Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in Daughter Tubers of Volunteer Potato Plants

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Abstract

Pánková I., Krejzar V., Čepl J., Kůdela V. (2007): **Detection of** *Clavibacter michiganensis* subsp**.** *sepedonicus* **in daughter tubers of volunteer potato plants**. Plant Protect. Sci., **43**: 127–134.

Daughter tubers of volunteer potatoes were tested for their ability to maintain *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*). In different areas of the CR, volunteer potatoes were searched for in crops grown in rotation with potatoes and where one or two years before *Cms* had been detected and identified in samples of harvested seed or commercial potatoes using the test scheme in accordance to EC Directive 93/85/EEC. During May and June of 2005 and 2006, emerging or emerged plants of volunteer potatoes were collected at nine locations of Bohemia and transplanted to the experimental field in the Diagnostic Service Laboratory at Šluknov-Kunratice in Northern Bohemia. The daughter tubers of these plants were harvested and stored at 6°C for 1 month and then at 22°C for 3 months for multiplication of *Cms* cells. Samples of the daughter tubers were divided into 215 partial samples and tested for the occurrence of *Cms* at five terms which differed in length of storage time. The DAS ELISA test was used to detect *Cms* in the tuber samples. *Cms* was detected in eight of the nine potato volunteer tuber samples from different locations. The presence of *Cms* in positively tested tuber samples was confirmed using a pathogenicity test on eggplants (*Solanum melongena*). The optimal time for the detection of the pathogen in the harvested daughter tubers was between 4 and 10 weeks of storage at 22°C.

Keywords: potato; *Clavibacter michiganensis* subsp. *sepedonicus*; bacterial ring rot; potato volunteer plants; incidence of occurrence; DAS ELISA; Czech Republic

Clavibacter michiganensis subsp. *sepedonicus* (Spieckermann & Kotthoff 1914) according to Davis *et al*. (1984) (*Cms*), the causal agent of bacterial ring rot of potato, is a quarantine organism which constitutes a worldwide threat to potato growers and the potato industry. *Cms* can cause damage of different types – by direct crop loss during growth and storage, by rejection of infected seed lots and the cost of control measures, by loss of export markets or by difficulties experienced in opening new markets. In Europe, *Cms* annually causes an estimated 15 million Euro in economic damage (van der Wolf *et al.* 2005).

According to Straňák (1917), a disease named bacterial ring rot (BRR) occurred in potato crops around the Czech Lands at the beginning of the 20th century. Among the Czech Lands, South Bohemia was supposed to be most severely threatened by BRR. Towards the end of the 20th century in the CR, a relatively high percentage of potato tuber samples proved to be infected by *Cms*, namely 1.14% in seed potatoes in 1998 and 4.13% in com-

Supported by the Ministry of Agriculture of the Czech Republic, Project No. QF 4105.

mercial potatoes in 1999. Zero incidence of *Cms* in basic and certified seed potatoes and 0.19% in commercial potato lots was achieved in 2005. However, *Cms* was detected in 0.15% of seed potato samples and 0.23% of commercial samples a year later, in 2006 (Kůdela 2007). These results confirmed the experiences from other countries, i.e. if in one year and region the certified tuber samples tested negative for the presence of *Cms*, this did not mean that the pathogen had been eradicated there.

Only potato (*Solanum tuberosum* L.) is natural host of *Cms*. The primary sources of bacterial ring rot inoculum are infected tubers in storage or overwintered in the field, but seed lots containing infected tubers are the main source of dissemination of *Cms*.

Cms is controlled predominantly through the prevention of infection in compliance with EC Directive 93/85/EEC (Anonymous 1993). The planting of pathogen-free seed, such as those provided through potato certification services, is the primary means of prevention. Strict hygiene measures calling for sanitation practices, such as disinfecting farm machinery and storage facilities, are also important components in prevention. Yet even with these practices, *Cms* remains a problem (van der Wolf *et al.* 2005).

Besides infected potato tubers, the contribution of other factors which affect the survival and dissemination of *Cms* in the potato ecosystem is poorly understood. These factors include the ability to survive on materials and equipment, in the soil, volunteer potatoes (groundkeepers) and weeds and non-host plants, or factors such as dissemination in the soil, via surface and drain water and by insects and nematodes.

Volunteer potato (groundkeeper) is a perennial weed that is difficult to control in crop rotations. According to Pérombelon (1975) several thousand tubers (e.g. 124 000 tubers/ha) may remain in a field after the potato harvest. About one fifth of these are on the surface and the remainder are buried in the top 20 cm of soil. Large numbers of groundkeepers can survive for many years in a field. Thus, volunteer potato can be a host of serious pathogens and pests in potatoes and could become a source of inoculum and pests for subsequent potato crops.

In this paper, an attempt is made to determine the ability of daughter tubers of volunteer potatoes to maintain *Cms* and to be a potential threat for a subsequent potato crop.

MATERIAL AND METHODS

Plant material. Volunteer potatoes were searched in fields of winter barley, spring barley, winter wheat, oat, mixed spring crops, peas and clover grown in rotation with potato in different areas of the CR. Seed or commercial potatoes had been grown in these fields one or two years before and *Cms* had been detected and identified in samples of the harvest, using the testing scheme according to EC Directive 93/85/EEC (Anonymous 1993).

During May and June in 2005 and 2006, emerging or emerged volunteer potato plants were taken from nine locations of Bohemia and transplanted to the experimental field of the Diagnostic Service Laboratory at Šluknov-Kunratice in Northern Bohemia. Later, samples of daughter tubers of these volunteer plants, of eight different cultivars, were harvested by hand at optimal maturity. The daughter tubers were stored at 6°C for 1 month and then at 22°C for 3 months for multiplication of *Cms* cells.

From each of the nine locations, 50 (in some cases less) daughter tubers of volunteer plants were selected at random for further testing. Each sample was divided into five and four partial samples in 2005 and 2006, respectively. Each partial sample of 7–10 tubers was tested for the presence of *Cms* at five or four terms that differed in the length of storage time and storage temperature (Table 2). Altogether 215 partial samples were tested.

Detection of the pathogen. All tubers of test-samples were washed in running water. The periderm around the heel end (stolon end) of each tuber was cut off with a disinfected scalpel (dipped in 70% ethanol and flamed). After a longitudinal section through the tuber, only the vascular tissue near the heel end was selectively cut out by scalpel.

An excess of 0.05M PBS buffer (pH 7.2) was added to the excised vascular tissues and these were homogenised using a blender (Waring, USA); afterwards they were shaken ca 18 h. Next day the homogenates were centrifuged at not less than 12 000 RPM to extract bacteria. The supernatants were decanted and discarded. The pellets were suspended in a coating buffer (pH 9.6) to give a total volume of approximately 1 ml. Each sample was divided into two equal parts and one part was retained for reference purposes by freezing at –20°C. A DAS ELISA kit (LOEWE Biochemica

GmbH, Germany) assigned for the detection of *Cms* in concentrated and diluted (1:10) pellets was used. A value of absorbance at 405 nm above 0.20 was regarded as a threshold characterising a positive reaction or proof of the presence of *Cms* in the tested sample. The highest absorbance values of concentrated suspended pellets were used to look for changes in the concentration of *Cms* cells in tuber samples when stored at 22°C (Figure 3).

To confirm the results, all DAS ELISA positive pellets obtained from homogenates of samples of daughter tubers were tested for pathogenicity on eggplants, *Solanum melongena* L. cv. Black Beauty. *Cms* collection strain NCPPB 3467 served as the positive and distilled water as the negative control in these tests.

Two plants (at a stage of three leaves) per variant were inoculated into the stems between the cotyledons and the first leaf using a syringe with a 19G needle. After inoculation, the plants were kept at optimal day/night temperatures of about 23°C/15°C for 40 days. After that, plants with symptoms of leaf wilting were recorded.

***Measure of** Cms **incidence**.* Two manners to express *Cms* incidence were used:

(*i*) *Cms* incidence at a tested location measures the proportion of cases in which *Cms* was detected in samples of daughter tubers of volunteer plants (numerator) compared to samples from all locations tested (denominator);

(*ii*) the cumulative incidence rate measures all new cases of *Cms* detection in samples of daughter tubers of volunteer plants (numerator) compared to all samples tested over the entire period of storing tuber samples (denominator).

RESULTS

Volunteer potato density

During May and June of 2005 and 2006 and at nine locations, the number of emerged volunteer potato plants were recorded in crops such as winter barley, spring barley, winter wheat, oat, mixed spring crops, peas, and clover grown in rotation with potato (Table 1). The density of volunteer potato plants varied from 0.005 to 0.1 per 1 m^2 (Figure 1).

Incidence of detection of *Cms* in daughter tubers of volunteer plants

The incidence of *Cms* in daughter tubers of volunteer plants was quite high in the tested samples. *Cms* was detected in the daughter tuber samples from eight of the nine locations (Table 2). When tubers were inspected at crosswise cut stem ends, the percentage of samples with characteristic bacterial ring rot symptoms increased with storage time. The pathogen was relatively effortlessly isolated from these symptomatic tubers. The presence of *Cms* in positively tested tuber samples was confirmed using the pathogenicity test on eggplants.

Influence of the length of storage time and storage temperatures on the incidence of *Cms* detection

Altogether 215 partial samples from nine locations were tested for the presence of *Cms* at five or four terms that differed by the length of storage

Table 1. History of potato volunteer plants collected to obtain daughter tubers for detection of *Cms*

Location No.	Village	Potato cultivar (year of growing)	Crop grown in 2005	Estimated number of volunteer plants/ha in 2005
1	Liboměřice	Santé (2003)	peas, clover	50
2	Lukavec	Krasa (2003)	winter barley	500
3	Frýdlant	Santé (2003)	winter wheat	200
4	Frýdlant	Karin (2003)	oat	200
5	Mnich	Dali (2003)	spring barley	500
6	Šternberk	Solara (2003)	spring barley	500
7	Šternberk	Rosella (2003)	oat	500
8	Květná	Kordoba (2004)	mixed spring crops	1000
9	Květná	Satina (2004)	mixed spring crops	1000

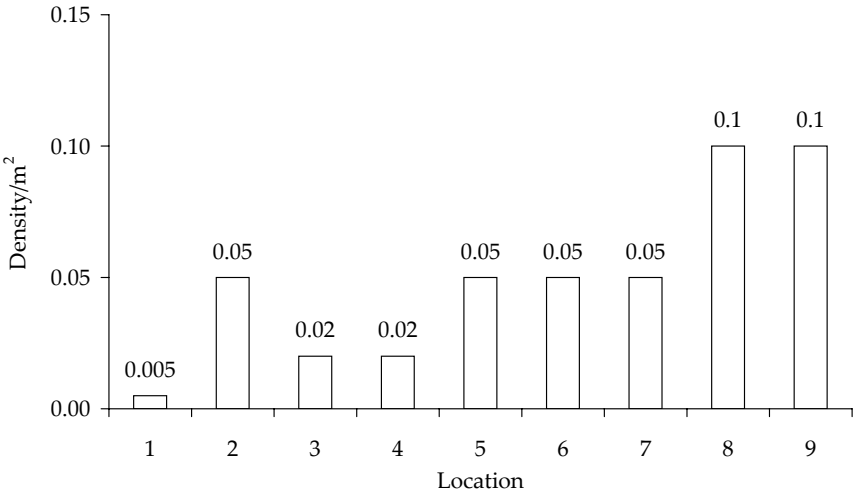

Figure 1. Density of volunteer potato plants per 1 m^2

time and storage temperature (Table 2). From the graph (Figure 2) showing the cumulative incidence of new cases of *Cms* detection during storage time, it is obvious that the optimal time for the detection of the pathogen in tubers lies between 4 and 10 weeks of storage at 22°C.

The value of absorbance at 405 nm above 0.20 was regarded as a threshold characterising a positive reaction or proof of the presence of *Cms* in tested samples and a criterion of the number of *Cms* cells. An increase of *Cms* detection in tuber samples with prolonged storage time was the result of favourable conditions for the multiplication of *Cms* cells when stored at 22°C (Figure 3).

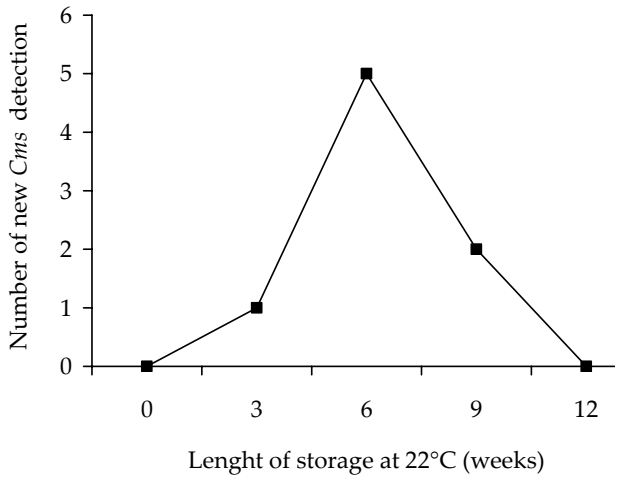

Figure 2. Cumulative incidence of new cases of *Cms* detection in volunteer daughter samples when stored at 22°C

Differences between absorbance values in concentrated and diluted suspended pellet samples

With prolonged storage time at 22°C, the absorbance values in most cases increased both in concentrated and diluted samples of suspended pellets; however, these values were higher in concentrated samples. Differences between absorbance values in concentrated and diluted suspended pellets ranged from 0.10 to 0.40 (Figure 4).

DISCUSSION

When assessing the ability of daughter tubers of volunteer potato plants to maintain *Cms* infection and to be a potential threat for a subsequent potato crop, the following parameters must be taken into account: (1) volunteer plant density, (2) the average number of daughter tubers of volunteer plants, (3) the incidence of *Cms* in daughter tubers.

Counts at nine locations in 2005 and 2006 showed that volunteer potato density varied from 0.005 to 0.1 plants per 1 m^2. Surveys of locations where potatoes had been planted with potatoes one or two years before, indicated that large numbers of volunteer potatoes can survive some years of crops grown in rotation with potatoes. Winter soil temperatures were not low enough to kill a high proportion of the buried tubers. The tubers need prolonged exposure at –2°C to be reliably killed (Eberlein *et al.* 1997). Nor was the control of potato volunteer plants effective enough to eradicate emerged volunteer plants.

Table 2. Incidence of *Cms* detection in sub-samples of volunteer daughter tubers in dependence on the length of storage at 22°C

No. of sample	Length of storage at 22°C																									IPR (%)
	before storing					3 weeks					6 weeks					9 weeks					12 weeks					
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	
1																				■						4.0
2														■										■		8.0
3									■				■		■	■	■	■								20.0
4														■										■	■	12.0
5																										0.0
6											■										■					8.0
7																■			■		■					12.0
8		not tested									■										■			■		15.0
9		not tested									■	■									■	■	■	■	■	35.0
IPR (%)		0.0					4.0					17.8					11.1					26.1				13.9*

grey coloured square means positive sample; IPR (%) – incidence of positive reaction in percentage; *average of incidence of positive reaction in percentage

In the study carried out in the years 2005 and 2006, volunteer potatoes were searched for and counted at locations where *Cms* had been detected and identified one or two years earlier in samples of harvested seed or commercial potatoes using the test scheme according to EC Directive 93/85/EEC (Anonymous 1993). Our test now detected *Cms* in eight of nine potato volunteer tuber samples from different locations. This result confirmed the known fact that the bacterial ring rot pathogen can survive several generations in volunteer potatoes (Gudmestad 1994). We also found that the incidence of infected volunteers' daughter tubers can be quite high, i.e. it ranged between 0% and 35% (Table 2). From this research the question arises: how serious is the risk of these infected tubers for the next potato crop? The tubers can either disintegrate and release *Cms* cells into the soil environment, or the tubers can give rise to new plants.

Different attempts to infect potato tubers by growing them in *Cms*-infested soil have failed.

There are no indications for plant-to-plant dissemination through soil. Only at low temperatures

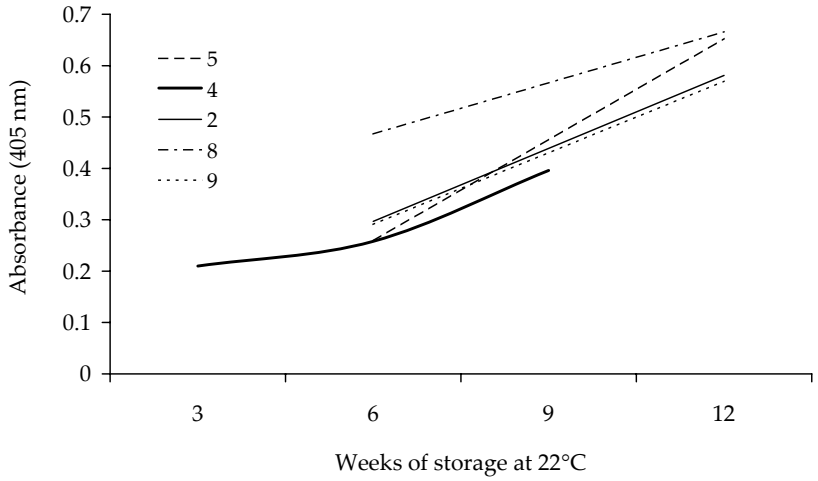

Figure 3. Changes in the highest absorbance values in tuber samples when stored at 22°C

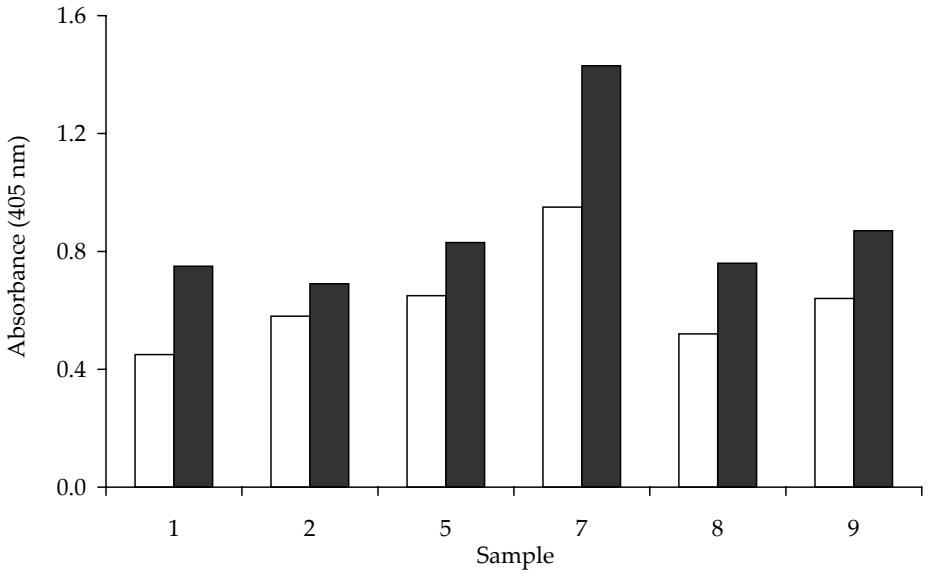

Figure 4. Differences between absorbance values in concentrated and diluted samples after 12 weeks stored at 22°C

do survival periods exceed one year. As soon as the temperature increases to 15°C, *Cms* can survive for only a few weeks. Therefore, *Cms* is not considered to be a soil resident (van der Wolf *et al*. 2005). In Poland, in one cold and wet year, plant-to-plant transmission occurred in only 0.42% of all the plants involved in a 4-year field experiment (Golenia & Pajewska 1977). Frazzoli *et al*. (1984) reported that *Cms* was not transmitted from infected plants to the tubers of healthy plants through their stolons. Sletten (1985) was not able to detect *Cms* in progeny tubers from control plots, despite their proximity to plots of infected plants. He suggested that the spread of bacteria in the field may not be common. According to Mansfeld-Giese (1997) plant-to-plant transmission may occur, but at a very low frequency (0.5%) and it is unlikely to play a significant role in comparison to the potential of transmission by shared potato handling equipment. In contrast, Dinesen (1987) observed a high frequency (40–60%) of plant-to-plant transmission. However, his study is the only one in which plant-to-plant transmission of the pathogen has been reported in field trials with a significant frequency as determined by detection of the pathogen in progeny tubers. During his trials in 1983 and 1984, infection of tuber progeny grown from healthy seed tubers planted adjacent to infected seed tubers was observed in 53% and 45%, respectively. Dinesen (1987), using a polyclonal antibody staining procedure, rarely found bacteria in the stems of infected plants derived from latently infected tubers or in the stems of infected plants derived from healthy seed tubers. It was thus concluded that the dispersal probably occurred through soil. Mansfeld-Giese (1997) used a more sensitive and specific monoclonal staining procedure and he consistently found bacteria in the stems of plants grown from artificially infected seed tubers and latently infected tubers as well as in the stems of two infected plants grown from healthy seed tubers. However, he concluded that

Table 3. Incidence of *Cms* infection in a sample size of 200 tubers consisting of a mixture of pathogen-free and infected tubers

Number of volunteer plants/ha	Number of daughter tubers/ha	Incidence of infection in a mixed sample (% at 50% infected daughter tubers)
100	300	0.005
500	1500	0.017
1000	3000	0.035
2000	6000	0.071

the low number of infected plants means that no conclusion as to the possible mode of transmission can be made.

New plants grown from infected daughter tubers of volunteer plants can be either healthy or infected. *Cms* can be potentially transmitted from the foliage of diseased plants to healthy ones by insects. The Colorado beetle (*Leptinotarsa decemlineata*) and green peach aphid (*Myzus persicae*) were mentioned as potential vectors of *Cms* (Christie *et al*. 1991). However, it is not known if *Cms* transmitted by insects to pathogen-free potato stems or leaves can be transferred to daughter tubers. Generally, the numbers of cells that are transmitted by insects is not sufficient to cause ring rot symptoms (van der Wolf *et al*. 2005).

Infected daughter tubers of volunteer plants surviving from previous potato crops in the locations where healthy seed potatoes were newly planted can cause problems. In the EU Member States, *Cms* is under strict statutory control (Anonymous 1993). A seed potato lot designated as contaminated by the pathogen is prohibited from subsequent planting. Normally, a sample of 200 tubers is taken per 25 tonnes of potatoes for laboratory testing. The recommended detection test allows a reliable detection of *Cms* at an incidence of higher than 1.5% infection level. It means that there is an 8.7% chance that *Cms* will be detected in a 200 tuber sample assuming that one infected tuber is in the sample which is a representative of the total lot (van der Wolf *et al*. 2005).

According to our results, the incidence of infected volunteers' daughter tubers ranges between 0 and 35%. Assuming that: (*i*) healthy seed tubers were planted at a location and they produced healthy daughter tubers, e.g. 20 t/ha containing more than 666 000 tubers with an average weight of 30 g/tuber; (*ii*) at the same location volunteer plants are present in a density from 100 to 2000/ha and they produce 300 to 6000 tubers; (*iii*) about 4% of the daughter tubers of volunteer plants are infected; (*iv*) a sample size of 200 tubers containing a mixture of pathogen-free tubers and infected tubers produced by diseased volunteer plants is taken; (*v*) the testing scheme according to EC Directive 93/85/EEC (Anonymous 1993) to detect *Cms* is used. With these conditions taken into account, the incidence of infected tubers in such a mixed sample is below the *Cms* detection threshold.

It can be concluded that under the conditions of the Czech Republic: (*i*) infected volunteer plants and infected daughter tubers do not constitute an important source of dissemination of the pathogen in the crop during the growing season; (*ii*) the incidence of infected daughter tubers of volunteer plants at the location where *Cms* had been detected in samples of harvested potatoes one or two years before is evidently too low to be detected using the test scheme pursuant to EC Directive 93/85/EEC; (*iii*) volunteer plants undoubtedly contributed to the survival of *Cms* in the potato production system under the detection threshold in spite of strict statutory control measures; (*iv*) to a certain extent, the existence of diseased volunteer plants at a low incidence explains why eradication of *Cms* in a certain contaminated area is hardly possible when using the current diagnostic methods with limited sensitivity of *Cms* detection in potato seed samples (Table 3).

This paper presented the results of *Cms* detection performed on samples of tubers at five terms that differed by the length of storage time. The DAS ELISA test was used for the detection of *Cms* in tuber samples. It is obvious that the optimal time for the detection of the pathogen in tubers fell into the period between 4 and 10 weeks of storage at 22°C. It reveals that the probability of detecting latent infections in a lot of potatoes is dependent not only on such key parameters as the size of the sample, the incidence of infection and the sensitivity of the diagnostic method, but also on when the samples are tested. This has practical implications. The effort to provide growers, exporters and importers of seed potatoes with the results of testing for the presence of *Cms* as soon as possible after harvest can have a negative consequence. Some samples with latent infection can be officially identified as negative, although if testing of the same sample had been done some weeks later after keeping it at higher temperature it could be confirmed as positive. According to Kaemmerer *et al*. (2006), there is any neither increase nor decrease in the number *Cms* cells in diseased potato tubers during five months lasting storage at controlled temperature 4°C (eventually at temperature between 2°C and 16°C in the cellar).

The real aim of BRR management is to prevent the spread of *Cms*. To achieve this, the most important control measure is using zero tolerance of *Cms* in the framework of the seed potato certification system, although this step does not guarantee absolute freedom from *Cms* in tested seed potato lots. However, this control method

is effective enough to prevent economically important losses in the potato industry. Further, our results show that the risk of infected volunteer plants and infected tubers for the next potato crop is not epidemiologically important. Therefore, other strict statutory cropping restrictions for the next 3 years (or longer) from the start of the next growing season in a field where *Cms* is confirmed (Anonymous 1993) do not seem to be justified and should be abolished.

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Received for publication July 15, 2007
Accepted after corrections October 18, 2007

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