Euphresco

Final Report

Title and acronym
The effects of disinfection on *Clavibacter michiganensis* subsp. *sepedonicus* and other bacterial pathogens of potato in Europe (CMS-Disinfect)

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2. Executive Summary

Project Summary

Several bacterial plant pathogens are known to spread via infected seed potato tubers. Disease spread via this route may occur by emergence of diseased plants from tubers bearing a systemic (frequently latent) infection, or by disease transfer through contamination from rotted and macerated tubers that produce bacterial ooze, forming a biofilm on healthy potato tubers or components of the potato production system, such as: machinery, premises, and storage facilities. Healthy potato tubers that come into contact with those contaminated materials may subsequently become infected.

The Euphresco project CMS-Disinfect focuses especially on the EU regulated (Council directive Council directive 93/85/EEC amended by Commission Directive 2006/56/EC) *Clavibacter michiganensis* subsp. *sepedonicus* (Cms, the causative agent of ring rot), the bacteria *Dickeya dianthicola* (Dd), *Dickeya solani* (Ds) and *Pectobacterium atrospeticum* (Pa) which cause blackleg. Elimination of disease transmission via bacterial ooze biofilm from infected potato tubers would lead to a significant reduction of losses in potato production caused by these bacterial infections. In case of Cms, this would mean a significant reduction of the burden for NPPO's resulting from (ring rot) outbreaks, which usually require great efforts to contain the infection and to trace the source of the infection.

The main objective of this Euphresco project was to find an effective method for disinfection of wooden potato storage crates and other matrices relevant in potato production systems, which are contaminated by Cms or the blackleg causing bacteria. To achieve this, it is necessary to find an effective active substance and the best procedure to apply the disinfectant. Within this project two studies were performed, each dealing with different aspects of disinfection, namely (1) Testing the efficacy of a range of disinfectants against Cms, Dd, Ds and Pa on wood, rubber or stainless steel under laboratory conditions, and (2) testing of commercially available disinfection products for their efficacy against Cms on wooden potato storage crates using an automated crate washer with jet cleaning.

(Study 1) Initial tests showed that Cms could survive on a range of surfaces of different materials for periods exceeding one year. Quantification of viable Cms cells indicated that populations did decline to very low levels after only 24 weeks. Wood was found to act as a better surface for Cms survival when compared to metal and rubber. Larger numbers of bacteria were detected on metal than rubber. Colonies appeared to be washed from the surface of rubber and metal more easily than wood. In contrast, Dd, Ds and Pa did not survive well and were undetectable after only a few days.

The efficacy of a range of disinfectants was determined against Cms, Dd, Ds and Pa, namely: Sodium Hypochlorite, AgriGene™ (Quaternary ammonium), Jeyes Fluid™ (cresol), Vanodox™ (peracetic acid, acetic acid, hydrogen peroxide), Q'sol™ (olefin sulphonate), Halamid™ (chloramine), Virkon S™ (oxone), FAM 30™ (iodophor), Sorgene 5™ (hydrogen peroxide), Jet 5™ (peroxyacetic acid) and Shift™ (sodium hydroxide/alcohol); and Dd, Ds & Pa, namely FAM 30™ (iodophor), GPC8™ (glutaraldehyde), Halamid™ (chloramine), Jet 5™ (peroxyacetic acid), Jeye's Fluid™ (cresol), Mikrozid AF™ (ethanmol/propan-1-ol), Sodium hypochlorite, V18™ (iodophor), Vanoquat™ (quaternary ammonium) and Virkon S™ (oxone). These disinfectants have various modes of action. In general, all disinfectants were equally effective at reducing Cms, Dd, Ds, and Pa to below detectable levels after only a few minutes providing that the manufacturer's recommended dose was used. In the case of Dd, Ds, and Pa many of these products remained effective even at lower concentrations.

(Study 2) Four commercially available disinfection products were tested for their efficacy (when tested at their authorized concentration) against Cms on wooden potato storage crates. Each of these products represented a different class of biocide, namely organic acids (benzoic acid), peroxygenous compounds (potassium peroxysulfate), quaternary ammonium compounds (didecyldimethylammonium chloride) and hypochlorite-generating compounds (sodium-p-toluenesulfochloramide). The target objects consisted of small wooden panels that were smeared with potato tuber pulp homogenized with a high inoculum of Cms. By dipping the inoculated panels

into the disinfectant product solutions and by subsequently determining the number of colony-forming units, the relative efficacy of the products was established. The two products that exhibited the highest relative efficacy were then studied in a follow-up experiment for their disinfection performance when applied as cleaning agent in a conventional automated crate washer. In the two final experiments the single best disinfectant was tested using detailed time intervals and by using a "natural" Cms inoculum prepared from symptomatic tubers instead of the artificial Cms inoculum that was used in the initial experiments. The results showed that jet cleaning in a crate washer for 2 minutes using the authorised dose of the product containing sodium-p-toluenesulfochloramide is an effective method (and ready to use for practical operations) for disinfection of wooden potato storage crates which are contaminated by Cms containing ooze from infected potato tubers.

The conventional automated crate washer for application of the disinfectant product is known to operate by recirculation of the disinfectant solution. This implicates pollution of the disinfectant solution by organic material that is washed from the wooden crates, which on its turn would enhance dissipation of the active substance from the disinfectant solution during practical operation. It is therefore recommended to adequately clean the crates prior to disinfection to remove organic material from surfaces. In addition, the crate washer should have an installation for filtering off organic material and for monitoring and supplementing the concentration of active substance.

Application of the recommended (broad spectrum) disinfectant substances for bacteria will have great benefits for the potato production sector because this will eliminate the pathogen transmission route via contaminated potato storage crates and other matrices relevant in potato production systems. Both, potato producers and NPPO's, will benefit when the spread of Cms can be slowed down amongst production places by effective disinfectants besides the application of other proper hygiene practices.

3. Report

Introduction

Potato is one of the major food crops in the world. Nowadays potato is cropped worldwide in nearly all major areas of crop production, including production areas in Northern Europe and the Mediterranean region. Potato crops are attacked worldwide by various plant pathogens which spread via different pathways. Many bacterial plant pathogenic diseases are known to spread via infected seed potato tubers. Disease transmission via this route can occur by emergence of diseased plants from tubers bearing a systemic (frequently latent) infection, or by disease transfer through contamination from rotted and macerated tubers that spill bacterial ooze, which forms a biofilm on healthy potato tubers or components of production systems, such as: machinery, premises, and storage facilities. Healthy potato tubers that subsequently come into contact with these contaminated materials can become infected. These infections are mainly established via cracks and wounds (for example broken off sprouts).

Persistence and viability of bacterial cells in such biofilms varies between bacterial species and depends on the contaminated type of material or matrix, and the biological and physical environment. Several vascular plant pathogenic bacteria, including the ring rot bacterium (*Clavibacter michiganensis* subsp. *sepedonicus*; Cms), are known to form a self-produced extracellular matrix of polysaccharides inside the host plant that protects the bacterium against unfavourable conditions (Marques *et al*. 2003; Ramey *et al*. 2004). Bacterial cells within microbial biofilms generally exhibit a significantly higher tolerance towards antimicrobials than their planktonic analogues (Howard *et al*. 2015). Under most favourable conditions for their survival, viable Cms cells could be detected for years after contamination took place (Nelson 1978, 1979, 1980; Nelson and Kozub 1990; Ward *et al*. 2001).

Besides Cms, other bacterial potato pathogens such as the blackleg causing bacteria *Dickeya dianthicola* (Dd), *Dickeya solani* (Ds) and *Pectobacterium atrospeticum* (Pa) have been recognized to spread through contamination by biofilm forming ooze from rotted and macerated tubers. Soft rot and blackleg bacteria such as Dd, Ds and Pa do not survive well in the environment (Pérombelon & Kelman, 1980), and their survival is highly dependent on temperature and humidity levels. Survival can be longer in the presence of potato debris but even under favourable conditions, survival is restricted (Pérombelon & Hyman, 1989). *Dickeya solani* does not survive well in soil in the absence of its host (Toth *et al*., 2011) and earlier studies have suggested it is unable to persist beyond 3 weeks, irrespective of soil type, temperature and humidity (van der Wolf *et al*., 2007).

Phytosanitary management for prevention of the occurrence and spread of ring rot focuses not only on prohibition of the use of infected seed potatoes and land, but also demands strict hygiene measures. In case of disease detection, thoroughly cleaning and disinfection of all machinery and materials (e.g. wooden storage boxes, plastic and rubber conveyer belts, storage rooms) is necessary to prevent further infestations. Wooden potato storage boxes which are re-used and shared amongst production places within several European countries have been recognized as a major potential source to transfer Cms contaminations between production systems and crops. Besides Cms, other bacterial potato pathogens (e.g. Dd, Ds and Pa) may spread via this route. Therefore, control of bacterial potato pathogens in general is likely to benefit from the application of hygiene measures.

In practice there is awareness of the need for adequate disinfection, but there was a lack of relevant information regarding the efficacy of disinfectants and application techniques. To facilitate decision making by farmers on pursued investments in preventive disinfection, it is necessary that the efficacy of disinfection methods is substantiated by research data. However, no recent research data were available regarding the cleaning and disinfection of machinery and materials with legally registered disinfectants. Available disinfection protocols were based on expert judgement and not on research.

Within this Euphresco project two studies were performed, namely:

(1) Testing a range of disinfectants to study their efficacy against Cms, Dd, Ds and Pa on wood, rubber or stainless steel under laboratory conditions; and
(2) Testing of commercially available disinfection products for their efficacy against Cms on wooden potato storage crates using a conventional automated crate washer with jet cleaning.

The focus of the project is on hygiene treatment to eliminate the risk of disease transfer (of Cms, Dd, Ds and Pa) through contamination by biofilm forming ooze from rotted and macerated tubers on potato tuber storage crates and other parts of potato production systems such as machinery, premises, and storage facilities.

Objectives

The overall objective of this project was to investigate the efficacy of disinfectants on bacterial pathogens of potato on matrices relevant in potato production systems and to test effective disinfection products with an application technique used in practice.

(1) The initial objective for this work was to study the efficacy of a range of disinfectants focusing on Cms and blackleg causing potato pathogens on a number of solid matrices commonly found in potato production, specifically wood, rubber and stainless steel.

(2) The second objective was to find an effective method for disinfection of Cms-contaminated wooden potato storage crates. For that purpose, an effective active substance and an optimal disinfectant application procedure were needed. Eventually, this should be tested under conditions encountered in practice, thus by using an automated crate washer with jet cleaning.

Methods

The two studies were both performed by checking the effect of selected disinfectants in their legal application concentrations on populations of Cms or potato black leg causing pathogens (*Pectobacterium* and *Dickeya* spp.) applied as a coating on matrices relevant in potato production systems (wood, rubber or stainless steel).

Testing of disinfectants against Cms on wood, rubber and stainless steel

Procedures followed to study the effect of disinfectants on the viability of Cms and black leg causing potato pathogens are as follows. In the case of the studies involving Cms three different approaches were performed and these include: paper disc agar diffusion, a quantitative suspension test and a surface test method.

(1) For the *paper disc diffusion assay* paper discs were soaked in known concentrations of disinfectants and then placed on agar plates seeded with Cms. The resultant zone of inhibition was measured after incubation.

(2) The *quantitative suspension test* was used to determine the effectiveness of the disinfectants over a range of concentrations and contact times by suspending actively growing Cms in solutions of known concentration of each disinfectant.

(3) Finally the s*urface test method* was used to measure the effectiveness of the disinfectants on pre-soaked carrier materials (wood, metal (stainless steel) and rubber) found typically in grading equipment or storage containers. The carriers were cut into 1 cm^2 pieces, sterilised by autoclaving and suspended in a Cms cell suspension (10^8 CFU/mL) for one day, then removed from the suspension and allowed to dry for a further 2 days. The contaminated carrier was then suspended in disinfectant solution at the lowest active range (general use) and allowed to stand for a contact time of 5 and 10 minutes. The carrier was then rinsed with 900µl of sterile water to dilute the disinfectant and elute the bacteria from the carrier. Sterile water was used for controls. The carrier was placed into 15 mL of MNTA broth and incubated for 2 days, 1 mL of broth was then pipetted into an Eppendorf and 100µl plated out onto YGM plates. The carriers were then placed onto YGM plates and pressed down to ensure good contact with the agar surface. Plates were then incubated at 21°C for 7 days prior to being scored.

In total, nine disinfectants were tested against Cms, namely: Sodium Hypochlorite, AgriGene™ (Quaternary ammonium), Jeyes Fluid™ (cresol), Vanodox™ (peracetic acid, acetic acid, hydrogen peroxide), Q'sol™ (olefin sulphonate), Halamid™ (chloramine), Virkon S™ (oxone), FAM 30™ (iodophor), Sorgene 5™ (hydrogen peroxide), Jet 5™ (peroxyacetic acid) and Shift™ (sodium hydroxide/alcohol), at either the manufacturer's recommended dose or at more diluted concentrations to create conditions similar to their use in agriculture (see Tables 1-3 for details).

Testing of disinfectants against Dd, Ds and Pa

We evaluated whether *D. solani* MK13, *D. dianthicola* 2260 (A5309) and *P. atrosepticum* NCPPB549 can survive on materials commonly used in potato grading and storage. Five materials (aluminium,

hessian, rubber, steel & wood) were used. These materials were cut to approximately 4 cm^2 and were autoclaved prior to use. A cell suspension of $1x10^8$ CFU/mL was made using sterile distilled water (SDW) and an overnight culture grown on nutrient agar. SDW was used as a negative control. The materials were incubated overnight in the bacterial suspension at 36°C (25°C for Pa) and removed from the suspension and allowed to dry overnight. The materials were rinsed with 1 mL SDW and incubated in pectate enrichment media (PEM) for 48 hours at 36°C (25°C for Pa). The SDW that was used in the rinsing was also plated (100µl) onto CVPM and incubated for 48 hours at 36°C (25°C for Pa*)*. After incubation, 100µl of the PEM suspension was plated onto CVPM and incubated at 36°C (25°C for Pa) for 48 hours.

A revised method was also used with cell suspensions as previously described. However, PEM rather than SDW was used. After incubation for 48 hours at 36°C the materials were allowed to dry for 4 hours and then placed into PEM overnight at 36°C (or 25°C for Pa*)*. The materials were not rinsed with SDW prior to incubation in PEM. 100µl of the PEM suspension was plated onto CVPM and incubated at 36°C (25°C for Pa*)* for 48 hours. In addition, cell suspensions were also mixed into mashed-up potato material and then the paste was applied onto the materials and treated as described above.

Ten disinfectants (Fam30, GPC8, Halamid, Jet-5, Jeye's Fluid, Mikrozid AF, Sodium hypochlorite, V18, Vanoquat and Virkon S) commonly used in agriculture or in laboratory were tested for their effectiveness in controlling the growth of Dd, Ds and Pa. A cell suspension of 10^8 cells/mL was made using an overnight culture. Various concentrations were tested. Initially these were: (1) Lowest active range (LAR; i.e. recommended dilution for general use), (2) 50% dilution of LAR concentration, and (3) 25% dilution of LAR concentration.

Dilutions of the disinfectants were tested against the bacteria by adding 100 µl of suspension to 900 µl of the disinfectant solution. The mixture was vortexed and left for the required incubation time (5, 10 or 30 minutes). Prior to the end of the contact time the suspension was vortexed again. The suspension was centrifuged at 13,000 rpm for one minute and the disinfectant (the supernatant) removed and 1 mL of sterile distilled water (SDW) added and vortexed to rinse the bacterial pellet. The solution was centrifuged again for 1 min at 13,000 rpm and the supernatant removed. The pellet was resuspended in 1 mL of SDW and 100 µl of the sample plated out onto CVPM and incubated for 48 hours at 36°C (25°C for Pa). Initial results showed no growth at the lowest active range (LAR), 50% LAR and 25% LAR. Further dilutions of 10% LAR, 5% LAR and 1% LAR also showed no growth. Dilutions of 0.5%, 0.4%, 0.3%, 0.2% and 0.1% LAR were chosen for comparison as some growth appeared at these concentrations. The disinfectants were also tested against Pa and Dd at the lower concentrations.

Selection of disinfectants on small wooden panels

Small wooden panels (planks) derived from wooden potato storage crates were initially used as target objects for testing under laboratory conditions. The aim of the three experiments that were carried out on wooden panels was to select the two most effective disinfectants out of four candidates. To create the inoculum a streptomycin-resistant mutant of Cms (IPO 1873, mutant from NCPPB 4053) was cultured on YGM agar medium containing 100 mg/L of streptomycin and 100 mg/mL of cycloheximide (van der Wolf and Beckhoven, 2009). For details on reagents, material and disinfectants used in this study we refer to the paper by Stevens *et al*. (2017).

The wooden target objects were smeared with potato tuber pulp homogenized with a high concentration of Cms. By disinfectant application and subsequent determination of the densities of remaining colony-forming units, the efficacy of the tested disinfectant products was established. Identity of Cms isolates grown from the treated wooden target objects was confirmed by TaqMan PCR (Schaad *et al*., 1999). Four commercial products were studied for their efficacy for disinfection of Cms-contaminated wooden panels. In this report the disinfection products are referred to as products A, B, C, and D. The active substance in the four respective products are: (A) benzoic acid, (B) pentapotassium bis(peroxymonosulphate) bis(sulphate), (C) didecyldimethylammoniumchloride, and (D) sodium-p-toluenesulfochloramide.

Cms disinfection using a conventional automated crate washer

For the operational disinfectant application experiments under practical conditions, we used a conventional automated crate washer with jet cleaning. The inoculated target objects were mounted in a potato storage crate. Three separate experiments were performed.

In the first experiment, the products A and D (see above) were tested in a practical setting using an automated crate washer with jet cleaning, and in parallel, comparative testing was performed by submerging the wooden panels in the disinfectant solution. The aim of this experiment was to confirm the efficacy of the two selected disinfectants (product A and D) when applied (at time intervals of 2 and 5 minutes) by means of jet cleaning using a conventional crate washer, and to compare this with the efficacy by manual (submerging) application of the disinfectants.

The second experiment was performed using product D in the same experimental set up as described above, while using more intervals of exposure time (30 sec, 1, 2, and 5 minutes) of the (Cms-contaminated) target objects to the disinfectant.

Experiments 1 and 2 were performed with artificial inoculum freshly prepared by homogenisation of tuber pulp with a streptomycin resistant Cms mutant (IPO 1873, mutant from NCPPB 4053). The third experiment was performed with product D (which showed the best efficacy) to verify whether this artificial inoculum constituted a realistic representation of naturally infected tuber material with regard to its susceptibility to the disinfectant. Therefore, an experiment was performed using an inoculum derived from tubers showing symptoms of bacterial ring rot. In this experiment manual disinfectant application (submersion) was performed on panels smeared with the naturally infected derived inoculum in parallel to the same treatments applied to panels smeared with artificial inoculum.

Results and discussion

Laboratory testing of a range of disinfectants against Cms and black leg causing potato pathogens

With regards the work with Cms, three approaches were investigated to determine the optimal approach for efficacy testing, specifically; a paper disc agar diffusion method, a quantitative suspension test and a surface test method.

Table 1. Results from the paper disc agar diffusion method using a range of commercial products against Cms.

Commercial Product (Recommended Dilution Rate)	Average Zone of Inhibition (mm)							
	Rec. Dilution		0.50% Dilution		0.25% Dilution		0.125% Dilution	
	10 days	20 days	10 days	20 days	10 days	20 days	10 days	20 days
Sodium Hypochlorite (10% solution)	33.75	33.75	30.75	30.75	19.50	19.50	20.25	20.25
AgriGene (1:50)	19.75	19.75	17.88	17.88	14.13	14.13	12.38	12.38
Jeyes Fluid (5:100)	18.00	15.00	16.25	12.25	9.00	7.63	6.63	3.38
Vanodox (1:100)	15.63	15.63	13.50	13.50	12.25	12.25	9.63	9.63
Q'sol (5 mL per 5 L)	12.00	12.00	10.00	10.00	7.00	7.00	6.75	6.75
Halamid (1% solution)	11.25	11.25	10.25	10.25	9.38	9.38	8.88	8.88
Virkon S (1:100)	11.25	11.25	11.25	11.25	7.00	7.00	2.88	2.88
FAM 30 (1:180)	9.75	7.63	9.63	7.63	8.00	1.38	6.25	0.00
Sorgene 5 (1:100)	9.00	9.00	8.38	8.38	7.88	7.88	7.88	7.88
Jet 5 (1:125)	9.00	9.00	8.38	8.38	7.50	7.50	7.50	7.50
Shift (1:250)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

The aim of the *Paper Disc Agar Diffusion* method was to measure the effectiveness of the disinfectants at different concentrations. Sodium hypochlorite had the largest zones of inhibition at all dilutions compared to the other disinfectants (Table 1). This may have been due to the recommended dilution being the most concentrated of all the disinfectants. However, even at the highest dilution (1.25% solution) the zone of inhibition was greater than the other disinfectants at their recommended dilutions. It should also be noted that zones of inhibition for sodium hypochlorite were uneven. Jet 5 and Sorgene 5 had very similar zones of inhibition at all dilutions. There were no large decreases in zone size as the dilutions increased. Sorgene 5 had slightly higher zone sizes at the two higher dilutions. This may had been due to the recommended dilution being a higher concentration. Some products also showed signs of regrowth after 20 days incubation, specifically Jeyes Fluid and FAM 30,

indicating that the active ingredient had become denatured/exhausted or that resistance had been induced in the test strain. The detergent Shift exhibited no inhibition of Cms under these test methods. It should be noted that zone of inhibition alone may not be a true reflection of the disinfectants effectiveness against Cms and may be due to the disinfectants ability to diffuse through the agar.

The aim of the *Quantitative Suspension Test* was to determine the effectiveness of the disinfectants over a range of concentrations and contact times. Sorgene 5, FAM 30, Jeyes Fluid, Virkon S, Vanodox, Halamid, Hypo clean and Hypo clean thick all tested negative for Cms at the lowest active range of the disinfectant (general use), 0.50% and 0.25% dilutions after contact times of 5, 10 and 30 min (Table 2). One colony was detected using Jet 5 at the 0.50% dilution after a contact time of 30 min. No colonies were detected using AgriGene at the general use and 0.50% dilution after contact times at 5, 10 and 30 min. However, at the highest dilution rate, colonies were found after contact times of 5, 10 and 30 min. As the contact time increased, the number of colonies decreased. Colonies were detected using Q-sol detergent at the general use dilution (1:1000) after a contact time of 5 min. However, no colonies were detected at the general use dilution rate after 10 and 30 min showing that as contact time increases the number of colonies decrease. Colonies were detected at 0.50% and 0.25% at 5, 10 and 30 min contact times. Colony numbers increased as the dilution rate increased and contact time decreased. Similar results were found using Shift power wash liquid. However, more colonies were detected at each of the dilutions and contact times than Q'sol. Two colonies were detected at the general use dilution for Shift after a contact time of 10 min. It should be noted that disinfectants may show high activity level in suspension tests but these may be reduced on contaminated surfaces.

The aim of the *Surface Test Method* was to measure the effectiveness of the disinfectants on pre-soaked carrier materials (wood, stainless steel and rubber) found typically in grading equipment or storage containers. The carriers were suspended in a Cms cell suspension (10^8 CFU/mL) for 1 day and then removed from the suspension and allowed to dry for two days. The contaminated carrier was then suspended in disinfectant solution at the lowest active range (general use) and allowed to stand for a contact time of 5 and 10 minutes. The carrier was then rinsed with 900µL of sterile water to dilute the disinfectant and elute the bacteria from the carrier. Sterile water was used for controls. The carrier was placed into 15mL of MNTA broth and incubated for 2 days, 1mL of broth was then pipette into an Eppendorf and 100µL plated out onto YGM plates. The carriers were then placed onto YGM plates and pressed down to ensure good contact with the agar surface. In the first element of this experiment two positive controls were employed; one substituted a disinfectant for water and the other was 100µL taken from the wash water of the positive carrier (Column entitled '900µl H_2O'; Table 3). An average of 115 colonies were detected after using Shift power wash liquid to treat wood for a contact time of 10 min. These numbers were less than those obtained after treating wood with distilled water for 10 min (~ 850 CFU). High numbers of colonies were also recovered from rubber treated with Shift after contact times of 5 and 10 min, and metal treated with Q-Sol for 5 min. Colonies were also recovered from metal treated with Shift for 5 min. However, as with Q-sol the colony numbers greatly decreased when the contact time was doubled. Colonies were recovered from rubber treated with Jeyes Fluid and Q'sol at both contact times, the increase in contact time having little or no effect on colony numbers. No colonies were detected on wood, rubber or metal after using Sorgene 5, Virkon S and FAM 30 at general use dilution rate for a contact time of 10 min. After incubating the carrier in MNTA broth growth was only observed in broth after treating rubber or metal with either Shift or Q-Sol for 5 min. In addition, application of the carrier to YGM agar shows that Sorgene 5 and Fam 30 exhibited no growth. Growth was observed when using shift and Q'sol, Jet 5 and Jeyes Fluid on the wood carrier for 10 min and Virkon S when using at a contact time of 5 min.

Table 2. Results from the quantitative suspension test using a range of disinfectants against Cms. Test conducted in triplicate.

Commercial Disinfectant (General use dilution)	Contact Time (min)	Colony Count								
		General Use			50%			25%		
		1	2	3	1	2	3	1	2	3
Jet 5 (1:250)	5	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0
	30	0	0	0	1	0	0	0	0	0
Sorgene 5 (1:200)	5	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	0	0
FAM 30 (1:180)	5	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	0	0
Jeyes Fluid (5:100)	5	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	0	0
Virkon S (1:100)	5	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	0	0
Vanodox (1:300)	5	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	0	0
Halamid (0.3% solution)	5	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	0	0
Q'sol (1:1000)	5	2	2	0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	10	0	0	0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	30	0	0	0	7	3	5	~550	~550	~550
Q'sol (1:1200)	5	10	3	6	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	10	1	0	0	TNTC	TNTC	TNTC	~400	~400	TNTC
	30	2	2	0	~360	~360	~450	83	36	~1200
Shift (1:500)	5	1	1	0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	10	2	0	0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	30	0	0	0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
Agrigene (1:200)	5	0	0	0	0	0	0	TNTC	TNTC	TNTC
	10	0	0	0	0	0	0	TNTC	TNTC	TNTC
	30	0	0	0	0	0	0	74	85	59
Hypo Clean	5	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	0	0
Hypo Clean Thick	5	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	0	0
Positive Control	5	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	10	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	30	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

TNTC; Too numerous to count.

Table 3. Results from the surface test method using a range of commercial products against Cms on three substrates.

Commercial Product (General use dilution)	Contact Time (min)	Colony Counts														
		900µl H2O						MNTA Broth						Carrier		
		Wood		Rubber		Metal		Wood		Rubber		Metal		Wood	Rubber	Metal
		1	2	1	2	1	2	1	2	1	2	1	2			
Jet 5 (1:250)	5	ND	ND	0	0	0	0	ND	ND	0	0	0	0	ND	0	0
	10	0	0	0	0	0	0	ND	ND	0	0	0	0	1	0	0
Sorgene 5 (1:200)	5	ND	ND	0	0	0	0	ND	ND	0	0	0	0	ND	0	0
	10	0	0	0	0	0	0	ND	ND	0	0	0	0	0	0	0
FAM 30 (1:180)	5	ND	ND	0	0	0	0	ND	ND	0	0	0	0	ND	0	0
	10	0	0	0	0	0	0	ND	ND	0	0	0	0	0	0	0
Jeyes Fluid (3:100)	5	ND	ND	11	16	0	0	ND	ND	0	0	0	0	ND	0	0
	10	0	0	17	22	0	0	ND	ND	0	0	0	0	1	0	0
Virkon S (1:100)	5	ND	ND	0	0	0	0	ND	ND	0	0	0	0	ND	+	+
	10	0	0	0	0	0	0	ND	ND	0	0	0	0	0	0	0
SHIFT (1:500)	5	ND	ND	105	116	28	30	ND	ND	22	28	0	0	ND	6+	+
	10	~110	~120	78	83	2	2	ND	ND	0	0	0	0	~300+	+	0
Q-Sol (1:1200)	5	ND	ND	6	8	72	73	ND	ND	0	0	4	3	ND	0	4+
	10	0	0	16	25	3	0	ND	ND	0	0	0	0	25	0	0
+ve Control Water	5	ND	ND	37	40	~300	~280	ND	ND	1	2	38	39	ND	14	70 +
	10	~850	~850	17	20	~150	~150	ND	ND	0	4	28	26	~800+	4+	21+
+ve Control wash water	5	ND	ND	8	16	~400	~400	NA	NA	NA	NA	NA	NA	NA	NA	NA
	10	~180	~180	2	3	~160	~145	NA	NA	NA	NA	NA	NA	NA	NA	NA

ND; No data
+; Colony growth around carrier

Testing of disinfectants against Dd, Ds and Pa

Initial results showed no growth of Dd, Ds and Pa from any of the storage materials and an apparent loss of the pathogens during the process, so revisions were made to the experimental method. Using the revised method in which bacteria were applied to surfaces in enrichment media rather than sterile distilled water, all three pathogens were able to persist and could be grown from all five storage materials. Subsequent experiments using mashed up potato material as an alternative media applied to all materials showed that Ds did not survive on any material studied. Taken together these results indicate that Dd, Ds and Pa do not survive well in the absence of the host and appear to be poorly adapted to surviving on surfaces for even relatively short periods of time.

It is clear from the results presented in Appendix 2 that all 10 disinfectants studied as part of this investigation were equally and highly effective at controlling Dd, Ds and Pa if applied at the concentration recommended by the manufacturer for general use. However, the effectiveness of each disinfectant varied widely if diluted further down to 0.5, 0.4, 0.3, 0.2 and 0.1% of the manufacturer's recommended general use dilution. It is clear from these results that sodium hypochlorite and Vanoquat were the only disinfectants effective against all three pathogens even at 0.1% of the manufacturer's recommended general use dilution. Halamid also appears to be highly effective even though a small number of Ds colonies were beginning to appear on the 0.2% and 0.1% dilutions when only 5 minutes of contact time was employed, indicating that at these dilutions and contact time the effective range of the product had been reached. As the contact time increased to 10 minutes and beyond, effective control was re-established. At the opposite end of the spectrum, diluting Jeye's Fluid to 0.5% of the manufacturer's general use dilution was ineffective at controlling any of the thee studied pathogens, regardless of the contact time applied. Some of the disinfectants appeared more effective at controlling some species, but not all. This was particularly true for Jet 5, Mikrozid AF, V18 and Virkon S. *Dickeya dianthicola* was highly sensitive to these products in contrast to *D. solani* and *P. atrosepticum* that showed strong resistance. In the case of Jet 5 and Virkon S increasing the contact time produced greater control of the latter species, whilst increasing contact time was ineffective in the case of Mikrozid AF and V18.

Selection of disinfectants on small wooden panels

For details and figures of results from the study using an automated crate washer with jet cleaning and for the discussion of those, we refer to the paper by Stevens *et al*. (2017). The results from the experiments where Cms-infected panels were manually disinfected show that product D exhibited the best disinfection performance, followed by product A, product B and product C, respectively. On the basis of these results, product A and D were selected for further evaluation with application by an automated crate washer.

Cms disinfection using a conventional automated crate washer

Three separate experiments were carried out using a conventional automated crate washer. The results from the first experiment showed that after application of disinfectant A and D at time intervals of 2 and 5 minutes, no viable cells were recovered from treated target objects. However, given that treatment by manual application (submergence) for 2 minutes was less effective for product A than product D, we selected the latter disinfectant for further evaluation in the second and third experiment.

The results from the second experiment showed that when the target objects were subjected (at time intervals of 30 sec, 1, 2, and 5 minutes) to 1% of product D in the crate washer, no viable Cms was detected in any of the samples. These results confirm the observations of the initial experiment, revealing that 2 and 5 minutes of exposure to product D (1% concentration) is sufficient to completely eradicate Cms, and suggest that even 30 seconds or 1 minute might be sufficient.

In the first and second experiment, the coated potato material which was pre-smeared on the wooden panels was visibly removed after jet cleaning in the automated crate washer. This was not the case when the wooden panels were manually treated (submerged) in the disinfectant solution; the potato material coating on the target objects was not visibly reduced. The results obtained with the control jet treatments using water (with no disinfectant added) showed that the cleaning procedure by itself leads to a significant removal of Cms from the inoculated target objects. However, the results also show that total eradication of Cms does require application of a disinfectant. In the third experiment, inoculum was prepared from symptomatic ring rot-infected tubers. Using this inoculum, no viable Cms was recovered after submersion for 2 min in 1% of product D. These results show that product D exhibited

the same disinfection efficacy towards the artificial Cms inoculum as towards the Cms inoculum prepared from symptomatic infected tubers.

Conclusions

The experiments performed within this project were aimed at verifying the effects of selected disinfectants (at their legally required or recommended application concentrations) on populations of Cms or potato black leg causing pathogens (Dd, Ds and Pa) when these bacterial pathogens are applied as a coating on matrices relevant in potato production systems (wood, rubber or stainless steel).

For Cms, wooden surfaces yielded the largest colony counts across a range of experiments. Larger numbers of colonies were detected on the positive metal carrier than the rubber carrier. More colonies were detected on the positive metal and rubber carriers after 5 min contact time with distilled water than 10 min. Colonies appeared to be washed from the surface of rubber and metal more easily than wood. Under normal conditions, without the need to add enrichment media, it was clear that Dd, Ds and Pa are not able to persist on a range of surface for long time. The clear message from this work is that a range of products are available to control the spread of Cms, Dd, Ds and Pa if used properly and adhering to the manufacturer's recommended concentration for general use. It is also clear that some disinfectants have a greater 'safety net' than others, and this is particularly true for sodium hypochlorite and Vanoquat.

It is recommended that when disinfecting metal and rubber surfaces, these should remain wet with the disinfectant treatment for up to 10 min, and if possible, this treatment should be repeated. Steam and pressure washing is recommended on wood surfaces prior to disinfecting, and/or repeating disinfectant treatment. Contact time was found to be an important factor affecting the activity level of disinfectants. It is important that disinfectants are used at the recommended dilution rate and contact time for specific tasks. Organic material is likely to reduce the efficacy of disinfectants against bacteria, and in particular sodium hypochlorite and iodine based disinfectants. Detergents should not be used instead of disinfectants and care should be taken using products such as Jeye's Fluid as there is evidence that diluted solutions of the product have minimal effect on any of the pathogens tested here. Finally, it should be noted that some disinfectants are corrosive to metals (e.g. hypochlorite solution).

The experiments on Cms from disrupted biofilms, mixed with tuber material and present on wooden crate surfaces, show that Cms can be eradicated by the exposure to (submergence in) a conventional disinfectant within 2 minutes. Application of the disinfectant by means of a crate washer is preferable to submersion, because the treatment inside the crate washer provides a strong combination of mechanical jet cleaning and chemical disinfection. Since Cms biofilms generally exhibit a significantly higher tolerance towards antimicrobials than their planktonic analogues (Howard *et al*. 2015), disinfection of crate surfaces should be preceded or accompanied by disruption of the biofilm matrix. This was confirmed by the observation that the benzoic acid product was effective when applied by an automated crate washer for 2 minutes, while this product was insufficient for eradication of Cms when it was applied for 2 minutes by submersion alone. Among the four chemical disinfectants tested, the most effective product was based on the active substance sodium-p-toluenesulfochloramide. Since 2 min of mere submersion into the authorized concentration of this product did not leave any detectable viable Cms, exposure to sodium-p-toluenesulfochloramide for 2 minutes (when applied in the authorized concentration; 1% w/v) by means of jet cleaning inside a conventional crate washer is considered as a reliable method for disinfection of Cms-contaminated wooden potato storage crates.

All experiments using a conventional automated crate washer with jet cleaning were performed with freshly prepared product solutions. Under practical conditions, conventional crate washers generally recirculate the cleaning solution. During recirculation, the active substance is inevitably dissipated by the reaction with organic material which is washed from the wooden crates during jet cleaning. It is therefore recommended to provide crate washers with an installation for filtering off organic material and for monitoring and supplementing the concentration of active substance.

Paper

Stevens LH, Lamers JG, van der Zouwen PS, Mendes O, van den Berg W, Tjou-Tam-Sin NNA, Jilesen CJTJ, Spoorenberg PM, van der Wolf JM (2017) Chemical Eradication of the Ring Rot Bacterium *Clavibacter michiganensis* subsp. *sepedonicus* on Potato Storage Crates. Potato Research ISSN 0014-3065, 1-14p.

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Appendices

Appendix 1: Frequently used abbreviations:

Cms = *Clavibacter michiganensis* subsp. *sepedonicus*

Dd = *Dickeya dianthicola*

Ds = *Dickeya solani*

Pa = *Pectobacterium atrospeticum*

Appendix 2; (sheet 1/ 6): Susceptibility of Dd, Ds and Pa to a range of disinfectants.

Commercial Product (General use dilution)	Strains	Contact times (minutes)	Colony Count					
			%					
			100	0,5	0,4	0,3	0,2	0,1
FAM 30 (1:180)	*D. solani*	5	0	0	0	0	510	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	TNTC	TNTC
	D. solani	10	0	0	0		104	TNTC
	D. dianthicola	10	0	0	0	0	0	1
	P. atrosepticum	10	0	0	0	TNTC	TNTC	TNTC
	D. solani	30	0	0	0	0	0	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	TNTC	TNTC
GPC8 (1:35)	*D. solani*	5	0	0	2	1	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	2	231
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0

TNTC; Too numerous to count.

Appendix 2; (sheet 2/ 6): Susceptibility of Dd, Ds and Pa to a range of disinfectants.

Commercial Product (General use dilution)	Strains	Contact times (minutes)	Colony Count					
			%					
			100	0,5	0,4	0,3	0,2	0,1
Halamid (0.3:100)	*D. solani*	5	0	1	2	0	1	3
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
Jet 5 (1:250)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	1
	D. solani	10	0	156	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	4	TNTC	TNTC	TNTC
	D. solani	30	0	0	0	0	70	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	1	16	TNTC

TNTC; Too numerous to count.

Appendix 2; (sheet 3/ 6): Susceptibility of Dd, Ds and Pa to a range of disinfectants.

Commercial Product (General use dilution)	Strains	Contact times (minutes)	Colony Count %					
			100	0,5	0,4	0,3	0,2	0,1
Jeye's Fluid (5:100)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
Mikrozid AF liquid	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	TNTC	TNTC	TNTC	TNTC	TNTC

TNTC; Too numerous to count.

Appendix 2; (sheet 4/ 6): Susceptibility of Dd, Ds and Pa to a range of disinfectants.

Commercial Product (General use dilution)	Strains	Contact times (minutes)	Colony Count %					
			100	0,5	0,4	0,3	0,2	0,1
Sodium hypochlorite (14:100)	*D. solani*	5	0	0	0	0	0	0
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
V18 (1:500)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	TNTC	TNTC	TNTC	TNTC	TNTC

TNTC; Too numerous to count.

Appendix 2; (sheet 5/ 6): Susceptibility of Dd, Ds and Pa to a range of disinfectants.

Commercial Product (General use dilution)	Strains	Contact times (minutes)	Colony Count					
			%					
			100	0,5	0,4	0,3	0,2	0,1
Vanoquat (1:300)	*D. solani*	5	0	0	0	0	0	0
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
Virkon S (1:100)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	1
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0

TNTC; Too numerous to count.

Appendix 2; (sheet 6/ 6): Susceptibility of Dd, Ds and Pa to a range of disinfectants.

Commercial Product (General use dilution)	Strains	Contact times (minutes)	Colony Count					
			%					
			100	0,5	0,4	0,3	0,2	0,1
Negative control (water)	*D. solani*	5	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	5	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	10	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	10	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	30	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

TNTC; Too numerous to count.