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RING ROT BACTERIUM: AN OVERVIEW OF ITS GENERAL CHARACTERISTICS, PATHOGENICITY FACTORS AND DETECTION METHODS

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ABSTRACT

The aim of this literature review is to provide an overview of recent advances in our understanding of known pathogenicity factors implicated in ring rot disease caused by *Clavibacter michiganensis sepedonicus (Cms)*. Several genetic determinants with possible functions in plant-pathogen interaction were identified. Functional analysis of genes involved in pathogenicity offered us some clues regarding the infectious process. Successful eradication of the ring rot disease relies on prevention by the exclusive use of certified, ring rot-free seed potatoes, sanitation and early detection. An important factor that contributed to the persistence of the ring rot disease in potato cultivation areas in northern Europe and North America is the occurrence of latent infections and limitation of detection technique. Early detection of the bacterium is a fundamental element of eradication program; however, screening tests do not provide the needed information about the potential pathogenicity or virulence of the detected bacteria. Recently developed molecular based detection protocols were demonstrated to be fast, sensitive and specific. Their use could improve the first line screening for ring rot in potato tubers. We anticipate that further genomic studies will allow the identification of pathogenicity or virulence markers useful in the advancement of new diagnostic tools as alternative strategies to the time-consuming pathogenicity tests.

Keywords: ring-rot bacterium, pathogenicity, detection methods.

INTRODUCTION

Clavibacter michiganensis sepedonicus (Cms) (Spieckermann A and Kotthoff, 1914; Davis et al., 1984) is a bacterial plant pathogen that produces bacterial ring rot (BRR), an important regulated disease of potato. Worldwide strict regulations have helped immensely in controlling the dissemination of the pathogen, which is considered to be the most dangerous bacterial disease affecting potato. BRR was for the first time described in 1906 by Appel after an outbreak in Germany (Franc, 1999) and drives his name from the characteristic yellow to brown necrotic discoloration of the vascular cylinder revealed by a cross section of the tuber. *Cms* is a wilt-inducing pathogen that infects intercellular spaces of the cortical and vascular parenchyma tissues and penetrates into xylem vessels and subsequently produces a systemic infection (Kado, 2010). The *Cms* host range is primarily restricted to solanaceous species (potato, tomato and eggplant and some solanaceous weeds). *Cms* affects vegetative propagated potatoes but not tomato and egg plant, which are propagated via true seed. Although tomato is considered a host upon artificial inoculation, recently a natural infection of *Cms* in tomato plants was reported (Van Vaerenbergh et al., 2016).

It was considered that *Clavibacter* genus comprises plant pathogens; however recent investigations are indicating the occurrence of nonpathogenic bacterial strains (Hahn et al., 2003; Zaluga et al., 2014; Zinniel et al., 2002).

These strains are designated as *Clavibacter* sp. (Yasuhara-Bell and Alvarez, 2015).

Strains of *Cms* have been shown to exhibit marked differences in virulence on host plants and are described as being virulent or pathogenic and avirulent or nonpathogenic.

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Virulent strains elicit a typical hypersensitive response in tobacco (Nissinen et al., 1997). Also, bioassay tests on eggplant (*S. melongena* L.) may be employed to evaluate measurable differences among pathogenic strains of *Cms* (Bishop and Slack, 1987).

Molecular investigations based on genomic fingerprinting (CHEF analysis of high-molecular weight DNA, rep-PCR) analysis revealed variations among virulent and avirulent strains of *Cms* (Brown et al., 2002; Fousek and Mráz, 2003).

The genome of *Cms* strain ATCC 33113 has been sequenced; the circular chromosome consists of 3,258 Mb and has a high GC content of 72.56%. In total 3058 coding DNA sequences were detected. The adaptation of the pathogen to the narrower niche of vascular system is indicated by the number of pseudogenes (3.4% of predicted coding sequences). Gene alterations occurred in several loci encoding for catabolic enzymes that are no longer necessary for survival of the pathogen (Bentley, 2008).

Zero tolerance regulation within the certified seed industry reflects the view that BRR is regarded as the world's most important disease of seed potatoes. Although the economic loss associated directly with the disease is low, the rejection of the infected crop and on farm control measures costs can be very high. It was estimated that it is responsible for 15 million euro of annual loses in EU alone due to eradication phytosanitary measures and compensation to producers (Pankova, 2007).

There is no evidence of type III-secretion (T3S) system in any gram-positive plant pathogen. It is presumed but has not yet been demonstrated that members of the Pat-1 family are secreted through a general secretion (Sec)-dependent pathway (Hogenhout and Loria, 2008). Currently, two factors are known to be associated with virulence of *Cms*. The cellulolytic activity and the ability to induce a nonhost hypersensitive response (HR) (Nissinen et al., 1997) are the two major pathogenicity factors described. The role of extracellular polysaccharides (EPS) in pathogenicity is still unclear (van der Wolf et al., 2005).

GENERAL CHARACTERISTICS OF *Cms* AND PATHOGENICITY FACTORS

The genus *Clavibacter* comprises corynebacterial phytopathogens that cause important economic losses in a variety of agriculturally important plants (Carlson and Vivander, 1982). Members of the genus *Clavibacter* are Gram-positive, aerobic, non-spore-forming, coryneform bacteria that were previously grouped within the genus *Corynebacterium* (Eichenlaub et al., 2006). *Clavibacter* belongs to the gram-positive and high-GC family *Microbacteriaceae* in the *Actinomycetales* (Park et al., 1993). It contains one species, *Clavibacter michiganensis* which is phytopathogenic. Based on plant host specificity and biochemical features, its subspecies are as follows: subsp. *michiganensis* (tomato canker), subsp. *sepedonicus* (potato ring rot), subsp. *insidiosus* (wilt of alfalfa), subsp. *nebraskensis* (wilt and blight of maize) and subsp. *tessellarius* (leaf freckle and leaf spot of wheat) (Jahr et al., 1999; Metzler et al., 1997). More information regarding the molecular basis of this classification is still needed (Davis et al., 1984; Carlson and Vidaver, 1982; Arthur et al., 1995; Louws et al., 1998). The genus *Clavibacter* may warrant recognition at species level, a view expressed more recently by several researchers. This modification would drive the additional benefit of enabling the formal description of saprophytic strains, as the current classification places a major emphasis on phytopathogenicity (Whitman et al., 2012).

The importance of *C. m.* subsp. *michiganensis*, *Cms*, and *C. m.* subsp. *insidious* as plant pathogens is highlighted by the fact that these pathogens are quarantine organisms under the European Union Plant Health Legislation and this is also the case in many other countries (Eichenlaub et al., 2006; Eichenlaub and Gartemann, 2011; Gartemann et al., 2003). *Cms* is an aerobic, non-sporulating, non-motile, Gram positive plant pathogenic bacterium. It is the causal agent of bacterial ring rot (BRR) in potato and was for the first time described in 1906 (Franc, 1999) by Appel after an outbreak in Germany. The bacterium was originally named *Bacterium sepedonicum* by Spieckermann and Kotthoff in 1914 and

over time it was changed to *Aplanobacter sepedonicum*, *Phytomonas sepedonica*, *Corynebacterium sepedonicum*, *Mycobacterium sepedonicum*, *Pseudobacterium sepedonicum*, *Corynebacterium michiganense* pv. *sepedonicum*. Carlson and Vivander (1982) elevated the pathovar to subspecies and the genus was modified to *Cms* by Davies et al., in 1984.

Morphologically and biochemically it is very similar to other Gram positive *Clavibacter michiganensis* subspecies able to cause plant diseases. The bacterial cells are pleomorph, mostly wedge shape with occasional rods, curved or straight, of about 0.5-1.0 μm in size, that tend to group in L or V formations according to "bending division". In infected plant extracts coccoid shape is observed. Bacterial wall consists of peptidoglycan based on diamino-butyric acid (van der Wolf et al., 2005). *Cms* isolates vary in disease-causing ability, colony morphology on nutrient rich media, production extracellular enzymes and extracellular polysaccharides, and the capacity to elicit a hypersensitive response (HR) in tobacco (Baer and Gudmestad, 1995).

Colony morphologies of *Cms* include the mucoid type, intermediate- and non mucoid-type. On complex media, the colonies appear after five days and are small, convex, smooth, glistening, pale yellow or whitish cream depending on the agar medium (Kado, 2010). It requires growth factors for *in vitro* cultivation: biotin, thiamine, nicotinic acid, histidine, purines and pyrimidines. The optimal growth temperature is 21ºC. It utilizes acetate and succinate as carbon sources, produces acid from mannitol and sorbitol and hydrolyses potato starch (Kado, 2010). By using biochemical API 50CH and API ZYM systems it has been found that *Cms* isolates from different geographic locations exhibited a high level of homogeneity in carbohydrate utilization and enzymatic activity (Palomo et al., 2010).

The full genome sequence of *Cms* (ATCC 33113) is now available (Bentley et al., 2008). It consists of a single circular chromosome (3.26 Mb), a circular plasmid pCS1 (50 kb), two linear plasmids: pCSL1 (94 kb) and pCSL2 (140 kb) totalling 3.35 Mb. The G+C content is high, ranging from 72.5% in the chromosome to 67-68% in the plasmids. The genome contains 3242 coding sequences (CDSs) (3058 CDSs on the chromosome, 67 on pCS1 and 117 on pCSL1). The plasmids are apparently related because they contain regions of homology. The biological functions of these plasmids have not been elucidated. The analysis of genome sequences revealed 106 insertion sequence elements. The increased number of pseudogenes is indicating the adaptation of *Cms* to a narrower niche. Chromosome rearrangements resulted in disruption of genes coding for catabolic enzymes, regulation and transport factors and pathogenesis-related determinants (Bentley et al., 2008).

PATHOGENICITY FACTORS

Cms is a vascular wilt pathogen; it gains entry into its plant hosts via root system, invades, multiplies and colonizes mainly the vascular tissues and causes wilting of the plant host (Kado, 2010). Host colonization and disease symptom expression have been correlated with the ability of *Cms* isolates to induce a hypersensitive response (HR) on non host plant tobacco (*Nicotiana tabacum*) and cellulase expression. It was demonstrated that successful invasion and disease induction requires both HR inducing proteins and cellulase expression (Nissinen et al., 2010).

Cms strains that are unable to induce an HR are nonpathogenic and unable to multiply *in planta*. The HR phenotype is associated with one or more proteins secreted by the pathogen, but the identity and roles of these proteins in disease development remains to be elucidated (Nissinen et al., 2009).

Genome sequencing of ring rot pathogen indicated several genetic determinants with possible functions in plant-pathogen interaction. Thus, *Cms* harbours several chromosomal and plasmid-encoded homologues (*cph* and *php* genes, respectively) of the pathogenicity (*pat-1*) locus from *C.m.* subsp. *michiganensis*. The genome of *Cms* encodes eleven members of the Chp family of serine proteases. *Cms cph-7* is probably the *C.m.* subsp. *michiganensis pat-1* gene with 82% amino acid identity (Bentley et al., 2008). It has been found to be necessary for HR elicitation in tobacco and for

virulence of *Cms* in eggplant and potato (Nissinen et al., 2009). The role of HR induction ability in pathogenicity was studied on a non-virulent, non-HR inducing *Cms* strain. *In vivo* experiments performed on eggplants did not result in multiplication of the pathogen *in planta* and disease symptom expression. These results suggest that the HR inducing proteins are essential for pathogen multiplication and subsequent host colonization. Investigations using knockout mutant *chp3*, *cph8*, generated via targeted mutagenesis showed a significant reduction in virulence *Cms* in both eggplant and potato plant. This underlines the notion that serine proteases are important for interaction of *Cms* with plants (Eichenlaub and Gartemann, 2011). Mutation in *php3* did not determined changes in virulence of *Cms* or HR elicitation. Additional members of the *chp* and *php* gene family are known in *Cms*, yet neither the roles of these genes nor their contribution to virulence of ring rot bacterium have been established. Using quantitative real-time PCR it has been showed that during BRR infection and in liquid culture, the expression of genes encoding cellulase and xylanase and of two homologues *pat* genes was up-regulated suggesting a possible involvement of several proteins in *Cms* pathogenicity (Holtsmark et al., 2008). Genome analysis showed also that ring rot bacterium has the genetic capacity to withstand low iron and oxidative stress that may be encountered during infection process (Bentley et al., 2008).

Cellulase is an important pathogenicity factor for *Cms*, being essential for symptom development (Ichim et al., 2016). Cellulase deficient mutants of *Cms* are able to multiply *in planta* but do not produce disease symptoms. Thus, cellulase activity is not required for host colonization but is essential for disease symptom expression [30]. The *celA* gene was identified on *Cms* plasmid pCS1 (Bentley et al., 2008). The gene corresponds to a secreted β-1,4-endoglucanase (CelA) that is necessary for virulence and contains a C-terminal α-expansin like domain (Laine et al., 2000).

Extracellular polysaccharides (EPS) are produced by many plant pathogenic bacteria and are generally considered a pathogenicity factor without being absolutely required for pathogenesis and without any discernible host specificity (Alfano and Collmer, 1996). Chemically they are a complex of biopolymers consisting of polysaccharides, proteins, nucleic acids, lipids and humic substances (Vu et al., 2009). EPS enables bacteria to attach to certain substrates. EPS are thought to protect free-living bacteria from a variety of environmental stresses and may aid pathogenesis by sustaining water soaking of intercellular spaces, altering the accessibility of antimicrobials or defense activating signals and blocking the xylem and thereby producing wilt symptoms (Alfano and Collmer, 1996). Sugar composition of *Cms* EPS was reported (Henningson and Gudmestad, 1993; Westra and Slack, 1992; Shafikova et al., 2006). *Cms* in culture was shown to synthesize EPSs of two types, acidic and neutral. The monosaccharides composing EPSs include glucose as major compound and mannose, rhamnose and arabinose as minor compounds. Bacterial EPSs content is known to diversify greatly according to strain and conditions of its cultivation (Shafikova et al., 2006). Some *Cms* strains synthesize EPSs in which fucose, galactose, rhamnose and ribose are present together with glucose and mannose (Westra and Slack, 1992).

The *Cms* chromosome contains four gene clusters for biosynthesis of exported polysaccharide but only two are functional. It appears that *Cms* has partially lost its capacity to synthesize EPSs because of gene disruption (Bentley et al., 2008). A clear role for EPSs has not been experimentally proven. *Cms* usually exhibit a highly mucoid phenotype, so mucoidy has been considered as a potential pathogenicity factor. However, informal laboratory observations with *Cms* demonstrated no obvious correlation between mucoidy and virulence, as some highly virulent strains have a completely dry, non-mucoid phenotype (van der Wolf et al., 2005).

Molecular tools advances may provide novel insights into mechanisms of pathogen-host interaction. Moreover, functional gene assays along with phenotype analysis could be used for identification of potential virulence effectors as well as virulence and pathogenicity markers useful in development of new diagnostic

assays that could provide information about the potential pathogenicity or virulence of the detected bacteria.

HOST RANGE

Cms has a narrow host range primarily restricted to solanaceous species (potato, tomato and eggplant and some solanaceous weeds). *Cms* affects only vegetatively propagated potatoes, not tomato and eggplant, which are propagated via true seed. *Cms* cannot colonize *Solanum dulcamara* (bittersweet) and *S. nigrum* (black nightshade), two important solanaceous weeds in Europe. *Cms* was reported to be able to colonize sugar beets, particularly the root tissue of the plant. To date, this is the only plant-pathogenic coryneform found to have an association outside a single genus of host plant (Bugbee et al., 1987; Bugbee and Gudmestad, 1988). After stem inoculation, *Cms* can induce disease symptoms in rape (*Brassica napus*) and stinging nettle (*Urtica dioica*). It persists for some weeks at low levels in a several stem-inoculated weeds and crops, but cannot invade and colonize plants.

THE DISEASE

The disease is particularly damaging in temperate areas (growth temperature optimum of ring rot bacterium is 21ºC). It has been reported in a number of EC Member States. Concerns over the pathogen continue to significantly impact global potato export markets, as states often restrict importation of seed from countries in which the disease is present (Kůdela. 2007).

Disease expression is known to be affected by several factors: potato cultivar, pathogen strain, initial inoculum dose and environmental conditions (Bishop and Slack, 1987; Manzer et al., 1987; Nelson, 1982; Nelson and Kozub, 1983). In the early stages of BRR infection of potato plants, lower leaves begin to wilt, and slightly roll at the margins. Infected leaves will appear a lighter green than healthy leaves. The interveinal spaces turn yellow. Leaves margins become brown and fragile. As the infection progresses, leaves become necrotic. By cutting and squeezing the stem just above the seed piece of an infected plant, a creamy or cheesy-white secretion may emerge. When leaves are heavily infected with *Cms*, necrotic lesions start to develop, which are only expanded in susceptible potato cultivars, but not in resistant ones, due to a hypersensitivity response (Romanenko, 2002). A few stems may show yellowing, and sometimes there are no above-ground symptoms at all. Symptoms in the tubers usually do not occur until the plant has wilted. Tubers develop a characteristic ring rot. The pathogen invades the vascular system of the tuber. Initially, the vascular ring turns glassy. As infection progresses, a milky ooze is observed when the tubers are cross cut and squeezed (stolon end). The vascular tissue becomes creamy yellow/brown and cheesy in texture. The pathogen systematically invades the vascular tissues and proliferates so that the bacterial ooze can be squeezed out from a cut end of an infected stem. The rot is odourless. The skin will form sunken areas and cracks. Secondary infections may cover typically BRR symptoms (van der Wolf et al., 2005). Often, infected potato plants do not exhibit the extreme symptoms described above. For several cultivars, visual inspections for disease symptom proved to be insufficient for detection (De Boer and McCann, 1990; Kawchuk et al., 1998; Manzer and Kurowski, 1992; Manzer and McKenzie, 1988).

LIFE CYCLE

Infected volunteer plants derived from buried infected potato tubers serves as primary inoculum (De Boer, 2008). The pathogen can persist in dried polysaccharide (“slime”) for two to five years (Kado, 2010). It does not survive for long periods of time in soil in the absence of undecomposed plant debris (De Boer, 2008). An additional inoculum source is represented by contaminated tuber storage facilities and farm equipment. They can serve as source of infection, even at temperatures below freezing (van der Wolf et al., 2005; Nelson, 1980). Potato seeds cutting operations are a major avenue by which pathogen spreads. After cutting an infected tuber, 20-30 healthy tubers may be infected

(Kůdela, 2007). During the growing season, chewing insects such as Colorado potato beetle, green peach aphid, and potato flea beetle (Stevenson et al., 2001) may transmit bacteria from infected plants to healthy plants.

DETECTION AND IDENTIFICATION OF RING ROT BACTERIUM

Cms is categorized as a quarantine organism by Council Directive 2000/29/EC. Several tests have been developed to detect *Cms* with high sensitivity and specificity. Serological assays were first tests developed, with *Cms* – specific monoclonal antibodies used in immuno-fluorescence assay (IF) (De Boer and Wieczorek, 1984) and enzyme-linked immunosorbent assay (ELISA) (De Boer et al., 1988). IF is preferred as first laboratory screening test and it is widely used in Europe for detection of bacterial pathogens in plant material for the presence of *Cms* (Nader et al., 2009). It has proven itself to be a robust, cheap and reasonably specific method. The detection limit for *Cms* with IF is near to 10^{-4} cfu mL^{-1}. Recently, a new type of polyclonal antibody that specifically detects *Cms* bacteria irrespective of their EPS level was developed (Przewodowski and Przewodowska, 2017).

ELISA has been approved for diagnosis of *Cms* in North America. Using monoclonal antibodies, this assay enables the detection limit ranges from 1×10^5 to 5×10^6 bacterial cells/ml. However, it has not yet been accepted by the EPPO as a reliable and functional detection method for *Cms*. It was demonstrated to be ineffective in the detection of nonmucoid or nonfluidal strains of *Cms* (Baer and Gudmestad, 1993; Henningson and Gudmestad, 1992).

The official testing methods on the control of potato ring rot describe standard tests for the detection of *Cms*. These tests include immuno-fluorescence (IF) testing or/and conventional polymerase chain reaction (PCR) as first screening tests (EC, 2006). For IF assays, the suspended pellet prepared from the cores of 200 potato tubers is applied to a microscope glass and fixed by heat or alcohol fixation. Fluorophore-conjugated antibodies are combined with and bind to the outer cell wall of any *Cms* bacterial cells present on the glass, and the fluorescent cells are visualized using epifluorescence microscopy. IF technique has several advantages, however its performance is influenced by the quality of the antisera and personnel training (Vreeburg et al., 2016). Non-specific reactions often generate false positive reactions. The novel modified official EC test scheme include a compulsory second rapid screening test based on DNA technology, which can help identify false positive IF results (van der Wolf et al., 2005).

Several DNA-based protocols involving DNA probes in DNA hybridisation [63-65] or involving primers based on cloned DNA sequences in polymerase chain reaction PCR (Rademaker and Janse, 1994; Lee et al., 1997; Li and De Boer, 1995; Drennan et al., 1994; Xiang and De Boer, 1995; Mills et al., 1997; Pastrik, 2000; Schaad et al., 1999; Schneider et al., 1993) and imunocapture assay (Baer et al., 2001; Mills and Russell, 2003) have been developed to specifically detect the ring rot bacterium.

Conventional PCR techniques employing primers based on the sequences of randomly cloned DNA probes, of plasmid DNA probes and from the 16S-23S rRNA spacer region are more sensitive than serological procedures and DNA hybridisation assays (Lee et al., 1997). The PCR methods have a detection level of 10^2 cfu ml^{-1} in pure cultures [76-77] and 10^3 cfu ml^{-1} with spiked potato core fluid (Mills et al., 1997). However, because these assays also detect other closely related bacterial strains, there have been critical defects in the diagnosis and identification of *Cms* isolates. The most appropriate PCR assays validated (in EU ring tests) on the basis of specificity and sensitivity are those of Pastrik (2000).

The sensitivity of detecting *Cms* in potato tissues by direct PCR may decrease because of the presence of potential reaction inhibitors in potato tissue extracts (Cho et al., 2015). An advantage of real-time Taq-Man PCR is that the multiplication of DNA during early stages can be visible, so that positive samples can be detected before inhibitory compounds in the extract block the PCR reaction.

TaqMan PCR exploits the 5′ nuclease activity of *Taq* DNA polymerase in conjunction with fluorogenic DNA probes.

Each probe, designed to hybridise specifically to the target PCR product, is labelled with a fluorescent reporter dye and a quencher dye. During PCR amplification, the probe is digested by *Taq* DNA polymerase, separating the dyes, resulting in an increase in reporter fluorescence. Repeated PCR cycles result in exponential amplification of the PCR product and a corresponding increase in fluorescence intensity (Weller, 2000).

BIO-PCR combined with the TaqMan automated closed detection system proved to be a rapid, reliable method of assaying large numbers of potato tuber extracts for *C. m.* subsp. *sepedonicus* [72]. Also, based on the cellulase A (CelA) gene sequence two other protocols were advanced. CelA real-time PCR and Cms50/72a PCR assays have been demonstrated to be more sensitive than IF in detecting *Cms* in infected potato tissue. The CelA primers were able to detect the pathogen at low level in potato tubers without disease symptom [80]. Using specific sequence region based on sequence alignment search methods a SYBR green real-time qPCR was developed for the specific detection of the putative phage-related protein from the *Cms* ATCC 33113 genome sequence. The sensitivity, simplicity and reproducibility of the developed method make it suitable as a screening test to detect and quantify *Cms* in potato samples, particularly for *Cms* outbreaks, allowing for more rapid monitoring (Schaad et al., 1999).

In recent years, several real-time PCR protocols have been developed for the detection of *Cms* in potato tubers that can be used as an additional first line screening method (Vreeburg et al., 2016; Cho et al., 2015; Neil et al., 2009). Current EU legislation does not include the use of real-time PCR in routine screening for ring rot bacterium in potato tubers. However, recent studies revealed that this technique is a good addition for routine screening of potato tuber for (latent) infections of *Cms*, since it has shown to be fast, sensitive and specific detection method (Vreeburg et al., 2016). 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 (van der Wolf, 2005). Following positive results in screening tests, a bioassay in eggplants (*Solanum melongena*) is used to assist in the isolation of the ring rot bacterium. Bioassays are based on the inoculation of the potato extract pellet into the stems and incubation under quarantine glasshouse conditions at 18-24ºC for up to 28 days (van der Wolf et al., 2005). Bioassays are used to differentiate between *Cms* from bacteria with similar morphologies and to determine the relative virulence level [10]. During this period, the plants are observed for the development of typical wilting symptoms and isolation of the bacterium from symptomatic and asymptomatic plants are attempted (Nissinen et al., 1997). The bioassay is performed also for isolation of *Cms*. It is considered to be a semi-selective enrichment assay that further aids multiplication of the bacteria on agar media. Additionally, the official protocol recommends it as confirmatory host test (Vreeburg et al., 2016).

Direct isolation of *Cms* form potato tubers is often difficult because of its fastidious nature and inherent susceptibility to antibiotics and inhibitors and is not performed in routine screening for ring rot bacterium in potato tubers. Knowledge regarding the complex nutritional requirements of *Cms* and the variability of such requirements among strains is needed. Developed semi-selective media often use mannitol or glucose due to pathogen limited ability to utilize carbon source. Mannitol apparently is the only sugar alcohol utilized by the bacterium and it is more selective in supporting bacteria growth. Only a few inhibitors were useful due to sensitivity of *Cms* to these compounds. Lithium chloride, polymyxins, cycloheximide, tellurite, sodium dichromate, nalidixic acid are among the selective components used in the development of selective media: M-20, D-2, 4-d, NCP-88 (de la Cruz, 1992). The estimated minimum detection level of *Cms* with NCP-88 is between 10^4 and 10^5 cfu/g of plant tissue (fresh weight). Furthermore NCP-88 may not

be very useful for isolation of the pathogen from complex microbial environments such as soil and decaying plant tissues (de la Cruz, 1992). A semi-selective medium, MTNA which contains mannitol, trimethoprim, nalidixic acid and amphotericin B, has proven effective at recovering Cms from artificially inoculated and naturally infected symptomless tubers (Jansing and Rudolph, 1998) as has NCP-88. Although isolation on culture media is a sensitive technique, false negative results may occur due to low density of pathogenic target bacteria or problems with overgrowth by more rapidly growing associated saprophytes.

Reliable assays for identification of presumptive isolates of *Cms*, in addition to the conventional nutritional and enzymatic bacteriological tests (Lelliott and Stead, 1987), include fatty acid methyl ester and protein profiles and protocols for these techniques are described (Stead, 1992). Fatty acid profiles are a very good alternative to other means for the identification of *Cms*. A fatty acid, 15: 1 anteiso A, comprises > 4% of the profile for all *Cms* strains (EPPO Standards, 2016). Biochemical API 50CH and API ZYM systems have been used for identification *Cms* isolates (Palomo et al., 2006). They could constitute alternative identification tools to the conventional nutritional and physiological identification tests currently included in the official methods employed in the European Union to detect and identify this bacterium (Palomo et al., 2006).

The detection methods developed for *Cms* have each advantages and disadvantages. Serological assays are widely used to detect bacterium ring rot; they are relatively fast and cheap. The use of monoclonal antibodies has significantly improved the specificity of IF however, but in the same time it has also led to reduced sensitivity of detection and can result in false negative results. Methods based on DNA technology can also produce false positive or false negative if PCR inhibitors are present. Direct plating of infected potato plant tissues is possible, though the pathogenic bacteria can be rapidly overgrown by saprophytes. Bioassay on eggplants facilitates isolation on agar media and is required for final confirmation of a diagnosis of *Cms* and for assessment of virulence of isolates identified as *Cms*.

PHYTOSANITARY MEASURES

There are two important aspects of BRR management in potato production: employment of certified, disease-free seed potatoes and good sanitation measures. Unfortunately, zero tolerance for BRR was not effective for preventing the disease since ring rot infections can remain symptomless (Ichim et al., 2017). In addition to seed potatoes guaranteed free from *Cms*, the next most important BRR control measures concern the maintenance of strict on-farm hygiene. It is considered that unexpected outbreaks most probably arise through transfer of *Cms* via shared or hired equipment used elsewhere on infected crops (van der Wolf et al., 2005). Similarly, the use of the same equipment and machinery to grade/process ware and seed potatoes poses a very high risk of cross-infection, particularly when the potatoes originate from different growers. Agricultural disinfectants employed for decontamination of different surfaces, machines and other equipment have to be effective in removing the pathogen, which is known to persist long periods in dried slime from infected potato tubers (Nelson, 1980). Control strategies based on thorough sanitation should take into account the communal existence of the pathogen (Howard et al., 2015). *Cms* originating from disrupted biofilms, mixed with tuber material and present on wooden crate surfaces can be eradicated within 2 min by the exposure to conventional disinfectants (Stevens et al., 2017).

CONCLUSIONS

Cms remains a very dangerous bacterium infecting potato due to lack of effective phytosanitary measures. Process exclusion has been insufficient for BRR long-term management due to latent infections and limitations in diagnostic methods. Recently advanced real-time PCR protocols demonstrated to be fast, sensitive and specific and have been suggested to be included in the first line screening methods for ring rot disease. Advancement of rapid, sensitive and reliable detection methods that provide the needed information about the potential pathogenicity of the detected bacteria is

required. Genomic analysis offered some clues regarding the pathogenic features of the organism and further functional analyses of virulence genes could provide more information about the mechanisms underlying host-pathogen interactions. We anticipate that additional knowledge regarding the mechanisms of pathogenicity of *Cms* will support new prospects for BRR management.

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