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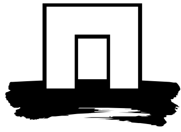

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Table of contents

page

Preface 1

Summary 3

1. Introduction 5

1.1 Bacterial ring rot as quarantine disease 5
1.2 The bacterium 5
1.3 Diversity 5

2. Damage and economic losses 7

2.1 Symptoms on tubers 7
2.2 Symptoms in the field 7
2.3 Factors influencing disease development 8
2.3.1 Introduction 8
2.3.2 Temperature 8
2.3.3 Moisture in soil 8
2.3.4 Light input 8
2.3.5 Soil composition 8
2.4 Economic losses 9

3. Virulence factors 11

4. Dissemination 15

4.1 Via seed potatoes 15
4.2 In soil 15
4.3 Via surface and drain water 15
4.4 Via insects and nematodes 16

5. Survival 17

5.1 On materials and equipments 17
5.2 In soil 17
5.3 In potato volunteers 18
5.4 In weeds and non-host plants 18

6. Management of bacterial ring rot 21

6.1 Introduction 21
6.2 Inspection and testing 21
6.2.1 Visual inspections 21
6.2.2 Sampling 22
6.2.3 Detection and Identification 23
6.3 Prevention of infections 26

page

6.4 Hygiene measures 27
6.4.1 Chemical disinfection 27
6.4.2 Physical control 28
6.4.3 Biological control 28
6.5 Use of resistant cultivars 28
6.6 Current EC legislation 28

References 31

Conclusions and recommendations for control 37

Preface

The quarantine organism *Clavibacter michiganensis* subsp. *sepedonicus (Cms)*, the causative agent of bacterial ringrot in potato, poses a constant threat to potato growers in Europe. *Cms* causes yearly an economic damage of an estimated 15 million euro, due to eradication campaigns and compensation to growers. Despite intensive inspection and testing programmes a full eradication has not been established.

Within the EU FAIR programme (PL98-4366), from 1999 – 2003 epidemiological studies were performed, to improve measures for control and eradication of Cms. By studying factors affecting survival and dissemination of *Cms* in the potato ecosystem, we have enlarged our knowledge for practical measures to be taken by EU Plant Health Authorities and farmers.

In this literature survey, we integrate results from our four-years project with data from literature on *Cms* and give recommendations to eradicate the organism, to prevent its establishment and to avoid dissemination.

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Summary

Introduction

Bacterial ring rot in potato is caused by quarantine pathogen *Clavibacter michiganensis* subsp. *sepedonicus.* In the field, it causes wilting, chlorosis in the interveinal spaces and necrosis, which starts at the margins. As the disease progress the plant can collapse. Tubers develop characteristic ring rot symptoms. When tubers are cut and squeezed, milky droplets can erase which sink down upon relief. As disease progress, the vascular tissue becomes soft cheesy in texture. Disease development is dependent on the susceptibility of the cultivar and favored by high temperatures at the end of the growing season. Infections remain easily unnoticed when leaf is destructed to avoid virus infection or masked by other diseases or natural senescence of the foliage.

In Europe, the economic damage caused by direct crop losses is low, but costs due to rejection of infected seed lots, for control measures and by loss of export markets are high. When seeds are damaged by cutting or by using picker type planters, the infection percentage can be up to 80%. Reports of findings came from 31 countries distributed over five different continents. Fortunately, in The Netherlands and Scotland, the main seed producing areas in Europe, *Cms* has no or only sporadically been found.

Relative little is known on the pathogenicity factors of Cms. For full virulence cellulase, a cell-wall degrading enzyme, needs to be produced. Also proteins that induce a hypersensitivity response in non-hosts are required for systemic infection of the pathogen. If extracellular polysaccharides (EPS) play a role in pathogenicity is still unclear.

Epidemiology

Infected seed lots are the main source of (long distance) dissemination of Cms. *Cms* cannot penetrate an intact tuber peel and needs natural openings or wounds to enter. During activities that damage potato tubers, such as cutting of seed tubers, presprouting, planting, harvesting, grading and storage handlings *Cms* can be easily spread. Potentially, *Cms* can also be disseminated by contaminated insects, that create wounds, such as aphids and the Colorado beetle, although their role in the epidemiology is unclear.

There are no indications for plant-to-plant dissemination via soil. *Cms* can survive for ca. 10 days in surface water. During this period population densities will decrease drastically by dilution and dying of bacteria. The chance that *Cms* may be spread via irrigation by surface water is considered as low, unless a weed growing in or along waterways is found, which can host and multiply Cms.

Cms can persist for long periods (> 2 years) on surfaces of different materials, including iron, wood, rubber, plastic and rubber. Survival is strongly favored by a low relative humidity of 10% and a temperature below 10 °C.

Cms is not considered a soil resident. Only at low temperatures survival periods exceed for one year, but as soon as the temperature increases to 15 °C *Cms* can survive only for a few weeks. Persistence is favored by a low moisture content, although temperature seems to be the major factor. Survival is not or only little affected by repeated freezing and thawing. Survival is little dependent on the type of soil (clay, peat, sand). Different attempts to infect potato tubers by growing them on a Cms-infested soil have failed.

Cms has a limited host range which is restricted to potato, tomato and egg plant and some solanaceous weeds. *Cms* causes disease problems only in vegetatively propagated potatoes, but not in tomato and egg plant, which are propagated via true seed. *Cms* cannot colonize *Solanum dulcamara* (bittersweet) and *S. nigrum* (black nightshade), two important solanaceous weeds in Europe. After stem inoculation, *Cms* can induce disease symptoms in rape *(Brassica napus*) and stinging nettle (*Urtica dioica*) although population densities were low in symptomatic plants. *Cms* can maximally persist for some weeks at low levels in a number of stem-inoculated weeds and crops, but cannot invade and colonize plants. *Cms* cannot persist in these plants after root inoculations.

Control

In all EU member states the quarantine pathogen *Cms* is under strict statutory control laid down in council directives 2000/29/EC and 93/85EEC, aiming to prevent entry and spread of *Cms* and eradicate it from the EU. Exclusion of

Cms from a region relies heavily on the availability of efficient measures to inspect and test potatoes, the use of healthy seed and there handling under strict hygienic measures.

Field inspections are important to monitor crops, but will not be able to detect latent infections. Symptoms often occur late in season and may remain unnoticed by haulm destructions. Symptoms can also be masked by other diseases or natural senescence.

EC Directive 93/85/EEC recommends for the laboratory test a sample size of 200 tubers. This sample size is normally taken per 25 tonnes of potatoes and allows a reliable detection of ring rot ($P<0.05$) at an infection incidence of higher than 1.5%. It is obvious that freedom of a potato lot from *Cms* can not be guaranteed on the basis of sampling and testing alone. In an outbreak situation there may be occasions when it will be necessary to undertake more intensive sampling and testing of individual stock.

The current testing protocol starts with a prescreening of *Cms* with immunofluorescence cell staining (IF). IF-positive samples are injected in stems of eggplants for growth and enrichment of the pathogen. Eggplant samples are plated on a semi-selective medium and colonies with a typical morphology are plated to pure cultures. *Cms* is characterized to full identity with a number of techniques, including PCR, fatty-acid profiling and a pathogenicity assay on eggplants, from which *Cms* is reisolated in case typical symptoms are observed.

Cms is genetically, serologically and biochemically relatively homogeneous. The main variation between strains is the amount of extracellular polysaccharides (EPS), the bacterial slime produced. Serological assays based on polyclonal antibodies will react with nearly all *Cms* strains, but show cross-reactions with other bacteria found in tuber extracts. Monoclonal antibodies have been produced with a higher specificity. Those suitable for ELISA, based on detection of EPS, will not react with non-mucoid strains. For detection and identification of *Cms* in tuber extracts also reliable molecular techniques have been developed, including an *in situ* hybridisation technique, in which cells are stained with specific DNA probes and different type of nucleic acid based amplification techniques ((real-time) PCR and NASBA). Amplification techniques are still prone to false-positive results by cross-contamination and false-negative results by inhibiting compounds after extraction of plant samples. Under the current revision of the detection scheme, PCR will be allowed in the screening fulfilling special requirements which ensure a reliable amplification.

The most effective means to prevent bacterial ring rot is through exclusion of the pathogen from the potato production system. Only certified seed should be used, selected from a place where ring rot is known not to occur. Potatoes cannot be guaranteed free of Cms, but intensive and repeated surveillance increase the confidence in a ring rot free area status.

A strict on-farm hygiene is the next most important ring rot control measure. In particular, equipments and machineries that are shared or hired, should be carefully disinfected, for example with glutaraldehyde, hypochlorite, quaternary ammonium-, iodophore based-, peroxygen- and phenol compounds. Activity of disinfectants can be reduced by organic materials and soil. A heat treatment for 5 min at 82 °C was found to kill the pathogen effectively too. Control of volunteers is another important hygiene measures. Waste dumps can be a source of infection and should be avoided. Effective control of insects is important to circumvent initial infections. Crop damage during culturing practices such as disease- and pest control, fertilization and irrigation should be avoided as much as possible to avoid spread of the disease. Potato cultivars, resistant to *Cms* are not available and the use of tolerant cultivars is not recommended as they will mask infections.

If infections are found, EC Directive 93/85/EEC aims to prevent further spread of *Cms* and eradication of the pathogen. Therefore a whole range of measures must be taken. If an infection is confirmed an extensive trace back of the origin and a trace forward of possible spread has to be done. Infected potato lots and lots designated as probably contaminated are prohibited for planting. All plant material, machineries and other objects that have been in contact with the infected lots should be considered as potentially contaminated. Machineries and equipments have to be cleaned and disinfected. Zones will be demarcated based on identification of potentially contaminated areas. Extensive measures must be taken on the farms for several years, concerning growing plants or potatoes, use of seed, surveys and cleansing and disinfection.

1. Introduction

1.1 Bacterial ring rot as quarantine disease

Bacterial ring rot (BRR) is caused by the plant pathogenic bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (Spiekermann & Kotthoff, 1914) Davis *et al.*, 1984 (Cms). The disease was reported for the first time by Appel (1906) after an outbreak in Germany. Spieckermann (1913) introduced the name 'Bakterienringfäule' whereas the pathogen was described for the first time in 1914 (Spieckerman & Kotthoff, 1914). The pathogen has been described under the following names: *Corynebacterium sepedonicum, Bacterium sepedonicum, Aplanobacter sepedonicum, Phytomonas sepedonica, Mycobacterium sepedonicum* and *Pseudobacter sepedonicum*. The name *sepedonicus* means causal organism of rot.

C. michiganensis subsp. *sepedonicus* is listed as an A2 quarantine pest by EPPO (OEPP/EPPO, 1978). It is considered of quarantine significance throughout the Old World, for example by APPPC and IAPSC, but also in the New World (COSAVE, JUNAC). Several seed-potato-producing countries in the EPPO region are free from the pest, and also Mediterranean countries exporting ware potatoes towards the north. While the direct economic impact of ring rot may only be moderate, especially with modern production systems, it would constitute a major extra constraint on seed potato production in countries where it does not occur, with considerable indirect effects on trade.

1.2 The bacterium

Cms is a Gram-positive bacterium and is morphologically and biochemically very similar to the other Gram positive Clavibacter michiganensis subspecies able to cause plant diseases. *Cms* is a pleomorph, slightly club-shaped short rod of about 0.5 – 1.0 µm in size. *Cms* contains a cell wall with predominantly peptidoglycan, based on diamino-butyric acid. It has a high G/C content of c. 70%. In immunofluorescence microscopy it is often seen in L or V formations resulting from 'bending division'. Sometimes coccoid (round) shaped cells are observed in infected plant extracts, but after growth on an agar medium, short rods are found again (Slack, 1987). De Boer (PEI, Canada, pers. comm.) observed these coccoids frequently in fresh isolates from egg plants. *Cms* is aerobic, but grows slowly at anaerobic conditions too; colonies become visible after five days and remain small. Colonies are creamy or light yellowish of color; they are smooth and often mucoid. *Cms* do not form spores and cannot resist high temperatures.

1.3 Diversity

Cms is relatively homogeneous, certainly in comparison to *C. michiganensis* subsp. *michiganensis* (Cmm), the causal organism of bacterial canker in tomato. Some diversity is found on agar plates, where a variation is seen in pigment formation and colony morphology. Colonies can be creamy to light yellowish and dry, mucoid and fluidal. Growth rate of dry strains is often reduced both on plates and in planta. Diversity is also seen in cell morphology (see 1.2).

In BOX-PCR, a genetic finger printing, based on the use of primers against repetitive sequences dispersed over the total bacterial genome, *Cms* strains form a tight group with a similarity over 80% (Smith *et al.*, 2001). BOX-PCR did not distinguish dry from fluidal strains, indicating that small genetic differences are responsible for the rate of fluidity. Studies with amplified fragment length polymorphism (AFLP) revealed a higher level of heterogeneity for *Cms* than found in BOX-PCR, but were more closely related to each other than to other subspecies of Clavibacter (Stead, CSL, unpublished results). No relation was found between colony appearance and finger printing pattern.

Also in fatty acid methyl esterase (FAME) analysis *Cms* forms a tight group which even overlaps with profiles of other subspecies within the species (Henningson & Gudmestad, 1991). Little variation in the serology of *Cms* is found too. Polyclonal antibodies generated against whole cells and a monoclonal antibody selected against a cell wall component reacted in immunofluorescence antibody-staining procedures with all strains tested (De Boer & Wieczorek, 1984). A monoclonal antibody against a soluble component, suitable for use in ELISA failed to react with dry non-mucoid strains, due to the absence of extracellulair polysaccharides (De Boer *et al.*, 1988).
BioLog, however, revealed a variation in the utilization patterns of different carbon sources for 22 *Cms* strains tested. This variation resulted in misidentification of *Cms* at even species level of between 21-55%, dependent on the BioLog database used (Van der Wolf, unpublished results).

2. Damage and economic losses

2.1 Symptoms on tubers

Tubers develop a characteristic ring rot. In the early stages of symptom development, before tissue maceration starts, the vascular ring becomes glassy and the vascular tissue at the heel end starts darkening. Later, when tubers are cut and squeezed, milky droplets of bacterial slime are erased. Thereafter the vascular tissue becomes creamy yellow and cheesy in texture. Ultimately the entire tuber can rot (Fig. 1). The rot is odorless. As rot progresses, surface cracks and dark blotches immediately beneath the periderm may become visible. Secondary infections can mask typical ring rot symptoms. Recently in Europe several cases have occurred where the central cortex rots at the same time as the vascular ring.

Figure 1. Bacterial ring rot symptoms in an early (left) and an advanced stage (right).

2.2 Symptoms in the field

In the early stages of symptom development on growing potato plants, leaves start to wilt and are slightly rolled at the margins (Fig. 2). The interveinal spaces of the leaves become light green to pale yellow. As the disease progresses, leaves become necrotic; necrosis starts at the margins (De Boer & Slack, 1984). Finally the total plant can collapse. Certain cultivars, such as Russet Burbank, can develop dwarf-rosettes early in season.
When leaves are heavily infected with Cms, necrotic lesions starts to develop, which are only expanded in susceptible potato cultivars, but not in resistant ones, due to a hypersensitivity response (Romanenko *et al.*, 2002).

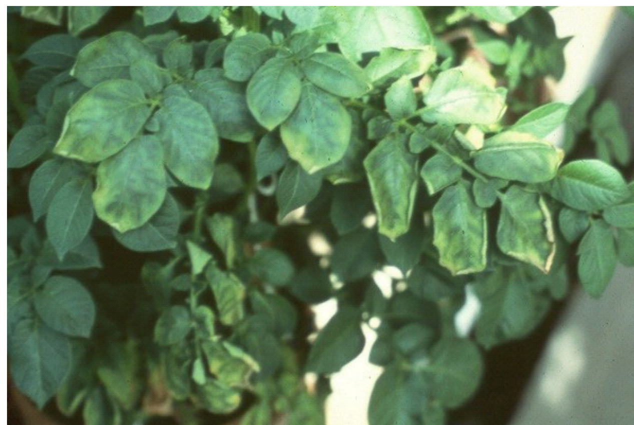

Figure 2. Leave symptoms: leavesshowing slightly rolled margins.

2.3 Factors influencing disease development

2.3.1 Introduction

In experiments to factors influencing disease development different methods for inoculation have been used. These have been found successful as long as *Cms* is transferred into the vascular tissue, indicating that for an induction of disease symptoms the bacteria has to enter the vascular tissue.
In Northern America and Western and Northern Europe first stem- and tuber symptoms are generally observed 90-100 days from planting. In tolerant potato cultivars (e.g. cv. Desiree) symptom expression is much less frequently observed than in susceptible varieties (e.g. cv. Hansa). Cell densities in stems of tolerant and susceptible cultivars are largely similar, but in tubers of tolerant cultivars cell densities are often lower (Hukkanen *et al.*, in press; De Boer *et al.*, 1992). The presence of high cell densities of *Cms* in tolerant cultivars emphasis the risks of using highly infectious tolerant cultivars with which high populations of *Cms* can be disseminated unnoticed. High densities in stems of tolerant and resistant cultivars make it worth considering to sample stems at the end of the growing season rather than tubers, although sampling of stems will be more laborious.

2.3.2 Temperature

Whereas low temperatures are favorable for survival of Cms, higher temperatures stimulate disease development. Symptom expression in greenhouse experiments occurred faster at 22-35 °C than at 16-18 °C or at 4 °C (Eddins, 1939). In experiments in climate chambers, wilting due to *Cms* infection was found earlier at a temperature regime of 24 °C for 24 h than at a regime of 24 °C for 12 h and 5 °C for 12 h (Bishop & Slack, 1982). After 45 days, the cell densities in the stem, however, were not different. In particular, high temperatures in August induce symptom development (cited from Manzer *et al.* (1987), unpublished data). Foliar symptoms generally do appear after flowering in midseason. In most places in Europe, it is unlikely that bacterial ring rot is often seen in seed potato production, because foliage is destructed relatively early to prevent virus transfer by aphids.
Also relative high soil temperatures favored disease development. Tubers were inoculated with *Cms* and planted in soil kept at constant temperatures of 16, 19, 21, 25, 28, 31 and 34 °C. Symptoms were induced most rapidly at 25 °C (Logsdon, 1967). However, Sherf (1944) found the most rapid disease development at soil temperatures between 18-22 °C, at temperatures of 26 and 30 °C the development was delayed. Variation may be due to soil type, soil conditions and *Cms* strain.

2.3.3 Moisture in soil

A five fold irrigation of a potato field in Wyoming (USA) resulted in a faster symptom development than a similar field which was not irrigated (Dykstra, 1941). In one experiment, irrigation resulted in a higher percentage of BRR infected plants (2-3 times higher), but not in another experiment. Irrigation may stimulate rapid transport of *Cms* through the vascular system of the plants.

2.3.4 Light input

Symptom development was delayed at a high total light input of 103 W.cm^{-2}.h compared with a lower light input of 45 or 74 W. cm^{-2}.h (Nelson & Kozub, 1983). It is likely that light has an indirect effect, via the physiology of the plant. The symptom development relative late in season is possibly related to this light dependency.

2.3.5 Soil composition

Abiotic factors in soil, such as composition of minerals and nutrients, have an effect on disease development, but interactions have not been carefully analyzed. High concentrations of phosphor and particularly nitrate decreases plant resistance.

2.4 Economic losses

Reports of findings came from 31 countries distributed over five different continents (Stead & Wilson, 1996). The disease has never been found in Australia. In Europe, the disease was found for the first time in Germany in 1906 and in North America in Quebec (Canada) in 1931. In 1940, BRR was present in all important potato-producing districts in Canada and the USA, indicating that without appropriate precautions the pathogen is rapidly disseminated via seed. In Europe, BRR is predominantly present in Scandinavia and Germany. Fortunately, in the Netherlands and Scotland, the two main seed potato production area's in Europe is rarely found. In Scotland the disease is not present, whereas in The Netherlands between 1996 and 2003 only 7 infected seed lots were found.

Within North America and the European Union, *Cms* is considered as a quarantine organism for which a zero-tolerance is established. A single infected tuber results already in big economic damage. *Cms* can cause damage in three different ways: 1. by direct crop loss during growth and storage, 2. by rejection of infected seed lots and the costs for the control measures, 3. by loss of export markets or by experiences difficulties to open new markets. For example, in recent history, Canada and USA had difficulties to export seed potatoes to Europe because of bacterial ring rot.

In the forties, in Canada and the USA, the direct damage by crop loss and by rejection was considerable. In particular the use of picker planters and the practice of cutting seed were largely responsible for the high incidence of BRR. The lack of adequate detection methods for seed testing hampered control of the disease in these years. In Maine (USA), in 1939 and in 1940, respectively 11.5 and 7.5% of all certified seed lots were rejected due to BRR (Baribeau, 1948). During surveys in Ottawa (Canada) from 1943 to 1947, 9-12% of the farms were infected, which covered 6-12% of the inspected area (Richardson & Goodin, 1949). In Quebec, in 1944 the disease was present in c. 16% and in 1945 in c. 9% of all lots of potatoes tested.
In the forties, incidentally, seed lots with BRR symptoms were rejected in the USA with an infection percentage up to 80% (Eddins, 1939; Kreutzer & Mclean, 1943). From latently infected tubers, still 1.5% BRR diseased tubers were harvested.

It is claimed that all statutory control measures have cost more than the actual yield losses (Stead & Wilson, 1996; De Boer, 1987). However, the direct damage caused by BRR should not be underestimated. In 1978 in the USA still 60% of the total rejected seed production areas were rejected due of BRR and between 1982 and 1985 between 0 and 15%, with an average of 4% (Slack *et al.*, 1979; De Boer, 1987). It is known that before symptoms are visible, tuber yield (number and weight) of tubers of diseased plants can be considerably lower than in healthy plants (Karjalainen, Helsinki, unpublished results).

Only by intensive, well-organized eradication campaigns, BRR infections can be reduced. After an intensive campaign, the percentage infections in Canada dropped down from 5.1% in 1983, to only 0.04% (1 latent infection) in 1992 (De Boer, 1999).

3. Virulence factors

Virulence factors are molecular species produced by bacteria during infection that are necessary for full virulence. They can have a variety of functions, such as allowing multiplication and spread of the pathogen, invasion of host cells, evasion of or resistance to host defense systems. They also can be responsible for symptom formation, which may play a role in the epidemiology of the pathogen, enhancing spread from plant to plant. Although in comparison to a number of Gram negative plant pathogens, our knowledge of molecular mechanisms of virulence for *Cms* is limited, roles have been established for two different pathogenicity factors: the extracellular cellulase and the hyper-sensitive response inducing protein, and these are described below in more detail. Mention will also be made of two other possible pathogenicity factors, EPS and *pat-1*, although neither has been shown to play a role in virulence for *Cms*.

Cellulase

Many pathogens produce lytic enzymes as virulence factors. These break down host tissues, and this usually removes mechanical barriers, allowing invasion of the host by the pathogen. In addition, the monomers produced by the breakdown of macromolecular plant polymers can often be used as carbon sources by the bacteria. Test with *Cms* showed that wild type strains produce an amylase (M. Metzler, unpublished data) and a cellulase. The cellulase was cloned from *Cms* using the cellulase from the related species *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*; Meletzus *et al.*, 1993) as a hybridization probe. The *Cms* gene was found to code for a protein of over 700 amino acids, and was very similar to the *Cmm* protein (>90% identity; Laine *et al.*, 2000).

Enzyme activity tests showed that the cellulase is produced by nearly all wild-type strains (M. Laine & M. Metzler, unpublished observations). Chemical mutagenesis was done on wild type strains, and various mutants were isolated that no longer produced cellulase. Of these, several were shown to grow to wild type levels *in vitro*. When inoculated into eggplant, these mutants produced reduced symptoms, suggesting that cellulase is important in virulence (K. Lehtilä & M. Metzler, unpublished data). However, mutants produced by chemical mutagenesis can have mutations in other loci that the one of interest, and such secondary mutations could account for the reduced virulence. Therefore, we used an unusual, naturally-occurring strain, P45, which was isolated from ring rot infected tubers in Canada many years ago and does not express cellulase activity. This isolate also was found to be nearly non-virulent on eggplants. The cloned cellulase gene was transformed into this strain, causing it to express the cellulase, as demonstrated by activity tests. Inoculation of the transformed P45 into eggplant gave significantly enhanced disease symptoms, demonstrating that the cellulase is an important virulence factor for *Cms* (Laine *et al.*, 2000).

The cellulase itself warrants special mention. It is a large protein, of about 750 amino acids, which consists of three different domains. This is very normal for cellulases, many of which have been described, and which very often consist of more than one domain. The largest, N-terminal domain of the *Cms* cellulase is clearly catalytic, responsible for the hydrolysis of the cellulase polymer to dimeric subunits. The next, middle domain is a cellulose binding domain (CBD). CBDs have been shown in other systems to substantially enhance cellulase activity, probably by helping the catalytic domain bind to the substrate. Both of these functions can clearly be ascribed to the different subunits based on their amino acid sequence similarity to related cellulase catalytic domains and CBDs. However, the final, C-terminal domain of *Cms* cellulase is completely unlike other cellulase-associated proteins, showing most similarity to expansin proteins (Laine *et al.*, 2000). Expansins are plant proteins that are highly expressed in the cell walls of growing plant cells (Cosgrove 1998). It has been shown that expansins allow the expansion of plant cell walls during plant growth. Although the molecular mechanism is not understood, it appears to act as a kind of molecular grease, allowing the cellulose microfibrils to slide past each other during plant cell growth. The role of this expansin like domain in the *Cms* cellulase is not known, however, studies on the cellulase from the related species *Cmm* shows that interruption of the expansin like domains eliminates virulence in that pathogen, although the catalytic and CBD domains are left intact (Jahr *et al.*, 2000). Thus the third domain may be generally important in virulence for other *Clavibacter michiganensis* subspecies.

Hypersensitive response inducing protein

When a pathogen invades a host, it moves systemically through the host and causes disease symptoms. However, when a pathogen invades a non-host, it can trigger a resistance reaction that results in the hypersensitive response (HR). In the HR, the invaded tissue undergoes programmed cell death. The plant cells then collapse and die. This has the effect of preventing spread of the pathogen. Although the resulting necrotic lesions may appear similar to disease symptoms, the HR actually signifies that the plant has successfully defended itself from invasion by the pathogen, which is restricted to the site of invasion and does not spread systemically.

The hypersensitive response had been previously observed for *Cmm* when infiltrated into leaves of the non-host plant *Mirabilis jalapa*, and this ability had been suggested to correlate with virulence of the strain tested (Gitaitis, 1990). Therefore, numerous laboratory strains of *Cms* were inoculated into eggplant and potatoes, and a number of non-virulent strains were identified, that is, strains that did not produce symptoms on host plants. Virulent strains were found to produce a hypersensitive response on tobacco, which is a non-host plant for *Cms*. When non-virulent strains were inoculated into tobacco, no hypersensitive response was observed. Thus, virulence on host plants and the ability to induced the hypersensitive response on a non-host was found to correlate (Nissinen *et al.*, 1997).

Although these results suggest that, like gram-negative plant pathogens, *Cms* may encode a harpin and therefore have a *hrp*/type III pathogenicity island, repeated efforts to identify such a locus using reverse genetics, hybridization and PCR techniques have not been successful (M. Metzler & S. Beer, unpublished data). Additionally, although there are a number of gram-positive pathogens for which the entire genome has been sequenced, for none has a type III pathogenicity island been identified, including the actinomycetes *M. tuberculosis* and *M. leprae*, which are fairly closely related to *Cms*. For these reasons, it appears unlikely that *Cms* contains a type III locus.

Respective roles of cellulase and hypersensitive response inducer in pathogenicity of Cms

A non-virulent, non-HR inducing strain, Cs4, was studied further to elucidate the role of HR induction ability in pathogenicity. When inoculated into eggplants, this strain does not cause symptoms and survives but does not multiply. In contrast, the cellulase-minus strain P45, which also does not cause symptoms, does multiply in planta, to levels close to wild type. When the two strains are simultaneously inoculated into eggplants, symptoms are produced at near wild type levels. Although amounts of Cs4, as determined by immunofluorescence cell-staining, were not increased by co-inoculation, expression of cellulase by this strain appears to have enhanced symptom expression (Nissinen *et al.*, 2001). Based on this data, it appears that cellulase expression is important in symptom expression, but that the ability to induce the hypersensitive response on a non-host correlates with ability to multiply in planta.

Other potential pathogenicity factors

Extracellular polysaccharides have been shown to be important pathogenicity factors in some plant pathogens. *Cms* usually has a highly mucoid phenotype, so mucoidy has been considered as a potential pathogenicity factor for *Clavibacter*. In *Cmm*, EPS plays no role in symptom formation, as fully mucoid strains that do not produce cellulase colonize host plants without difficulty, although they do not produce wilt symptoms (Bermpohl *et al.*, 1996). However, non-mucoid isolates do not colonize plants well. Informal laboratory observations with *Cms* demonstrate no obvious correlation between mucoidy and virulence, as some highly virulent strains have a completely dry, non-mucoid phenotype (M. Metzler, unpublished observations).

For *Cmm*, another pathogenicity factor has been identified. This is the product of the gene called *pat-1*, and encodes a protein with apparent similarity to serine proteases (Dreier *et al.*, 1997). The vital role of *pat-1* in pathogenicity has been firmly established for *Cmm* (Dreier *et al.*, 1997), but it has not been cloned from the *Cms* genome. However, the *Cms* genome project shows that a *pat-1* locus exists in the *Cms* genome (R. Nissinen, pers. com.). Whether *pat-1* is important in virulence for this subspecies remains to be established.

In 2004, the complete genome of Cms has been sequenced. Cms has a genome of 3.5 Mb and ca. 3000 genes (Ishimaru, personal communication). The presence of a circular plasmi (pCS1) and a linear plasmid (pCSL1) has been confirmed. The current annotation indicates that several homologs of the pathogenicity (*pat-1*) locus from Cmm is present, although the genomic structure is very different between Cmm and Cms. The sequence information will be very helpful in identification of genes involved in pathogenicity and unravelling the host pathogen interaction.

4. Dissemination

4.1 Via seed potatoes

Infected seed is the main source of dissemination of Cms. Within a potato farm, *Cms* is rapidly spread by seed cutting, which is still a common practice in the USA and in some countries in Europe. If seed is contaminated, seed cutting results generally in a high disease incidence (Starr, 1940; Dykstra, 1941; Dykstra, 1942; Metzger & Binkley, 1940). For example, cutting of seed with a homogeneous tuber infection of only 0.1% will result in 1.5% of diseased plants (Starr, 1943). In comparison with the use of whole seed, cutting of infected tubers increased the infection rate from 23% to 72% (Starr, 1940). Contaminated knives will even infect the 10th sliced tuber (Starr, 1940).

Cms seems not able to penetrate the intact periderm, but according to some authors infection can occur via the eyes of undamaged tubers (Starr, 1949; Dykstra, 1941; Dykstra, 1942). Incubation of intact tubers in a suspension of *Cms* incidentally resulted in infected tubers (Dykstra, 1941; Dykstra, 1942). Others did not find any infection via eyes of intact tubers and even not of tubers from which the peels were carefully pulled off.

Recent studies in Germany indicated that in case of direct contact between healthy and infected tubers, planting of the so contaminated tubers resulted in an infected progeny, both under controlled and field conditions (Kakau *et al.* 2004).
Infection can also occur during mechanical planting. In the USA, the use of picker planters has resulted in high disease incidences in the field (Raeder, 1949; Dykstra, 1941). But also with cup-type planters, commonly used in Europe, *Cms* can cause tuber to tuber transmission. In particular, if excessive sprouting is allowed, injuries easily occur, exposing the sprout vascular tissue of healthy tubers to the inoculum from infected tubers during planting. An excessive sprouted seed lot (> 10 mm) spiked with 4% of heavily infected tubers resulted finally in ca. 37% infected plants. A clean sprouted seed lot planted immediately after the spiked seed lot, without disinfection of the planter, resulted in ca. 6% infected plants. A seed lot with short sprouts (< 5 mm) spiked with 4% heavily infected tubers resulted in ca. 9% infected plants, showing the effect of the sprouting. Disinfection of the planter with Bezalconium chloride and glutaraldehyde effectively removed the infective inoculum, and resulted in a healthy crop.

4.2 In soil

Most studies indicate that plant-to-plant dissemination in the field plays no role in the epidemiology of BRR (Dykstra, 1941; Dykstra, 1942; Mansfeld-Giese, 1997). In field experiments in Denmark, in which infected and non-infected tubers were planted adjacently, only at a very low frequency transfer via roots was observed (Mansfeld-Giese, 1997). If plants were separated by a subsurface barrier, no transfer of *Cms* was found at all.

Transfer of *Cms* from soil to potato was only described in one report, where spreading of the pathogen was found in a part of a potato field, in which drainage of the soil was poor (Iverson & Kelly, 140; Dykstra, 1941). In field experiments in Main (USA), a relative high percentage of apparently healthy tubers planted adjacently to infected tubers became infected (Bonde, 1939). In the same report it was described how in pot experiments in which pathogen-free and infected plants grown together free from insects, some pathogen-free tubers became infected. It was suggested that infection occurred during watering of the plants. However, from none of the older studies it can be excluded that the 'healthy' tubers were latently infected.

4.3 Via surface and drain water

To assess the risks for dissemination of *Cms* via surface water and infection of potato crops by irrigation (Van der Wolf & Van Beckhoven, 2004). *Cms* was able to survive for a maximum period of 7 days in non-sterile surface water at 10 °C, a period during which *Cms* can be transported over long distances, but will also be strongly

diluted. It is concluded that contamination of surface water with *Cms* can pose a threat on potato production only if aquatic host plants can multiply *Cms* in high densities.
Survival of a fluidal and non mucoid strain was also studied in sterile ditch water and simulated 'drainage water', in sterile MilliQ water, in tap water, in physiological salt and in artificial xylem fluid. In addition, the influence of temperature and low oxygen conditions on persistence of *Cms* in some of these diluents was studied. A maximum survival period of 35 days was found for *Cms* in sterile tap water at 20 °C, independent of the strain used. In the other diluents survival periods ranged between 0 and 21 days. Relatively poor survival was found in MilliQ water and artificial xylem fluid. Low temperatures of 4 °C do not favour survival as it does in soil. Oxygen depletion affected survival detrimentally.

Survival periods determined by agar dilution plating and a direct viable counting method, based on the use of indicators for esterase activity and membrane integrity were similar. Therefore it was concluded that under the experimental conditions studied, *Cms* did not form cells in a viable but non culturable state.

4.4 Via insects and nematodes

Although several insects have been identified as a vector of Cms, the actual significance of insects in the epidemiology of *Cms* is unclear. It is expected that transfer via insects maximally occurs at a low incidence, but it may be the source for some unexplained and unexpected outbreaks. No data are available on the persistence of *Cms* in insects.

The colorado beetle (*Leptinotarsa decemlineata*) and the green peach aphid (*Myzus persicae*), but not the aster leaf hopper (*Macrosteles fascifrons)*, were shown to be relatively efficient vectors of *Cms* (Christie *et al.*, 1991). One colorado beetle was able to transfer *Cms* in two hours to a pathogen-free potato plant after feeding for 2 hours on an infected plant. The green peach aphids transmitted *Cms* in 48 h after feeding on an infected plant for 48 h.
In another study, the colorado beetle, the grass hopper (*Melanopulus differentialis*) and the black blister beetle (*Epicauta pennsylvanica*) were able to transfer *Cms* from infected to pathogen-free plants (List & Kreutzer, 1942). In this study, the period in which the insects were fed on the plants was not given.

It is unknown if *Cms* transmitted by insects to pathogen-free potato stems or leaves can be transferred to daughter tubers. The numbers of cells that are transmitted are in general insufficient to cause ring rot symptoms.

The gumming diseases of grasses, caused by *C. toxicus, C. iranicus* and *C. tritici* are transmitted predominantly via seed gall nematodes (Anguina spp.). For *Cms* and other subspecies of *Clavibacter michiganensis* no data on transmission via nematodes are available.

5. Survival

5.1 On materials and equipments

Cms is relatively resistant to desiccation and survives well on many surfaces of materials, in particular at a low humidity. Survival periods of over two years were found on iron (corroded and varnished), wood rubber, plastic and cotton, at a humidity of ca. 10% at 5 °C. Survival is favored at a low temperature, the relative humidity is the dominant factor.

Survival periods are slightly strain dependent. Unexpectedly, non-mucoid strains seem to survive for longer periods than fluidal strains. At low temperatures, *Cms* from natural inoculum (rotting tuber tissue) persisted longer periods on different materials than cells grown on agar media. The opposite was true at high temperatures, probably by microbial competition of saprophytes present in natural inoculum.

Abdel-Kader *et al.* (2004) proved that Cms could be transmitted to seed potatoes by the use of contaminated crates. Contamination of healthy seed occurred even if crates were used, which were contaminated and stored for 5 months at 4 °C prior to use.

5.2 In soil

Cms is not really a soil resident. Infection of potato tubers from infested soil by this non-motile organism is therefore unlikely. Nelson (1979) reported a maximum survival period of 278 days in a silt loam kept at a wilting point and at temperatures of 0 °C or -10 °C. At 20 °C and field capacity, *Cms* survived only for 6 days.

Also from experiments in which tubers of susceptible potato cultivars were planted in fields from which ringrot infected tubers were harvested in previous season indicated a limited survival time. Bonde (1942) has found never ring rot infected tubers in seven subsequent years from 2-8 fields from which in previous year a heavily contaminated crop was harvested. Also in additional field experiments, in which rotten tubers were ploughed through different soils, thereby covering a field plot with straw during winter time, never ring rot infections were detected in next crops. In North Dakota, Dykstra (1941, 1942) has never found symptomatic ring rot infected tubers in three subsequent ring rot-free crops planted on fields from which a heavily infested soil was grown. In the first year, (partly) rotten tubers were harvested from this field. Dykstra, however, did not check tubers for presence of (viable) cells by a Gram-staining or dilution plating. Eddins (1939) could not find ring rot infections in a total of 1200 plants from seed planted in a field from which 64% ring rot infected tubers were harvested. In Ottowa, freshly cut seed was planted in a field in which in previous season (autumn) (partly) rotten tubers were ploughed through the soil. No ring rot infected tubers were harvested (Dykstra, 1942). Only when large numbers of tubers were stored during winter in pots with infested soil *Cms* could be detected in a low frequency via a bioassay (Eddins, 1939).

Recent studies using more sensitive detection methods (immunofluorescence colony-staining with antibiotic resistant mutants) showed that *Cms* could survive under dry and cool conditions for longer periods than reported previously (P. Müller, BBA, Kleinmachnow, Germany, unpublished results).The organism was able to survive in four soils at 4 °C at 50% water holding capacity for one year. Only in light loamy sandy soil *Cms* could not persist for more than 2 month. Persistence at temperatures below zero was even better. The non mucoid and intermediate strain could still recovered after 413 days from loam, held at 50% field capacity and at – 5 °C. The survival at fluctuating temperature (-10 / 0 / +10 °C) was dependent on the water holding capacity. At the permanent wilting point *Cms* could survive for a longer period than at field capacity. Survival at higher temperature (15 and 20 °C) was very short. Living bacterial cells could not be recovered at 20 °C after 15 days and was decreased drastically at 15 °C after 15 days.

Cms can survive for more than 25 years in sterile sand (S.H. de Boer, PEI, Canada, unpublished results), indicating that *Cms* can survive for long periods by turning down metabolic activity to almost zero. Long survival periods were also reported by Sneisko & Bonde (1943), although Dykstra (1942) found a maximum time of 4.5 month in sterile soil at 3 °C. Short survival periods in natural, non sterile soil at higher temperatures may be explained by starvation due to activation of cell metabolism. Alternatively, the presence of predators and antagonists in soil or by the presence of moisture may also result in cell death. However, survival of *Cms* in different types of soil (clay, peat or sand), which vary largely in microbial composition, was largely similar, indicating that starvation and not antagonism or predation is the major factor in cell death.

5.3 In potato volunteers

Cms can readily maintain in potato volunteers were they pose a threat for a next potato crop (Bonde, 1942). Plants and daughter tubers of infected volunteers, however, will not necessarily be infected with *Cms* (Haasis, 1940).

5.4 In weeds and non-host plants

Cms has a narrow host range, which seems to be restricted to potato, eggplant and tomato, these are all solanaceous plant species (Larson, 1944). During crop production, however, *Cms* causes disease problems only in potato, but not in eggplant and tomato. This may be explained by the efficiency in which transmission of *Cms* occurs by vegetatively propagated seed potatoes, whereas eggplant and tomato, propagated via true seed, transmit *Cms* less efficiently. Some reports indicate that after stem inoculation, *Cms* can induce symptoms in other solanaceous weeds. Knorr (1948) reported symptoms after stem inoculation of the following plant species: *Althenaea sp., Lycopersicon esculentum* (tomato*), S. antipoviczii, S. ballsii, S. chacoense, S. citrullifolium, S. commersonii, S. corymbosum, S. demissum atypicum, S. endlicheri, S. fendleri, S. jujuyense, S. melongena, S. mammosum, S. pampasense, S. parodii, S. radicans, S. tequilense, S. tlaxcalense, S. vavilovii, S. verrucosum, S. warscewiczii and S. tuberosum.* In this survey, no symptoms were found 2 months after stem inoculation of the following plant species: *Ainsodus luidus, Apium graveolens, Atropa belladonna, Beta vulgaris, Brassica napus, Brassica oleracea, Brassica rapa, Browallia americana, Capsicum annuum, Cuscuta sp., Datura metel, Datura meteloides, Datura quercifolia, Datura stramonium, Datura tatula, Daucus carota, Helianthus, Hyoscyamus albus, Hyoscyamus niger, Lacatuca sativa, Lupinus luteus, Lycium halimifolium, Lycopersicum humboldtii, Nicandra physaloides, Nicotiana acuminata, Nicotiana angustifolia, Nicotiana bigelovii, Nicotinana cerinthoides, Nicotiana chinensis, Nicotiana glutinosa, N. longiflora, N. multivalvis, N. noctiflora, N. quadrivalvis, N. repanda, N. rustica, N. sanderae, N. sylvestris. N. tabacum, Nierembergia hippomanica, Pelargonium zonale, Petunia violacea, Phaseolus vulgaris, Physalis angulata, Physalis alkekengi, P. aequata, P. heterophylla, P. lanceolata, P. longifolia, P. virginiana, Pisum sativum, Salpiglossis sinuata, Saracha procumbens, Schizanthus pinnatus, Schizanthus sinuata, S. wisetonensis, Soja max, Soja hispida, Solanum acaule, S. aculeatissimum, S. antigenum, Solanum balbisii, S. crolinense, S. ciliatum macrocarprum, S. dimissum, S. dulcamara, S. gilo, S. guyanense, S. indicum, S. neoantipoviczii, S. nigrum, S pyracanthum, S. pseudocapsicum, S. rostratum, S. spinosissimum, S. triflorum, S. tripartitum, Spinacia oleracea, Trifolium pratense, Vicia fab.* Slack (1987) listed the following plant species as a (potential) alternative host, but not indicating how plants were inoculated and tested: *L. pimpinellifolium, L. racemgerum, S. cardiophyllum* and *S. integrifolium*.

The potential role of alternative hosts in the epidemiology of *Cms* was recently assessed for crops and weeds growing in Europe (Van der Wolf *et al.*, submitted). Ten crops grown in rotation with potato, including maize, wheat, barley, oat, bush bean, broad bean, rape, pea and onion and five cultivars of sugar beet were tested by stem- and root inoculation. Six weeks after stem inoculation, *Cms* could be detected in low densities in all crops except onion and sugar beet, although densities were low in immunofluorescence cell-staining (IF) (< 2.10^6 cells per g of plant material). Cms was successfully reisolated from IF-positive stems of maize, bush bean, broad bean, rape and pea, but not from IF positive stems of the other crops. All root-inoculated plants were IF negative. Some stem-inoculated rape plants (Brassica napus) showed wilting symptoms.

Bugbee *et al.* (1987), isolated Cms from symptomless sugar beet roots. More detailed studies showed an endophytic association of Cms with sugar beets and the presence of Cms on sugar beet seed (Table 6, Bugbee & Gudmestad, 1988). In our studies Cms did, however, not colonize roots and stems of five different cultivars of sugar beet (Van der Wolf, unpublished results). Other research groups were not able to isolate *Cms* from sugar beet seed too (S.H. de Boer, PEI, Canada pers. com.; K. Mansfeld-Giese, Slagelse, Denmark, pers. comm., Pastrick *et al.,* 2004). De Boer stem-inoculated 50 sugar beet plants and found only three plants with low densities of IF-positive cells. All 50 egg plant stem inoculated plants, which served as a positive control, were *Cms* positive and showed symptoms. Sugar beet seeds sown on *Cms* infested soils resulted in IF negative plants.

Fourteen solanaceous plant species, nine weed species found along water streams and thirteen weed species commonly found in potato fields were tested as hosts of *Cms* by root- and stem inoculations (Van der Wolf, unpublished results). *Cms* did not persist well in *S. nigrum* and *S. dulcamara*, two *Solanum* species widely distributed in Europe. In *Urtica dioica* and *Solanum rostratum*, *Cms* was found in high densities through the whole plant, where it caused wilting, chlorosis or leaf necrosis. In many of the other plants, *Cms* could persist for four to eight weeks at low densities. In particular stinging nettle, growing along water edges may potentially serve as a source for Cms, releasing bacteria from infected roots into surface water. *Solanum rostratum* is the scientific name of buffalobur nightshade (synonymous to Kansas thistle, prickly nightshade) and is commonly found in disturbed and waste areas throughout large parts of North America. It is a drought resistant, aggressive weed, abundant around buffalo wallows and toxic for cattle (Bassett and Munro, 1986).

Zizz & Harrison (1991) analyzed weeds from a *Cms* infested potato field in the USA and found 14% of the plants of *Solanum saccharoides* to be infected with Cms, but no plants of *Chenopodium album* and *Amaranthus retroflexus*. In Europe, in two subsequent years, 43 weeds belonging to nine species were collected from ring rot infected field plots and 17 weeds belonging to 6 species from non-infested field plots (Van der Wolf, unpublished results). Plants were tested for the presence of *Cms* by dilution plating and AmpliDet RNA, an amplification method which detected specific 16S rRNA sequences of Cms. Only from infested field plots *Cms* infected weeds were collected. *Cms* was isolated from six *Elymus repens* plants with roots grown through rotten potato tubers. These plants may play a role in long term survival of Cms, if *Cms* can persist in *Elymus repens*. From the other weeds ca. 30% of the plants were positive in AmpliDet RNA, but negative in IF, indicating that possibly plants have been infected at a very low density.

The results of these studies indicate that in general weeds and crops do not play an important role in the epidemiology of bacterial ring rot in Europe. *Cms* can persist only for short periods in low levels in stems of non-hosts, but not in roots. The solanaceous weeds *S. nigrum* and *S. dulcamara* can be considered as non-hosts for Cms. The potency of stinging nettle and rape to host *Cms* needs further study. In Northern America *S. rostratum* may play a role in long term survival and dissemination of Cms.

6. Management of bacterial ring rot

6.1 Introduction

In all EU member states *Cms* is under strict statutory control laid down in Council Directives 2000/29/EC (Anon. 2000) and 93/85/EEC (Anon., 1993). These have the aims of preventing entry of the pathogen into the EU and, following any outbreaks, to prevent further spread and eradicate it from the EU.

At the moment there is no method of direct chemical or biological control available. Breeding for resistance has produced some (mainly) tolerant cultivars which are not much used (Manzer *et al.*, 1987; Manzer & McKenzie, 1988). In fact, certification schemes in the USA have prohibited the use of tolerant cultivars such as BelRus because they can act as symptomless carriers of the pathogen. The most important components of the control strategy are maintenance of areas for production of *Cms*-free seed following strict testing schemes (Nelson, 1984) and sanitation measures (Lynch *et al.*, 1989).

Ring rot can occur at low levels in potato production systems, and can cause latent infection of tubers. So phytosanitary measures aimed at potato consignments only are quite inadequate. Measures have to be aimed at the whole production system, at the material from which potato consignments are derived and at the place or area of production; for seed potatoes, in particular, they involve a series of multiple checks, each of which is considered by itself insufficient.

6.2 Inspection and testing

The most important mode of transmission of *Cms* is undoubtedly in infected potato tubers. Exclusion of *Cms* from a country or area therefore relies on the production and use of healthy seed potatoes which includes the availability of efficient measures to inspect and test potatoes (particularly seed potatoes) for the presence of the bacterium, even in symptomless latent infections. These measures must be applied to prevent the possibility of entry of infected potatoes to a *Cms*-free area or place of production. Furthermore, they must be applied during regular surveys of seed and ware potatoes so that the distribution of the pathogen can be accurately determined across all production areas at national and international levels. The reliability and transparency of such measures is fundamental to successful ring rot control.

6.2.1 Visual inspections

Although visual inspection is an important part of control, it cannot be used alone to monitor freedom of any crop from *Cms* due to the long periods of latency in both tubers and foliage. Visual inspection of growing crops is less reliable than inspection of harvested tubers since foliar symptoms often occur late in the season may remain unnoticed when leaf is destructed to avoid virus infections. Symptoms may also be masked by symptoms of other diseases or general senescence of the foliage. In addition, foliar symptoms may be restricted to stunting or reduced tuber number and are not specific to ring rot. The fact that expression of ring rot symptoms varies with initial inoculum density, environmental conditions and cultivar differences is particularly troublesome for field inspectors. Although some cultivars may often express symptoms when infected, others may never or only very seldom show symptoms or may only do so under specific environmental conditions. Finding symptomatic plants and tubers can speed up diagnosis, since it is usually easier to isolate the pathogen from such material.

6.2.2 Sampling

To overcome the problems associated with detection of latent infections it is necessary to conduct laboratory testing of tubers. The probability of detecting latent infections in a lot of potatoes is a function of the sample size and the incidence of infection. For pathogens such as *Cms* for which there is a zero tolerance, the desire to achieve statistically optimal sample sizes and frequencies must invariably be balanced with the practicality and cost-efficiency of sampling and subsequent testing. Sampling strategy (random sampling, sample size and frequency) is therefore a key issue in the success of both certification schemes and pathogen distribution surveys. The sample size recommended in EC Directive 93/85/EEC is 200 tubers of potatoes).

The probability of detecting the target organism in relation to the incidence of infected tubers in the sample unit has been investigated using ring rot as a case study (Clayton & Slack, 1988; De Boer, 1994). Assuming that (1) infected tubers are uniformly distributed amongst the population, (2) that sampling is completely random and (3) that the detection test used will always detect one infected tuber in the sample, then the probability of detecting the pathogen (PD) in a 200 tuber sample is shown in Table 6.1.3.1. In practice, infected tubers are usually quite rare within a lot and a new statistical calculation based on the Poisson-distribution may therefore be more appropriate.

Table 6.1.3.1. Probability of detection of ring rot in a 200 tuber sample at varying infection rates.

Incidence of infection (%)	PD
5.0	0.99995
2.0	0.98168
1.5	0.95021
1.0	0.86466
0.5	0.63212
0.2	0.32968
0.1	0.18127
0.05	0.09516
0.02	0.03921
0.01	0.01980
0.005	0.00995
0.002	0.00399
0.001	0.00200

Using the same assumptions, the effect of sample size on detection of *Cms* is shown in Table 6.1.3.2.

Table 6.1.3.2. Effect of sample size on probability of Cms detection at given infection rates.

Sample size (tubers)	1% infected tubers	0.1% infected tubers
	PD	PD
200	0.865	0.181
400	0.982	0.330
1000	0.999	0.632
4000	0.999	0.982

Furthermore, a statistical optimum sample size (n) can be determined at various probabilities of detection (PD) and different infection rates (Table 6.1.3.3).

Table 6.1.3.3. Statistically optimum sample sizes for different rates of infection.

PD	Level of infection in the sample unit (%)			
	1.0	0.5	0.1	0.05
0.99	460	921	4605	9210
0.95	300	600	2996	5992
0.90	230	460	2302	4604
0.50	70	140	700	1400

Finally, the probability of detection (PD) of a contaminated sample is directly related to the number of tubers taken from the lot (Table 6.1.3.4).

Table 6.1.3.4. Probability of detection of Cms depending on the number of tuber taken from the lot.

No. of tubers sampled	PD at 0.1%	PD at 1%
200 tubers	0.18127	0.86466
400 tubers	0.32968	0.98168
800 tubers	0.55067	0.99995
1200 tubers	0.69881	0.99999
1600 tubers	0.79810	1.00000

From the above it is obvious that freedom of a potato lot from *Cms* can not be guaranteed on the basis of sampling and testing alone. However, sample sizes can be optimised in order to maximise the probability of detecting the organism. In a compromise between the statistically optimum sample size and the practicality and cost of sampling and testing, EC Directive 93/85/EEC requires that testing is based on a 200 tuber sample. This gives an 87% chance of detecting a 1% level of infection, assuming that the sample is representative of the total lot. Much larger samples however need to be tested to detect low levels of infection in stocks and to give a high level of assurance that ring rot is not present in a stock. Recent work in Belgium (Lebrun, personal communication) has shown that up to 20 samples of 200 tubers had to be taken and tested to detect a very low level of infection in a large stock with less than 1% infection rates. In an outbreak situation there may be occasions when it will be necessary to undertake more intensive sampling and testing of individual stocks. For example, a larger sample size is recommended when screening potentially infected seed stocks or when investigating the primary source of an outbreak.

6.2.3 Detection and Identification

The current test scheme, as normally used in the EU member states , is shown in Fig. 6.1.4.1. The testing procedure is intended for detection of latent *Cms* infections in potato tubers. A positive result from at least two screening tests, based on different biological principles, must be complemented by the isolation of the pathogen; followed by, in case of isolation of typical colonies, identification of a pure culture as *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*). The procedure follows the requirements of EC directive 93/85/EEC (Anon. 1993). This scheme is currently under review to ensure that sensitivity and specificity of detection are optimized whilst the test

methods remain sufficiently robust to ensure that they can be used to equal effect across all EU plant health laboratories. To ensure that this is the case, harmonized protocols have been validated and fully evaluated in ring tests, involving the key EU laboratories.

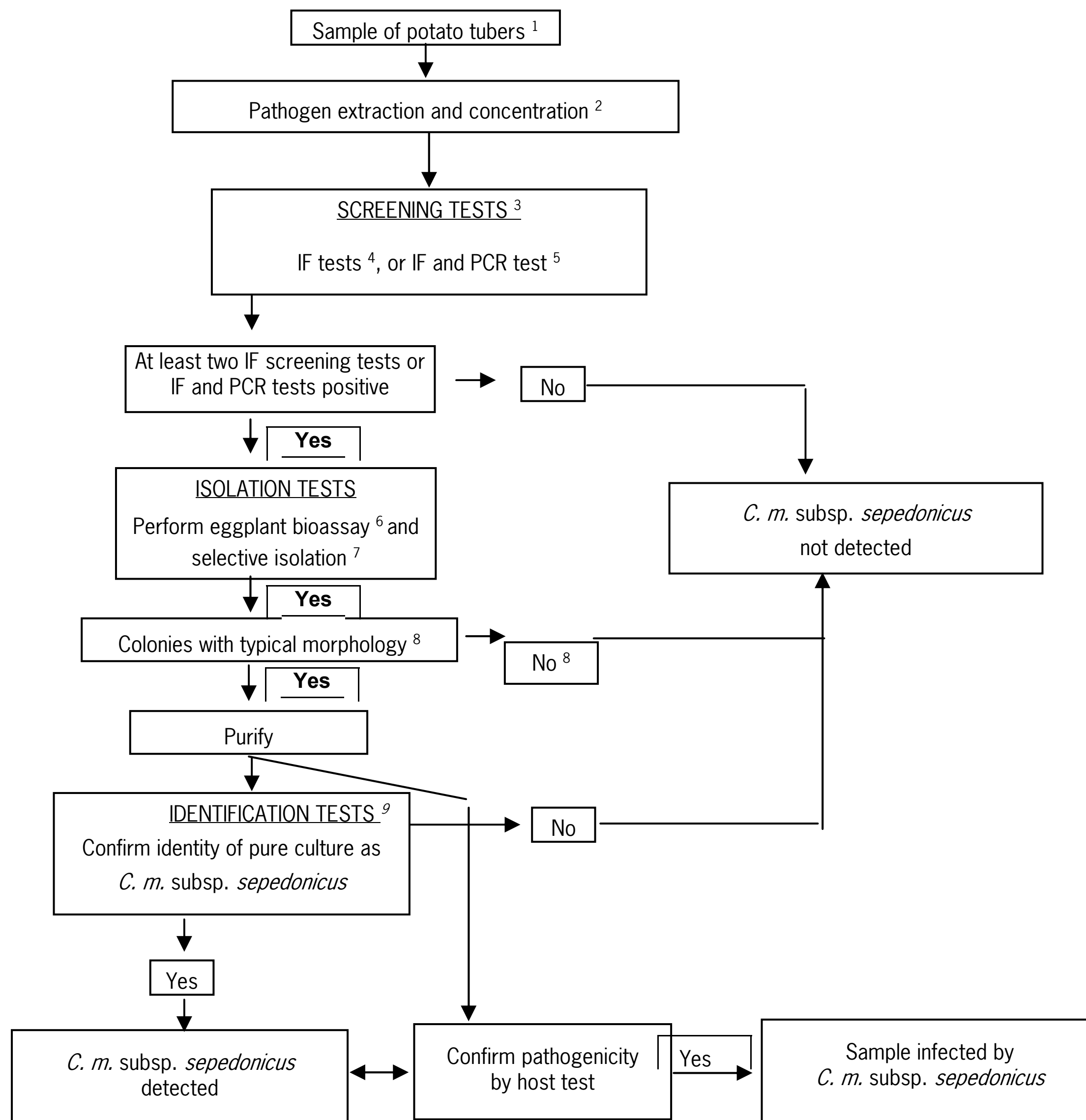

Fig 6.1.4.1. Scheme for detection and identification of Clavibacter michiganensis subsp. sepedonicus in samples of potato tubers.

Footnotes

1 The standard sample size is 200 tubers.

2 Cores containing vascular tissue are removed from the tuber heel ends processed and the resulting pellet is re-suspended in 1.5 ml 10mM phosphate buffer.

3 Two screening tests, preferably based on different biological principles (antibody and DNA technology) are performed.

4 The immunofluorescence (IF) test involves staining the target bacterial cells with specific antibodies to which are bound fluorescent markers (Janse & van Vaerenbergh, 1987). Selective binding of the antibodies to the cell walls of the ring rot bacteria allow them to be observed under UV microscopy. Two separate assays are used which incorporate either polyclonal or monoclonal antibodies. The pleomorph cells and the existence of coccoid shaped cells (apart from rods) may be confusing in IF microscopy.

5 A polymerase chain reaction (PCR) assay is performed as an additional confirmatory screening test. The PCR test involves highly specific and sensitive amplification and detection of DNA sequences (from ribosomal RNA gene targets) which are highly specific to the ring rot bacterium. This allows detection of potential false-positive IF results which sometimes occur due to non-specific binding of the antibodies to bacteria other than the ring rot bacterium.

6 Following positive results in screening tests, a bioassay in eggplants (*Solanum melongena*) is used to assist in the isolation of the ring rot bacterium. This involves injection of the potato extract pellet into the stems and incubation under quarantine

glasshouse conditions at 18-24 °C for up to 28 days. During this period, the plants are observed for the development of typical wilting symptoms and isolation of the bacterium from symptomatic and asymptomatic plants is attempted.

[7] A selective isolation medium (MTNA) is used for isolation of the ring rot bacterium from inoculated eggplants as well as directly from serial dilutions of the potato extract pellet (Jansing & Rudolph, 1998). Typical colonies can be obtained 3-7 days after plating but the slow-growing pathogen is easily overgrown by faster-growing saprophytic bacteria. It is therefore often more effective to isolate the bacterium after selective enrichment in the inoculated eggplants.

[8] Because of the fastidious nature of the ring rot bacterium it is sometimes difficult to obtain a pure culture of the pathogen. In such cases, following positive IF and/or PCR results, the stock should be resampled (if necessary more intensively) and the whole process repeated with a view to obtaining a pure culture.

[9] Once a culture of the bacterium has been obtained and purified, it is identified e. g. using IF-test, a combination of specific PCR assays, whole-cell fatty-acid profiling and a pathogenicity test on eggplants which involves injection of the pure culture into the stems, incubation at 18-24 °C, observation of typical symptoms and re-isolation of the organism from the diseased plants.

The key screening test is an immunofluorescence (IF) test which involves staining the target bacterial cells with specific polyclonal antibodies to which are bound fluorescent markers. Selective binding of the antibodies to the cell walls of the ring rot bacteria allow them to be observed under UV microscopy. To minimise the possibilities of false positive results, two separate assays are used which incorporate polyclonal or monoclonal antibodies to different antigens. Following a positive IF screening test, it is necessary to confirm results by isolating and identifying the causal organism. The pathogen is slowly growing and notoriously difficult to isolate on artificial growth media. To overcome this problem, a bioassay in eggplant seedlings is performed in which the sample is injected into the stem (15-25 seedlings), which are then incubated at around 21 °C for up to 28 days. Selective multiplication of the pathogen *in planta* then facilitates eventual isolation of the pathogen which can then be purified and identified. The eggplant bioassay is a slow test with timescale of up to 6 weeks and is often seen as an inconvenient bottleneck in confirming diagnoses.

Non-specific serological reactions sometimes lead to false-positive IF results Verification of an IF result can take several weeks when using the eggplant bioassay. New modifications to the official EC test scheme are therefore being introduced to include a compulsory second rapid screening test, based on DNA technology, which can help to identify false positive IF results within 24-48 hours. These involve either a polymerase chain reaction (PCR) assay (Pastrik, 2000) or a fluorescent *in situ* hybridisation (FISH) assay (Van Beuningen *et al.*, 1995). The PCR assay involves amplification and detection of a specific DNA sequence from the intergenic spacer region of the 16S–23S rRNA genes of *C. michiganensis* subsp. *sepedonicus*. It also incorporates an internal PCR control as a safeguard against false negative results which can sometimes occur due to the presence of PCR inhibitors in soil or potato tissue. The FISH assay also involves a fluorescently-labelled DNA probe from the 16S rRNA gene which selectively stains the pathogen cells and allows them to be detected microscopically. The use of either of these tests improves the reliability of screening so that potato consignments are only held when there is a high probability that they will be proven to be infected following the eggplant bioassay and subsequent pathogen isolation and identification.

New developments in diagnostic methods for *Cms* have recently been validated through ring tests in the various EU plant health laboratories (http://www.csl.gov.uk/science/organ/ph/diagpro/clavibacter.pdf). These include the use of a real-time quantitative PCR assay (Schaad *et al.*, 1999) which allows simultaneous pathogen detection, identification and quantitation and facilitates automation and high-throughput testing. This assay also incorporates an internal PCR control as a safeguard against false negative resulting from failure of the reaction in the presence of inhibitory compounds from plant or soil material. An improved selective medium (MTNA) is also available which may increase the chance of direct isolation of the pathogen (Jansing & Rudolph, 1998). A newly developed RNA amplification assay (NASBA) with a fluorescent detection system (Amplidet RNA) offers the potential for detection of only viable *Cms* populations but has yet to be validated by ring testing (Van Beckhoven *et al.*, 2002). Another real-time PCR assay which differentiates *Cms* from other subspecies of *C. michiganensis* has also been recently published but not yet validated (Bach *et al.*, 2003).

There have been a number of PCR assays published for detection of *Cms* (Schneider *et al.*, 1993; Firrao & Locci, 1994; Hu *et al.*, 1995; Karjalainen *et al.*, 1995; Li & deBoer, 1995; Slack *et al.*, 1996; Lee *et al.*, 1997; Mills *et al.*, 1997; Pastrik & Rainey, 1999, Pastrik, 2000). Many of these lacked specificity when tested against high numbers of isolates of related bacteria or even commonly-occurring non-related bacteria isolated from soil and tuber samples.

The most appropriate PCR assays validated (in EU ring tests) on the basis of specificity and sensitivity are those of Pastrik (2000). An additional assay in real-time quantitative PCR format (Schaad *et al.*, 1999) also performed well and has added advantages of being a less labour intensive and less prone to contamination between samples.

Reliable methods for identification of presumptive isolates of *Clavibacter michiganensis* subsp. *sepedonicus*, in addition to the conventional nutritional and enzymatic bacteriological tests (Lelliott & Stead, 1987), include immunofluorescence with specific monoclonal antibodies (De Boer & Wieczorek, 1984), whole cell fatty-acid profiling (Janse, 1991; Stead, 1992), PCR with *Cms*-specific primers (Schaad *et al.*, 1999; Pastrik, 2000), FISH (Van Beuningen *et al.*, 1995) and genetic fingerprinting using the repetitive BOX primer (Smith *et al.*, 2001). The latter method has been used to demonstrate that *Cms* isolates are genetically homogeneous despite often demonstrating phenotypic variation in colony morphology.

6.3 Prevention of infections

The most effective means to prevent ring rot disease is through exclusion of the pathogen from the potato production system. For this reason, Directive 93/85/EEC (Anon., 1993) requires that regular surveys (involving both visual inspections and laboratory tests for latent infections) are performed within the EU with the aim of clearly demarcating areas where the bacterium is present and establishing the limits of any potential spread. Furthermore, Directive 2000/29/EC effectively prohibits import of seed potatoes into the EU from third countries unless under a derogation which ensures that the requirements of Directive 93/85/EEC have been met.

The most important aspect of ring rot management for the commercial grower is to use only certified seed, carefully selected to originate in an area or place of production where ring rot is known not to occur. In all potato certification, a zero tolerance for the presence of *Cms* must be strictly applied. It is also important that the each potato lot must be clearly labelled and separated during the whole potato production procedure.

EPPO's specific quarantine requirements for *C. michiganensis* subsp. *sepedonicus* (OEPP/EPPO, 1990a) recommend that seed potatoes should only be imported from countries which can show, by surveys and tests, that they either implement an of EPPO-recommended national regulatory control system or have area freedom and operate a seed-potato production and distribution system free from ring rot. This should involve laboratory testing for latent infection by the EPPO-recommended method (OEPP/EPPO, 1990b). This can readily apply to countries where ring rot has never been recorded, but can also apply to countries where ring rot is locally present, but does not enter the particular seed-potato production system under consideration. In addition, all seed and ware potatoes should come from stock, and a place of production, found free from ring rot, and from a field inspected during the last growing season (or two growing seasons if the previous crop was also potatoes) and found free from ring rot. Finally, hygienic precautions should be taken in storage and packing houses, and packing material and containers should be new or disinfected. EPPO in fact proposes that countries may take even stronger measures; prohibition of seed and/or ware potatoes from countries where *C. michiganensis* subsp. *sepedonicus* occurs; requirement of proof by survey that the bacterium does not occur in a country.

Since latent infections must be detected, laboratory testing is an essential component of regulatory control. However, it is important to note that potatoes can not be guaranteed free from the ring rot bacterium on the basis of visual inspection or testing of samples for presence of the bacterium. Nevertheless, repeated surveillance over a number of years can increase the confidence in ring rot free area status and hence reduce the risks of spread of the bacterium. The reliability of such a scheme is heavily dependent on the vigilance of the industries and plant health authorities and the intensity of surveillance and testing throughout the EU.

Although the risk of pathogen introduction via ware potatoes is lower than for seed potatoes, it has to be considered. There have been regular findings in EU member states of ring rot in ware potatoes in some member states.

6.4 Hygiene measures

In addition to a supply of seed potatoes guaranteed free from *Cms*, the next most important ring rot control measures concern the maintenance of strict on-farm hygiene. Ring rot outbreaks have continued to occur in North America, Scandinavia and elsewhere, despite concerted efforts to eradicate it from seed multiplication schemes. The pathogen is known to survive long periods in dried slime from diseased potato tubers (Nelson, 1980) and it is thought that unexpected outbreaks most probably arise through transfer of *Cms* via shared or hired equipment used elsewhere on infected crops. Since actual infection rates arising from such contamination can be quite low, it can be several generations until the disease builds up to detectable levels or symptoms are observed. The importance of this mode of transmission may therefore have often been overlooked.

Control of volunteer potatoes (groundkeepers) is also a key control measure. The bacterium can survive several generations in volunteers (Gudmestad, 1994) , which are therefore an important source of inoculum. The pathogen can spread from groundkeepers to healthy tubers by direct contact or via contaminated surfaces during harvesting and grading, although direct transmission through the soil during the growing season is thought to be unlikely (Mansfeld-Giese, 1997). Waste dumps can similarly be a source of infection and should be avoided.

Directive 93/85/EEC (Anon., 1993) addresses the above issues by requiring disinfection of all machinery, grading equipment, stores, storage trays, boxes and packing materials , as well as boots and clothing (if applicable) which may have come into contact with infected or probably infected potatoes. Furthermore, any other crops which may have come into contact with infected potatoes or contaminated machinery or surfaces are designated as 'probably contaminated' and are therefore also subject to official control measures (see 6.6).

6.4.1 Chemical disinfection

A number of disinfectants have been shown to be effective against *Cms* under experimental conditions. Other limitations, such as safety and corrosiveness, may affect the choice of disinfectant to be used. Gluteraldehyde, quaternary ammonium and iodophore-based disinfectants all killed *Cms* within 10 minutes at recommended rates (Dinesen, 1984). Secor *et al.* (1987) showed that most disinfectants (in a range of 28) could effectively eliminate *Cms* from machinery surfaces given a contact time of at least 10 minutes. They noted that the efficacy of hypochlorite and iodine-based disinfectants was reduced by the presence of organic material. Easton and Nagle (1985) showed that aqueous sprays of copper 8-quinolinolate controlled *Cms* on contaminated wood, polyurethane foam, and urethane rubber surfaces found on potato seed-handling equipment in 6 yr of trials. Poschenrieder and Lohweg (1992) demonstrated that the activity of quaternary ammonium disinfectants (Mennoter Forte, Orbiplant Standard or Florasept) was less effective in the presence of soil than those based on organic acids (Venno Cycla 2), aldehydes (Orbiplant Spezial), phenol (Delegol) or 8-hydroxyquinoline (Albisal). Quaternary ammonium compounds at 300 and 1000 ppm inhibited *Cms* both in culture and on a metal surface (Sundheim, 1973). Of seven disinfectants tested on wood, metal and plastic surfaces, those based on iodine (Iobac P), aldehydes (Korsolin) and potassium persulphate-based peroxygen/organic acids (Virkon S) were the most effective against *Cms* (Kaponen *et al.*, 1992). At CSL in the UK, a range of disinfectants were compared to phenol in the efficiency at which they eliminated *Cms* in aqueous suspensions. According to the calculated phenol coefficients, several commercially available products were more effective than phenol when used at the recommended rates. These included disinfectants with active ingredients of chlorine dioxide (Oxysafe), dichlorphen plus alkali (Panacide), peroxyacetic acid (Clamarin 150), potassium persulphate-based peroxygen/organic acids (Virkon-S) and iodine-based sulphuric and organic acids (Fam 30). Niepold (1999) demonstrated the efficiency of percarbonic acid and peracetic acid formulations in removing viable Cms from contaminated water and sewage effluents. In Germany disinfectants for control of Cms are officially approved and registered. Currently a disinfectant based on benzoeacid is used (Kakau, 2002).

6.4.2 Physical control

As the bacterium is readily spread from infected to non-infected tubers during the cutting of seed potatoes, it is strongly recommended to avoid this practice. In cases where cutting cannot be avoided, the use of heated cutting knives, or complete cleaning and disinfection between potato stocks may reduce the risk.
Since the bacterium survives well in dried debris and the activities of several disinfectants are inhibited in the presence of organic material, washing or steam-cleaning of machinery, equipment, packing materials, storage boxes and surfaces prior to disinfection is recommended (Gudmestad, 1994). Thorough cleaning of surfaces on grading/ conveyor lines, machinery, containers, trailors and stores etc. will therefore improve contact between residual bacteria and disinfectant and also ensure that the disinfectant is fully effective. Steam cleaning itself can completely inactivate the pathogen provided that a minimum temperature of 82 °C for 5 min is maintained (Secor *et al.*, 1987). For seed potatoes only new, unused packaging material should be used.

6.4.3 Biological control

There are a few reports of studies on the biological control of Cms. Root inoculation with a *P. fluorescens* strain from the rhizosphere prevented growth of *Cms* (De la Cruz *et al.*, 1992). In addition, ring rot incidence was reduced when cut seed, infected with *Cms*, was subsequently inoculated with a strain of *Arthrobacter* (Gamard & De Boer, 1995). There is no evidence that biological control would have any value for eradication of the pathogen or under a zero tolerance production system.

6.5 Use of resistant cultivars

There are no currently available potato cultivars with immunity or useful resistance to ring rot. Several studies have shown that potato cultivars vary in their propensities to express ring rot symptoms (Sletten, 1985; Zielke & Naumann, 1987; DeBoer & McCann, 1990; Langerfeld & Bätz, 1992; Nelson *et al.*, 1992; Pastuszewska & Junosza-Kisielewska, 1992; Kawchuk *et al.*, 1998). However, much less variation between cultivars in their susceptibilities to latent infections was observed (De Boer & McCann, 1990). Fear that so-called tolerant cultivars, which tend not to develop symptoms, can act as symptomless carriers of *Cms* have led to their removal from seed certification schemes in North America (Manzer & McKenzie, 1988). The concept of cultivar tolerance to ring rot is not yet completely understood (De Boer & McCann, 1990) and little is known of the status of most commonly-grown European cultivars with respect to their susceptibility to infection and colonisation under varying environmental conditions. A recent study by Laurila *et al.* (2003) has demonstrated that an accession (PI472655) of the wild potato species *Solanum acaule* was susceptible to latent infection by *Cms* at 15 °C although it appears immune to infection at 25 °C. Temperature dependent colonisation and symptom expression in modern potato cultivars requires thorough investigation.

6.6 Current EC legislation

Council Directive 2000/29/EC (Anon. 2000) aims to restrict the movement of potatoes into the EU from third countries where ring rot, amongst other quarantine pathogens, is known to occur. Council Directive 93/85/EEC (Anon., 1993) deals more specifically with demarcation and containment of ring rot where it has been found within the EU and outlines measures to be taken towards its eradication. In both directives, the knowledge of *Cms* on disease incidence, sampling, inspection and detection strategies, epidemiology and hygienic measures is integrated into a solid package of measures to prevent introductions and further spread, and to achieve eradication. The annexes of this directive dealing with specific measures and validated methods for detection of the bacterium are currently under review.
Directive 93/85/EEC lays down measures to be taken by member states against potato ring rot, with respect to potato plants and tubers in order to:

- Locate it and determine its distribution.

- Prevent its occurrence and spread.
- If found, to prevent its spread and to control it with the aim of eradication.

Principal control measures according to EC Directive 93/85/EEC.
Annual surveys are conducted for the presence of *Cms* on tubers and plants of potato. If infections are found, possible sources and extent of spread are investigated. Actions by plant health inspectors include:
- Holding action in cases of suspect occurrences, including prohibiting the movement of consignments from which samples have been taken and results are awaited.
- Action to trace the origin of any suspect consignments.
- Investigation of the extent of risk or contamination as a consequence of clonal relations, pre- or post-harvest contact or through any production link with the suspect consignment.
- Where the organism is confirmed in potatoes on production premises, those premises, affected crops and fields, and associated machinery and equipment will be designated as contaminated.
- Potato material and associated machinery and equipment etc. known or likely to be contaminated will be subject to officially controlled disposal or cleansing and disinfection.
- On production premises designated as contaminated, specific controls will be introduced.
- Zones will be demarcated around designated premises, based upon identification of the contaminated items and/or area of land and an investigation into the probable extent of the contamination and possible risk of its spread, within which specific measures must be taken immediately and for at least 3 years after designation.
- Seed potatoes must comply with Plant Health Directive 2000/29/EC and derive in direct line from material obtained under an officially approved programme which has been officially tested and found free from the organism.
- Where ring rot is confirmed in seed potatoes, all clonally related potato stocks must be held under official notice and be tested for latent potato ring rot infection.

Further cropping restrictions on contaminated fields and other fields on the holding are applied as follows:

Cropping restrictions on contaminated fields

Either:
In the contaminated field or unit of protected cropping, for the next 3 years from the start of the next growing season (or longer if notice requirements are not complied with)
- Growers must eliminate volunteer potatoes.
- Potatoes must not be planted.
- The field or unit must be free from volunteer potatoes for at least 2 consecutive years prior to planting the first potato crop. For example, if the field is not free from potato volunteers in year 2 then the earliest that potatoes may be planted is year 5, provided that no volunteers are found in years 3 and 4.

In the first potato cropping season thereafter
- Officially certified seed potatoes must be planted and for ware production only.
- An official survey, including laboratory testing of the harvested tubers, must be carried out.

During the next potato cropping season,
- Officially certified seed potatoes must be planted for either seed or ware production.
- An official survey, including laboratory testing of the harvested tubers, must be carried out.

Or alternatively
For the next 4 years from the start of the next growing season (or longer if notice requirements are not complied with) in the contaminated field
- Growers must be restricted to bare fallow or permanent pasture (which must be kept close cut or intensively grazed).
- Growers must eliminate any volunteer potatoes.

In the first potato cropping season thereafter,

- Officially certified seed potatoes must be planted for either seed or ware production.
- An official survey, including laboratory testing of the harvested tubers, must be carried out.

Further restrictions in other fields on contaminated holdings

For the first year following confirmation of potato ring rot. In the other fields -

Either

- Growers can opt not to plant potatoes, but must take measures to eliminate volunteer potatoes.

Or alternatively

- Growers must plant officially certified seed potatoes but only for ware production, provided that they have eliminated the risks of volunteer potatoes.
- Growing crops will be officially inspected and any volunteer potato plants must be tested for the organism.
- An official survey, including laboratory testing of the harvested tubers, must be carried out.

In the second year following confirmation of potato ring rot in the other fields on contaminated holdings:

- Growers may plant only officially certified seed potatoes for seed or ware production.
- Growers must take measures to eliminate volunteer potatoes.
- An official survey, including laboratory testing of the harvested tubers, must be carried out.

In the third year following confirmation of potato ring rot in the other fields on contaminated holdings:

- Growers may plant only officially certified seed potatoes for seed or ware production.
- Growers must take measures to eliminate volunteer potatoes.
- An official survey, including laboratory testing of the harvested tubers, must be carried out.

In those production systems where complete replacement of the growing medium is possible:

- No tubers, plants or true seed shall be planted unless the production unit has been subjected to officially supervised measures to eliminate the organism and remove any potato or other solanaceous material. This must include, at least, a complete change in growing medium and cleansing and disinfection of the production unit, and all equipment. The production system must subsequently be granted approval for potato production by the official plant health service.
- Potato production shall be from officially certified seed potatoes, or from mini-tubers or micro-plants from tested sources.

On confirmation of a ring rot finding in a potato crop, notice will be served by the plant health inspector. This will demarcate the premises to be ‘a zone contaminated with *Clavibacter michiganensis ssp. sepedonicus* (Potato Ring Rot)’ and designate relevant items as ‘contaminated’ and ‘probably contaminated’. The notice will also prohibit the further planting of potato tubers designated as ‘contaminated’ or ‘probably contaminated’ and require approved disposal or use of contaminated and probably contaminated material.

Methods of disposal of any contaminated tubers or plants are:

- Incineration.
- Use of potato tubers as animal feed following boiling for at least 30 minutes – it is essential that the material is well cooked.
- Deep burial of tubers or plants
- Direct and immediate delivery of potato tubers for industrial processing to a site which operates officially approved waste disposal facilities
- some other use or disposal, provided that it is established that there is no identifiable risk of the organism spreading

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Conclusions and recommendations for control

1) To minimise the risks of entry of ring rot, phytosanitory measures have to be aimed at the whole production system. They involve a series of multiple checks, each of which is considered by itself insufficient because:
   a) Ring rot can occur at low levels in potato production systems and can cause latent infection of tubers.
   b) Symptom expression often occurs late in the season and may be masked by other diseases, natural senescence or haulm destruction.
   c) The bacterium can spread by contact with contaminated surfaces and is not always transmitted clonally via infected seed. Populations spread in this way may be low, dispersed and difficult to detect. Testing of tuber samples with current protocols (200 tubers per sample) will only trace relative high disease incidences (e.g. a 0.5% infection incidence will be detected with only 63% confidence). Larger sample sizes are required to detect low incidences with high (95%) confidence levels, and should particularly be used in investigations of potential source and spread of infection following an outbreak.

2) The most important aspect of ring rot management is the use of certified seed, preferably sourced from areas were ring rot in known not to occur.

3) If an infection is confirmed an extensive trace back of the origin and a trace forward of possible spread has to be done. Infected potato lots and lots designated as probably contaminated are prohibited for planting. All plant material, machinery and other objects that have been in contact with the infected lots are considered as potentially contaminated and need to be cleaned and disinfected.

4) Zones need to be demarcated, based on identification of potentially contaminated areas. Extensive measures must be taken on the farms for several years, concerning growing of potatoes, use of seed, surveys and cleansing and disinfection

5) After finding a ring rot infection, the recommended period of 3 to 4 years free from potatoes and potato volunteers, is considered sufficient as:
   a) Cms cannot survive in potato cropping regions under natural conditions for longer than 2 years in soil. Decline under higher temperatures (> 15 °C) and a high humidity (> 50% field capacity) is rather fast (months).
   b) The efficiency of spread of Cms from infected soil to potato tubers is low and has never been reported in practice.
   c) Cms has a restricted host range. It does not naturally infect roots and colonize weeds and crops, grown in rotation with potato.

Former publications describing sugar beet as a host could not be confirmed by four independent groups. Therefore, sugar beet is considered as safe in a crop rotation plan with potato.

6) Control of volunteer potatoes (groundkeepers) is essential as Cms can survive several generations in volunteers.

7) Waste dumps can be a source of infection and should be avoided.

8) Effective control of insects is recommended to circumvent initial infections

9) After infection of fields, it may be preferred to leave soil fallow than to grow grasses, as some indication was found that Cms may persist at a low level on roots of grasses.

10) Use of surface water for plant growth and application of pesticides does not harbor an identifiable risk as:
    a) Cms can survive only for short periods in water (max. 5 weeks) during which period densities considerably decrease. No indications of Cms cells in a viable but non culturable (dormant) state have been found.
    b) no weeds growing along watercourses have been identified as alternative hosts
    c) use of contaminated surface water in green house experiments for irrigation of potato plants in pots did not result in infection of progeny tubers.

11) The use of contaminated machines and equipment during planting, sorting, and storage carries a high risk for infection of tubers as:
    a) Cms can survive for long periods on different materials, in particular under dry conditions and at a low temperature
    b) for an effective infection Cms must enter the vascular tissue. The vascular tissue is exposed when tubers are damaged during harvest, sorting and grading, during cutting of seed and when presprouted tubers are sown.

12) Machines and other equipment and materials (boots, clothing) need to be rinsed and carefully disinfected, certainly when entering a new field. For disinfection a number of effective disinfectants are available. At least 10 minutes contact of cleaned surfaces with disinfectant is recommended.

13) Physical treatments may be used to disinfect cutting knives, although cutting of seed is not recommended. Equipments and materials should be treated with steam or water for at least 5 min at 82 ^{o}C.

14) No useful antagonists are known.

15) No potato cultivars with immunity are currently available. Cultivars with a tolerance should not be used, as they can act as symptomless carriers of Cms.