant leaf deformations and after two months parts of the leaves withered, regardless of symptoms (Figure 1B). The daughter

Table 1. Susceptibility levels of 52 potato cultivars to the causal agent of ring rot, *Clavibacter michiganensis* subsp. *sepedonicus* bacteria

Symptomless group *SI* ≤ 1				Moderate symptom group *SI* (1.01–2.99)				Severe symptom group *SI* ≥ 3					
[a]cultivar	[b]potato classification	[c]foliage symptoms/ water stress (week)	[d]daughter tubers total number/symptomatic (%)	susceptibility index (*SI*)	[a]cultivar	[b]potato classification	[c]foliage symptoms/ water stress (week)	[d]daughter tubers total number/symptomatic (%)	susceptibility index (*SI*)	[a]cultivar	[b]potato classification	[c]foliage symptoms/ water stress (week)	[d]daughter tubers total number/ symptomatic (%)	susceptibility index (*SI*)
Belana	ware	0/0	97.5/0.0	0.59	Adéla[a]	ware	8/4–7/4	74.3–40.5/25.1–30.2	1.45–1.88	Adora	ware	3/2	0.0/0.0	3.60
Bernard	ware, industrial	0/7	90.3/0.0	0.77	Agáta	ware	5/4	11.2/60.5	2.24	Agria[a]	ware	5/2	0.5–0.0/0.0	3.10–3.19
Carrera	ware	0/7	87.5/0.0	0.97	Albatros[a]	ware, industrial	4/3	8.1-4.0/72.5–80.5	2.42–2.80	Borek	industrial	0/2	0.0/0.0	4.19
David	industrial	0/0	100.0/0.0	0.39	Amado	industrial	5/4	8.3/75.0	2.45	Dali[a]	ware	3/3	0.0/0.0	3.44–3.77
Fabia[a]	industrial	0/6	92.5–94.3/0.0	0.87–0.95	Anuschka	ware	5/5	15.0/55.0	2.10	Granada	ware	0/2	0.0/0.0	4.55
Gala	ware	0/8	90.0/0.0	0.74	Arabela	industrial	8/5	90.3/9.8	1.29	Lady Claire[a]	ware, industrial	3/2	0.0/0.0	4.06–4.09
Krone	ware	0/8	90.5/0.0	0.79	Colette	ware	6/5	32.7/30.0	1.88	Rafaela	ware	4/4	0.0/0.0	3.57
Magda	ware	0/8	100.0/0.0	0.64	Ditta	ware	7/5	82.5/17.5	1.38	Ramses	industrial	5/2	0.5/100.0	3.21

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Table 1 to be contiued

Symptomless group *SI* ≤ 1					Moderate symptom group *SI* (1.01–2.99)					Severe symptom group *SI* ≥ 3				
[a]cultivar	[b]potato classification	[c]foliage symptoms/ water stress (week)	[d]daughter tubers total number/symptomatic (%)	susceptibility index (*SI*)	[a]cultivar	[b]potato classification	[c]foliage symptoms/ water stress (week)	[d]daughter tubers total number/symptomatic (%)	susceptibility index (*SI*)	[a]cultivar	[b]Potato classification	[c]foliage symptoms/ water stress (week)	[d]daughter tubers total number/ symptomatic (%)	susceptibility index (*SI*)
Pardál	industrial	0/0	97.5/0.0	0.39	Dominátor	industrial	7/4	28.7/30.0	1.92	Riviera	ware	4/3	0.0/0.0	3.39
Priamos	industrial	0/8	90.5/0.0	0.73	Elfe	ware	6/4	10.3/12.5	2.26	Rosara[a]	ware	3/3	0.0/0.0	3.48–3.61
					Eurostarch	industrial	5/4	0.0/0.0	2.47	Tomensa	ware, industrial	6/3	0.3/0.0	3.20
					Hermes	industrial	8/5	71.9/22.5	1.47					
					Impala[a]	ware	6/4–5/3	25.0–11.0/32.5–62.5	1.88–2.25					
					Krumlov[a]	industrial	6/4	27.2–28.7/32.5–37.5	1.94–1.98					
					Kuras	industrial	8/5	76.0/22.5	1.45					
					Laura[a]	ware	5/4	10.0–2.7/0.0	2.21–2.46					
					Marabel[a]	ware	7/5	65.0–68.2/30.0–32.5	1.56–1.57					
					Monika	ware	7/4	52.5/50.0	1.65					
					Musica	ware	8/6	93.8/0.0	1,18					
					Ornella[a]	ware, industrial	6/3–5/3	41.8–17.5	1.79–2.05					
					Poutník	industrial	8/6	87.5	1.34					
					Princess[a]	ware	5/4–5/3	49.6–15.0/80.0	1.69–2.01					
					Red Anna[a]	ware	8/7–8/5	89.1–77.5/5.4–6.8	1.08–1.45					
					Regent	industrial	7/4	50.9/53.3	1.69					
					Roberta	industrial	7/4	33.5/55.6	1.82					
					Saturna[a]	ware, industrial	6/3–5/3	12.5–8.0/0.0	2.13–2.43					
					Sirius	ware, industrial	4/3	7.0/0.0	2.54					
					Soraya	ware	8/6	97.9/0.0	1.17					
					Verne	industrial	4/3	0.0/0.0	2.56					
					Westamyl	industrial	4/3	0.0/0.0	2.34					
					Žofie	industrial	8/5	95.8/0.0	1.15					

[a]experiment was run twice using plants of 15 potato cultivars; [b]classification of potato cultivars – ware and/or industrial cultivar; [c]number of weeks after plant inoculation when the first bacterial ring rot foliage symptoms or water stress were observed; [d]percentage of daughter tubers formed after *Clavibacter michiganensis* subsp. *sepedonicus* plant inoculation in comparison to the total number of daughter tubers formed at control plants of the same potato cultivar, and proportion of symptomatic tubers

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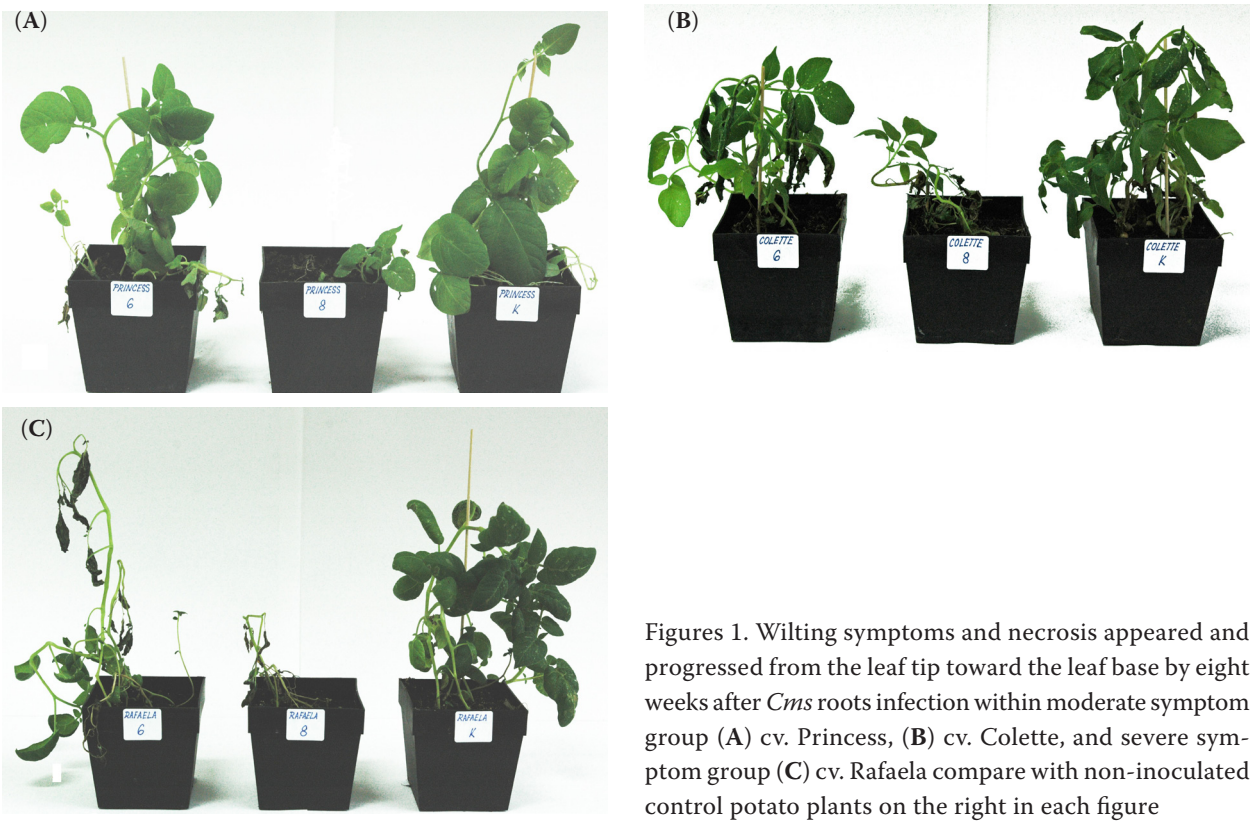

Figures 1. Wilting symptoms and necrosis appeared and progressed from the leaf tip toward the leaf base by eight weeks after *Cms* roots infection within moderate symptom group (**A**) cv. Princess, (**B**) cv. Colette, and severe symptom group (**C**) cv. Rafaela compare with non-inoculated control potato plants on the right in each figure

tubers were formed in up to 15% pots within the given cultivars (Table 1).

The third group of 11 cultivars (21.2%) showed significant differences between infected and control plants (Figure 1C). The main severe symptom of Cms infection was a high susceptibility to even a relatively moderate water stress (not shown). Within 3–6 weeks the health status of the infected plants, regardless of the *Cms* inoculums concentration, deteriorated sharply. Whole plants withered without

Table 2. Determination of *Clavibacter michiganensis* subsp. *sepedonicus* bacteria population densities in infected plants and daughter tubers of 52 potato cultivars

[a]Determination method	[b]10^8 CFU/ml				[b]10^6 CFU/ml				Water
	5 weeks		8 weeks		5 weeks		8 weeks		
	AS	S	AS	S	AS	S	AS	S	control
Number of cultivars	19	33	10	39	27	25	10	42	52
[c]DAS ELISA (A_{405} ± SD)	0.55 ± 0.10	0.75 ± 0.27	0.86 ± 0.25	1.48 ± 0.51	0.45 ± 0.10	0.60 ± 0.20	0.60 ± 0.25	1.30 ± 0.52	0.20 ± 0.05
[d]Real-time PCR PSA F/R (C_t ± SD)	22.89 ± 0.76	22.30 ± 1.01	21.76 ± 0.84	19.79 ± 1.03	23.00 ± 0.43	22.30 ± 1.90	22.39 ± 0.70	20.86 ± 1.76	27.48 ± 0.13
[d]Real-time PCR CelA F/R (C_t ± SD)	23.85 ± 0.51	22.35 ± 1.56	24.06 ± 0.85	20.38 ± 1.74	27.70 ± 0.34	25.30 ± 0.75	24.56 ± 1.09	21.63 ± 2.87	28.50 ± 0.25

[a]determination of *Clavibacter michiganensis* subsp. *sepedonicus* population densities in infected plants of 52 potato cultivars 5 and 8 weeks after reaction; [b]concentration of *Clavibacter michiganensis* subsp. *sepedonicus* inocula used for potato plants infecting; [c]mean absorbance values for all asymptomatic or symptomatic potato plants and daughter tubers regardless cultivar infected with 10^8 or 10^6 CFU/ml; [d]mean cycle threshold values for all asymptomatic or symptomatic potato plants and daughter tubers regardless cultivar infected with 10^8 or 10^6 CFU/ml; AS – asymptomatic plants; S – symptomatic plants

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Figure 2. A higher rate of plant recovery and regeneration within industrial cultivars – (**A**) Krumlov, (**B**) Dominátor, and (**C**) Eurostarch in moderate symptom group was observed

Note the dead or unthrifty shoots (1) on each plant next to a regenerated shoot showing significant recovery (2)

developing typical leaf symptoms (Figure 2B) and at the end of the experiment 50–90% of plants died. Daughter tubers formed rarely (Table 1). Exposure to the higher *Cms* concentration of 10^8 CFU/ml led to a 15% greater number of dead plants (Table 2).

The experiment was performed on 52 cultivars and then repeated with the 15 most interesting cultivars for breeders (Table 1). The same plant behaviour within a given cultivar was observed, and group categorisations were not changed (Table 1).

Of the 20 industrial cultivars, 25% were included in the first symptomless group, 65% in the moderate symptom group, and 10% in the severe symptom group. A greater ability to recover after infection was observed generally within industrial cultivars regardless of symptom group. After 5 weeks of growth, symptomless new leaves and even new stems were observed (Figure 2).

Susceptibility index. The calculated *SI* ranged from 0.39 to 4.55, and the value was 0.39 for two symptomless cultivars David and Pardal and 4.55 for the cv. Granada, for which nearly all of the inoculated plants withered (Table 1). The *SI* for the group of 10 cultivars without noticeable foliage and tuber symptoms was ≤ 1 (range 0.39–0.95 was calculated). For the second group of 31 cultivars with moderate disease symptoms, the *SI* range was 1.18–2.56, and for the group of 11 cultivars with severe foliage symptoms *SI* ≥ 3 (3.1–4.55) was determined. The group thresholds of tolerance/susceptibility have been set based on a comparison of foliage symptoms on infected and control plants. The ability of the inoculation method to consistently produce the same *SI results* in the 15 cultivars was determined twice. The differences between the two experiments ranged from 0.01 to 0.37, 0.08 for one cultivar from the symptomless group, from 0.01 to 0.25 for 10 cultivars from the moderate symptom group, and from 0.03 to 0.37 for 4 cultivars from severe symptom group (Table 1).

Cms concentration in plant tissues. The presence of *Cms* was determined in 85% of symptomatic and 80% of asymptomatic plants 5 weeks after inoculation and in 100% of symptomatic, 95% of asymptomatic plants, and 80% of tubers at the end of the experiment (Table 2). According to DAS ELISA absorbance values and real-time PCR C_t values the pathogen concentration inside the plants increased during the experiment, regardless of cultivar and disease symptoms (Table 2). The distribution of potato cultivars into three susceptibility groups at the end of the experiment (Table 3) showed a moderate correlation between determination methods mean A_{405} and C_t values and symptom group ($r \geq 0.25$; $P = 0.005$). The approximate concentrations of *Cms* in vascular tissues were set according to DAS ELISA and SYBR® Green real-time PCR calibration curves.

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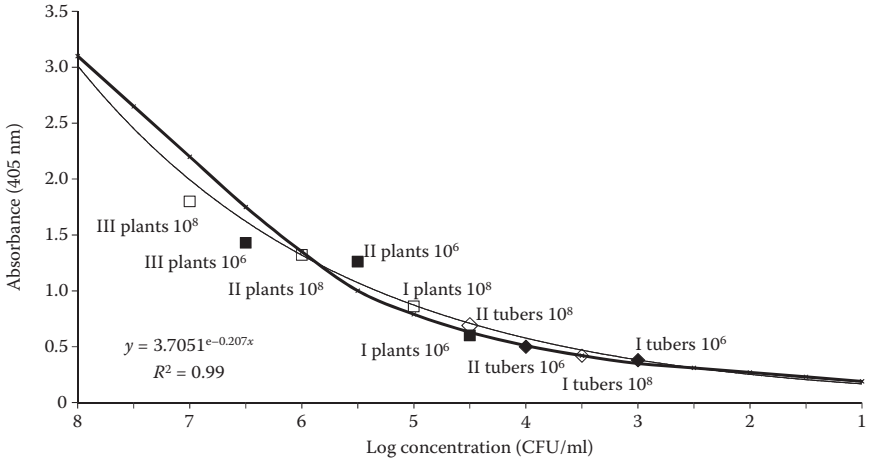

Figure 3. Concentration of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) in potato plants and tubers determined by DAS ELISA method at the end of the experiment – infected potato plants and tubers were evaluated for *Cms* concentration using the determination method DAS ELISA 8 weeks after inoculation

Cms concentration was determined according to standard calibration curves calculated individually for two initial inoculum concentrations, 10^8 and 10^6 CFU/ml, within symptomless group (I), moderate symptom group (II), and severe symptom group (III)

In the symptomless group, the *Cms* concentration in potato plants reached up to 5×10^4 CFU/ml for the initial inoculums 10^6 CFU/ml, and up to 10^5 CFU/ml for the initial inoculums 10^8 CFU/ml at the end of the experiment, while in the moderate and severe symptoms groups the levels in plants reached up to

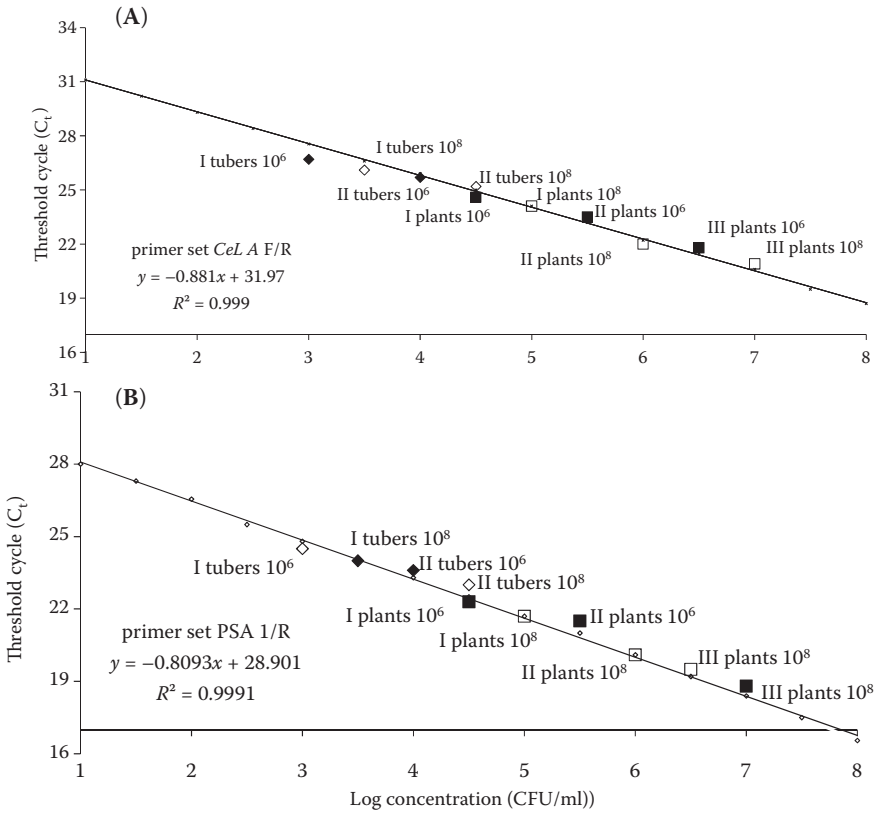

Figure 4. Concentration of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) in potato plants and tubers determined by SYBR® Green real-time PCR methods at the end of the experiment – infected potato plants and tubers were evaluated for *Cms* concentration using a real-time PCR primer set (A) *CelA* F/R and PSA1/R 8 weeks after inoculation (for explantion see Figure 3)

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Table 3. Determination of *Clavibacter michiganensis* subsp. *sepedonicus* population densities in infected potato plants separated into three disease symptom groups

	Symptomless				Moderate symptom				Severe symptom		Negative control plants		Positive control 10^5 CFU per ml bacteria
Inoculum concentration	10^8 CFU/ml		10^6 CFU/ml		10^8 CFU/ml		10^6 CFU/ml		10^8 CFU/ml	10^6 CFU/ml	water		
Determination method/plant tissue	plants	daughter tubers	plants	daughter tubers	plants	daughter tubers	plants	daughter tubers	plants	plants	plants	daughter tubers	
[a]DAS ELISA (A_{405} ± SD)	0.86 ± 0.25	0.42 ± 0.09	0.60 ± 0.25	0.38 ± 0.10	1.32 ± 0.12	0.69 ± 0.20	1.26 ± 0.06	0.50 ± 0,18	1.80 ± 0.21	1.43 ± 0.44	0.20 ± 0.05	0.15 ± 0.05	0.75 ± 0.10
[b]Real-time PCR primer set PSA F/R (c_t ± SD)	21.76 ± 0.84	24.00 ± 2.31	22.39 ± 0.70	24.51 ± 2.03	20.15 ± 0.67	24.00 ± 3.01	21.53 ± 1.09	25.20 ± 2.61	19.42 ± 0.56	20.18 ± 0.78	27.48 ± 0.13	27.90 ± 0.30	21.59 ± 0.10
[b]Real-time PCR primer set CelA F/R (c_t ± SD)	24.06 ± 0.85	26.06 ± 1.83	24.56 ± 1.09	26.66 ± 1.33	21.90 ± 0.52	25.00 ± 2.03	23.50 ± 1.00	25.70 ± 2.03	18.85 ± 0.75	19.76 ± 1.08	28.50 ± 0.25	28,96 ± 0.35	24.16 ± 0.06

[a]mean absorbance values for all infected potato plants in three disease symptom groups regardless cultivar infected with 10^8 or 10^6 CFU/ml; [b]mean cycle threshold values for all infected potato plants in three disease symptom groups regardless cultivar infected with 10^8 or 10^6 CFU/ml

5×10^5–10^6 CFU/ml and up to 5×10^6–10^7 CFU/ml, regardless of the determination method used (Figures 3–5). The *Cms* concentration in potato tubers in the symptomless group reached up to 10^3 CFU/ml for the initial inoculum 10^6 CFU/ml and up to 5×10^3 CFU/ml for the inoculum 10^8 CFU/ml at the end of the experiment, regardless of the determination method used. In the moderate symptom group the levels reached up to 10^4 and 5×10^4 CFU/ml, respectively (Figures 3 and 4).

In the 15 cultivars that underwent the experiment twice, a small difference between the results was found only for the group with severe disease symptoms, in which the final *Cms* concentrations in plant vascular tissues reached up to 5×10^7 CFU/ml. No significant correlation was found between a higher starch content in the cultivars, which is relevant for industrial processing, and the plants' and tubers' pathogen concentration ($P < 0.001$). The absorbance values and C_t values for plants of ware and industrial potato cultivars within all of the symptom groups, and for tubers within symptomless and moderate symptom groups, were equal.

DISCUSSION

The susceptibility index (*SI*) serves to facilitate the discussion of foliage and tuber symptoms expression and susceptibility level. Cultivar responses to bacteria vary owing to several factors, such as growing conditions and inoculums size. All of the plants were inoculated by removing the plants from the growth medium and emerging the roots for 2 s in *Cms* inoculum. Planting *in vitro* cultures to soil in greenhouse, mechanical root disruption, and bacterial infection activated local tissue responses to injuries and defence mechanisms throughout the plants and could significantly affect the development of ring rot disease symptoms (Juge 2006; Holtsmark *et al*. 2007). In our experiment all plants were inoculated under the same conditions but the expression of disease symptoms on foliage and tubers of individual cultivars was different. Altogether 21.2% of cultivars were sensitive to water stress and potato plants suddenly died without expressing any typical symptoms. They formed the severe symptoms group. We assume that in practice infected plants of these cultivars will express certain suspected symptoms, such as slow growth or water stress, which would lead to the elimination of plants from the propaga-

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tion process. Plants of almost the same number of cultivars (20%) on the other side of the SI scale did not express any disease symptoms during the experimental period; they formed the most dangerous symptomless group. Most of potato cultivars (58.8%) exhibited water stress reaction 2–3 weeks and the foliage ring rot symptoms 4–5 weeks after inoculation; they formed the moderate symptom group. According to Schuld *et al*. (1992) and our experience, it is too late. Visual symptoms of *Cms* infection observed during the experiment may be readily overlooked in the professional propagating process, where the plants are multiplied by nodal stem cuttings every 3 weeks. For the symptomless and the moderate symptoms groups special *c*ontrol process has been introduced in recent years. Initial breeding materials are visually evaluated during the whole season and the presence of *Cms* is determined in mother tubers, potato plants, daughter tubers, and *in vitro* cultures (Pánková & Krejzar 2016).

Our results of *Cms* determination in plant tissue extracts during the whole experiment showed that the C_t values in the quantitative real-time PCR method (Laine *et al*. 2000) were in correlation with the DAS ELISA absorbance values (Figures 3 and 4). Hence the cheaper and easier serological method can be used for Cms determination during the procedures of potato breeding and the practice of multiplying stock plant materials.

The experiments demonstrated not only survival, but also the growth of the *Cms* population in potato plant tissues predominantly. Growing concentrations of *Cms* in plant tissues during the experiment were measured regardless of cultivar, concentration of inocula, and developed symptoms (Table 2). Thus, all potato cultivars should be considered susceptible. Resistant plants should have significantly less infection at equivalent inoculum loads than the other plants expressing disease symptoms (De Boer & McCann 1990) or the restriction of pathogen replication or invasion in plant tissues would be demonstrated (Manzer & Kurowski 1992). We recommend that cultivars within the symptomless group, the moderate symptom group, and the severe symptom group be designated slightly susceptible, moderately susceptible, and very susceptible, respectively.

Final concentrations of *Cms* in plants and daughter tubers correlated positively. In vascular vessels of daughter tubers 10–100 times lower concentrations of *Cms* were found compared to concentrations in plant stems and foliage. This may be in part due to the length of the experiment. However, even the lowest *Cms* concentrations measured within the experiment, up to 10^3–5×10^3 CFU/ml in tuber samples of symptomless group, cause significant noticeable foliage symptoms on eggplants 6–8 weeks after inoculation (Pankova, unpublished). Under experimental conditions the threshold concentration value for noticeable disease symptoms was 10^5 CFU/ml. In reality the *Cms* concentration in transferred symptomless potato tissue cultures is low, most of the time under the threshold limits of the determination methods. Well-watered and fertilized plants respond to infection by producing growth inhibitors that keep the population of pathogen below the threshold needed for macroscopic disease symptoms development (Hammerschmidt 1999). Based on the results of the experiment, the probability of selecting *Cms* naturally infected clones in the laboratory or in greenhouse according to ring rot disease symptoms in tissue cultures is very low (Schuld *et al*. 1992). Subsequently, under field conditions, the silent symptomless growth of pathogen populations to the determination limit (EPPO Bulletin 2011) takes 3–5 propagating cycles depending on the season temperature, potato cultivar, agricultural practices, and possibly other unknown factors. Therefore, extraordinary care must be taken to health of initial potato materials used for breeding new cultivars. Determination of pathogen in small breeding samples is more efficient and easier than in subsequent prospective clones.

More *Cms*-positive samples have recently been found by plant protection service among industrial potato cultivars in the Czech Republic. Our study did not find a significant correlation between the starch content and the disease symptoms or *Cms* concentration in plant tissues during and at the end of the study. However, the higher rate of plant recovery and regeneration within industrial cultivars was recorded for the moderate symptom group. Heavily infected parts of stems or whole stems died and new ones grew during the study (Figure 2). A higher level of recovery should help plants overcome the first critical multiplication steps. These results are consistent with the studies claiming a relationship of enhanced carbohydrate level associated with high average resistance to the major potato diseases (Nowicki *et al*. 2012; Diez-de-Medina Roldán *et al*. 2013). The significance of increased starch content for the survival of *Cms* infected *in vitro* cultures and potato plants during the first steps of the multiplication is a topic that requires further study.

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