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Effect of Temperature Treatments on the Viability of *Clavibacter sepedonicus* in Infected Potato Tissue

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Abstract

The objective of the present study was to establish whether exposure to temperatures of 55-70 °C results in eradication of the pathogen *Clavibacter sepedonicus* (Cs) in colonised potato tissue, in order to evaluate the efficiency of composting for disinfection of Cs-infected potato waste. Pre-sprouted potato tubers were inoculated and planted to produce Cs-colonised stem and tuber material. After incubation in temperature-controlled water baths, the infected potato tissue was analysed for the presence of culturable and pathogenic Cs. Additional experiments were performed with Cs-colonised potato stem tissue crushed and deaerated, thus simulating macerated stem tissue in the compost heap. To enable a comparison with bacterial cells that are not enclosed by their natural organic matrix, temperature treatments were applied to non-infected stem tissue crushed and deaerated, and spiked with freshly prepared Cs-suspensions. Cs settled inside colonised potato tissue, as well as Cs present as Cs-suspensions supplemented to potato tissue, was eradicated by exposure to heat, even after a treatment of 1 h at 55 °C, with the exception of one case in which the pathogen present in intact stem material escaped a treatment of 6 h at 60 °C, indicating that incidentally stems may provide niches in which the pathogen is protected against heat.

Keywords Disinfection of potato waste ring rot · Potato composting · TaqMan assay

Introduction

Clavibacter sepedonicus (Cs) causes ring rot in potatoes, which is a highly damaging potato disease. The bacterium is a quarantine organism in the European Union

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(2000/29/EC Annex IAII). For growth and symptom expression, Cs prefers relatively cool temperatures (Anonymous 2006a), but can also cause symptoms in warmer climates (De Boer 2007). Generally, exhibition of disease symptoms caused by Cs occurs late in the growing season (De Boer et al. 1992). Isolation of the pathogen is hampered by the relatively low growth rate of Cs on media and the concomitant risk of the bacterium being overgrown by saprophytes (Stead 1999). Cs colonises xylem in stems and roots, and forms biofilms causing plugging of the xylem vessels like most other vascular plant pathogenic bacteria (Marques et al. 2003).

The common practice of recycling plant material by means of composting and subsequent application of the produced humus to the environment, presents a risk of spreading Cs. The magnitude of this risk is affected by the temperature profile during the composting process, and by the sensitivity of Cs to these elevated temperatures.

Present knowledge about control of plant pathogens by means of exposure to high temperatures is largely provided by studies on disinfection of botanical seed. Heat treatments comprise application of heated moisture such as hot water or aerated steam, and dry heat. Water is twice as efficient as steam and five times more efficient as dry heat with respect to heat transfer (Maude 1996). Tomato seed infection with the causative agent of bacterial canker, *Clavibacter michiganensis* (Cm), can be eradicated by hot water (20 min at 52 °C; Fatmi et al. 1991), by aerated steam (30 min at 56 °C; Navaratnam et al. 1980), and by dry heat (1 hr at 80 °C; cited from Maude 1996). So far, no results have been reported about the effect of heat treatment on seed potato tubers infected with Cs.

During composting, the microbial biodegradation of organic substrate produces heat, generally resulting in temperature rises in the compost heap up to 50-60 °C or even more (Gurtler et al. 2018; Nozhevnikovaa et al. 2019). Heat transfer during composting occurs via water, moistened air and organic and inorganic materials. In a study on the viability of Cs during composting, Steinmöller et al. (2013) exposed encapsulated potato plant material spiked with freshly prepared Cs suspensions at a final concentration of 10^6 cfu/g of substrate to elevated temperatures by burying the filled capsules in compost heaps, and showed that composting for 6 days at maximally 70 °C and for 13 days at maximally 55 °C, or 90 min of pasteurisation at 55 °C, did not fully eradicate the bacterium.

Literature suggests that the efficacy of temperature treatments depends highly on the physical and chemical environment of the bacterium during temperature exposure; in particular, the presence of an organic matrix may provide significant protection to relatively high temperatures (Fatmi et al. 1991; Downer et al. 2008; Raviv et al. 2011; Steinmöller et al. 2013; Howard et al. 2015). The natural environment of Cs inside potato plants is constituted by their self-produced biofilm attached to the walls of xylem vessels (Marques et al. 2003). The extracellular polymeric matrix protects the bacterium against unfavourable conditions. In addition, bacterial cells immobilised inside biofilms are supposed to be more protected against adverse conditions than planktonic cells (Marques et al. 2003; Ramey et al. 2004; Howard et al. 2015). Eradication of Cs by means of pasteurisation or by controlled composting therefore requires knowledge about the effects of elevated temperature on Cs that is present in infected plant material.

The objective of the present study was to establish whether temperature treatments are able to eradicate Cs in its natural environment of colonised potato tissue, in order to evaluate the efficiency of composting for disinfection of Cs-infected potato waste. To

obtain the required Cs-infected plant material, pre-sprouted tubers were inoculated and planted to produce Cs-colonised stem and tuber material. The Cs-infected stem and tuber material was incubated in temperature-controlled water baths after which the viability of Cs cells was analysed by dilution-plating on an agar medium and by testing their capability to infect eggplants. To enable a comparison with bacterial cells that are not enclosed by their natural organic matrix, temperature treatments were also applied to suspensions of Cs and Cm. Additional experiments were performed with Cs-colonised potato stem tissue crushed and deaerated, thus simulating macerated and deaerated stem tissue in the compost heap. Furthermore, temperature treatments were tested on non-infected stem tissue crushed and deaerated, but artificially inoculated (spiked) with a freshly prepared Cs-suspension.

Materials and Methods

Bacterial Strains and Cultivation Media

Isolates of *Clavibacter sepedonicus* (Cs) and *Clavibacter michiganensis* (Cm) were grown for 72 h at 22 °C on yeast extract, mineral salts medium (YGM) and Trypticase Soy Agar (TSA), respectively. The spontaneous streptomycin resistant strain IPO1831 (NCPPB4053, a fluidal strain) was used for the experiments with Cs and the spontaneous rifampicin resistant strain IPO3356 (Syngenta, strain 88) for the experiments with Cm. The YGM agar contained 2.0 g/L bacto yeast extract (Difco), 2.5 g/L D-glucose (monohydrate), 0.25 g/L K_2HPO_4, 0.25 g/L KH_2PO_4, 0.1 g/L $MgSO_4.7\ H_2O$, 0.015 g/L $MnSO_4.H_2O$, 0.05 g/L NaCl, 0.005 g/L $FeSO_4.7\ H_2O$, 18 g/L purified agar no. 3 (Oxoid) and 1 mL/L of a stock solution of streptomycine was added to a final concentration of 100 mg/L. The TSA was a premade solution from Difco and 1 mL/L of a stock solution of Rifampicine was added to a final concentration of 25 mg/L. The antibiotics were added immediately before pouring the agar plates when the temperature of the medium was below 50 °C.

Production of Cs-Infected Potato Plant Material

To serve experiments 1, 2 and 3, which were performed in spring 2017, Cs-infected potato stem and tuber material were produced in 2016, as follows. Seed potato tubers (12 tubers of cultivar Kondor; 55 tubers of cultivar Bintje) were induced to sprout by placing them in the dark at 15 °C. After ca. 6 weeks, on 3 June 2016, the pre-sprouted tubers were inoculated with a suspension of the streptomycin resistant Cs strain (ca. 10^9 cfu/mL in Ringer's solution), freshly prepared from a one-week-old culture grown on YGM agar at 20 °C. Inoculation was done by piercing a syringe needle circa 1 cm deep under each sprout into the tuber and subsequently injecting 10-20 μl of the Cs suspension into the created channel while retreating the needle from the tuber. Two injections were administered per sprout. Control tubers were injected with sterile Ringer's solution. One day after inoculation, the tubers were individually planted in pots with potting soil (3-5 cm below the soil surface), resulting in young plantlets within one week after potting. Plants were grown in a greenhouse compartment using a 16 h light period at 24 °C (day) and 20 °C (night) and 70% RH. On 15 July 2016, each

plant was inoculated again as described above by administering Cs to the main stem directly above the soil, since no symptoms were visible at that moment. On 22 September 2016, the lower part (circa 20 cm) of each main plant stem was harvested and stored at -20 °C. On the next day, daughter tubers were harvested and stored at 5 °C in the dark. One week before harvesting, the above ground parts of each plant were inspected by eye for typical ring rot symptoms.

To serve experiments 4, 5 and 6, which were performed in November 2018, Cs-infected potato stem material was produced similarly as in 2017. On 24 July 2018, a group of 30 pre-sprouted tubers were inoculated, and a control group of 20 tubers was mock-inoculated with Ringer's buffer. On 6 September 2018, each plant was inoculated again as described above by administering Cs to the main stem directly above the soil. On 24 October 2018, the lower part (circa 20 cm) of each main plant stem was harvested and stored at -20 °C.

DNA Extraction from Inoculated Potato Stem and Tuber Material to Establish Infection Rate

Stem segments of 1-2 cm were put in Bioreba extraction bags and crushed using a rubber hammer. Subsequently, they were incubated for 15 min with 5 mL of a quarter strength Ringer's solution (Fluka) containing 0.02% diethyldithiocarbamic acid (Acros Organics). After homogenisation and incubation, 2 mL aliquots of extract were collected and spun down by centrifugation (6000 rpm for 15 min); the supernatant was discarded. The pellets were suspended in 300 μl of lysis buffer PVP (LGC Genomics) plus 0.5 μl of RNAse (2 mg/mL). After addition of 90 mg of glass beads and a metal bead of diameter 3.2 mm, the mixture was shaken in a TissueLyser II bead mill (Qiagen) for 40 s (frequency setting 20 per s). This suspension was clarified by centrifugation (6000 rpm for 15 min). DNA was isolated from the supernatant using the Sbeadex maxi plant kit protocol. A dilution series (ca. 10^8–10^1 cfu/mL) of Cs 1873 suspended in Ringer's buffer spiked with healthy potato stem extract served as positive control. Approximately 4 mm × 10 mm of daughter tuber material was separately taken from the tuber stem end and from the tuber bud end using a punch sampler. Each tuber sample was placed in a 2 mL tube with 300 μl of lysis buffer PVP (LGC Genomics) plus 0.5 μl of RNAse (2 mg/mL) and 2 metal beads of with a diameter of 3.2 mm. This mixture was shaken in a bead beater (Precellys Evolution, Bertin Biotechnologies) for two times 30 s at a speed of 8000 rpm with a pause of 10 s. This suspension was clarified by centrifugation (6000 rpm for 15 min). DNA was isolated from the supernatant using the Sbeadex maxi plant kit protocol.

TaqMan Protocols

Two TaqMan protocols were used. The Schaad protocol, described by Schaad et al. (1999) and used in experiments 1-6, was as follows: 2 μl of DNA-extract was added to a mix of ROXII (0.5x), Premix Ex Taq (1x) from Takara (Westburg, Leusden, The Netherlands), forward primer Cs 50-2F and reverse primer Cs 133-R (final concentration 0.3 μM each), probe FAM-Cs 50-53T (final concentration 0.1 μM) to a total volume of 25 μl (experiments 1-3) or 20 μl (experiments 4-6). The second protocol (Nytor protocol), described by Vreeburg et al. (2016) and used in experiments

4-6, was as follows: 2 μl of DNA-extract was added to a mix of ROXII (0.5x), Premix Ex Taq (1x) from Takara (Westburg, Leusden, The Netherlands), forward primer Cm-pan1-F2 and reverse primer Cm-pan1-R3 (final concentration 0.3 μM each), probe TP-Cs-pan1-198 (final concentration 0.1 μM) to a total volume of 20 μl. The real-time PCR amplification was performed using a real-time PCR system QuantStudio (Applied Biosystems BV) with the following cycling conditions: initial denaturation for 2 min at 95 °C; then 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. For analysis of plant material, Ct values lower than 35 were considered positive.

Colony TaqMan PCR

DNA-extracts were prepared by picking individual colonies using a tooth pick, suspending the sample in 50 μl Milli-Q water and subsequent boiling of the mixtures for 5 min at 95 °C. The extracts were subjected to the TaqMan protocols described above. As high numbers of bacterial cells were analysed, only colonies resembling Cs with a Ct-value lower than 23 and a high ΔRn-value of 1.75 were considered positive.

Exposure of Suspensions of Cs and Cm to Hot Water

To investigate the survival of Cs and Cm suspensions at temperatures of circa 60 and 70 °C, two temperature-controlled water baths (GFL water bath 1002) were used. Each water bath contained for each strain a separate beaker filled with circa 400 mL of water. Each beaker contained five closed tubes (volume 50 mL), which were filled with 19.80 mL of Ringer's solution. The experiments were started by adding 0.20 mL of freshly prepared Cs or Cm suspensions (OD_{600} 0.1, indicating 10^7-10^8 cfu/mL) to the tubes containing the preheated Ringer's solution. After 1 h at 68.7 °C and 6 h at 59.6 °C, samples were taken and spread-plated in duplicate undiluted on semi-selective agar media (100 μl sample per plate). Four tubes per strain, kept at room temperature (23 °C) and containing 0.20 mL of the inoculum in 19.8 mL of Ringer's solution, served as controls. The samples taken from these controls were cultivated after being diluted 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} in Ringer's solution (100 μl of diluted sample per plate). The temperature of each water bath was continuously monitored by two thermocouples (Microlog with external NTC temperature sensor with 100-mm needle probe, Fourtec). Colonies were counted after 4 days of growth at 25 °C and 8 days at 20 °C for Cm and Cs, respectively. The experiment was repeated once, while omitting the 10^{-3} dilution for the control.

Heat Transfer in Potato Tissue

Freshly cut potato tuber disks or whole potatoes were incubated in water baths in beakers with water equilibrated at 55 °C and 70 °C. The temperature development at the core of the disks and tubers was monitored with a needle probe containing at the needle end the thermo-sensor (Microlog with NTC temperature sensor, Fourtec). The exit opening in the tissue for the thermocouple was closed with Vaseline and emerged a few millimetres above the water surface during the experiment in order to avoid leaking in of water.

Exposure of Cs-Infected Potato Tuber Material to Hot Water (Experiments 1, 2 and 3)

Per experiment, stem ends and bud ends (1.5 cm) of five tubers that were tested Cs-positive in the TaqMan assay (Ct<35) were cut into 8 segments of equal dimensions (1.77 ± 0.45 g per piece). The resulting 40 segments from the stem end and 40 segments from the bud end were randomly distributed over 10 tubes (tube volume 50 mL). Tissue of non-infected tubers was added to complete the tuber mass per tube to a total of 20 g. The same procedure was followed to generate 12 segments from stem end and 12 segments from bud end of three non-infected healthy tubers, which were randomly distributed over 3 tubes and supplemented to a total mass of 20 g per tube. The tubers used in experiments 2 and 3 were surface-sterilised with 70% ethanol to minimise the number of background microorganisms. The tubes with the tuber segments were prepared the day before the temperature treatments were applied, and were kept in the dark overnight at room temperature. The temperature exposure of the tuber segments was started by dumping the content of each tube in a preheated glass bottle containing 200 mL of preheated demineralised water, and immediate transfer of these bottles to pre-equilibrated water baths (GFL water bath 1002) set at fixed temperatures of 55 °C, 60 °C and 70 °C. To prevent a temperature drop caused by the initial low temperature of the tuber segments, the start temperatures of the preheated incubation bottles were circa 5 °C above the target temperatures. The bottles containing the non-infected tuber tissue were provided with thermocouples which continuously monitored the temperature of the water inside these bottles; temperature equilibria arrived within circa 2 min to continue throughout the incubations at 55 °C ± 1 °C, at 59 °C ± 1 °C and at 69 °C ± 1 °C. To finish the temperature treatments, the bottles were taken from the water baths, and the water content was immediately replaced with 100 mL of fresh demineralised water equilibrated at room temperature (23 °C) which after 2 min was replaced by another 100 mL of fresh demineralised water equilibrated at room temperature (23 °C). After 3 min, the tuber segments were transferred to Bioreba bags, smashed using a rubber hammer and extracted for 5 min with 20 mL of Ringer's solution. The extract was plated in two-fold (100 μl per plate) on YGM-agar containing streptomycin. Colonies were counted after incubation for 8 days at 20 °C.

Exposure of Cs-Infected Potato Stem Material to Hot Water (Experiments 1, 2 and 3)

The stems used in experiments 1, 2 and 3 were disinfected beforehand on the outside with 70% ethanol to minimise the number of background microorganisms. The lower part (ranging from the soil to ca. 20 cm above the soil) of the main stem of five potato plants that tested Cs-positive (Ct≤35) in the Taqman assay were each cut crosswise in 10 segments of 2 cm each, resulting in 50 circa 2 cm long cylindrical shaped segments, which were randomly distributed over 10 tubes. Infected stem material was added to complete the stem mass per tube to a total of 3.5 g. The tubes with the stem segments were prepared on the day of the experiment and left for 1 h to reach room temperature before the start of the temperature treatments. The heat exposure of the stem segments was performed in bottles containing 200 mL of demineralised water placed in water baths essentially as described for the tuber segments. The addition of five stem

segments per bottle of 200 mL water preheated at 55 °C, 60 °C and 70 °C only led to a small and immediate temperature drop that took less than one minute to recover. Controls were kept outside a water bath at room temperature (23 °C). Temperature (55 °C ± 1 °C, 60 °C ± 1 °C and 70 °C ± 1 °C) was monitored throughout the incubation periods inside bottles containing preheated water (without tissue) placed in the water baths. To finish the temperature treatments, the bottles were taken from the water baths, and the water content was immediately replaced twice with 100 mL of fresh demineralised water equilibrated at room temperature (23 °C). After 5 min, the stem segments were transferred to Bioreba extraction bags, smashed using a rubber hammer and extracted for 5 min with 3.5 mL of Ringer's solution. The extract was plated in two-fold (100 μl per plate) on YGM-agar containing streptomycin. Colonies were counted after incubation for 8 days at 20 °C.

Encapsulation of Potato Stem Segments by Vacuum Sealing (Experiments 4, 5 and 6)

Five intact 2-cm segments of five different potato stems were cut from stored and defrosted potato stems, and sealed under vacuum in a stomacher bag (Bioreba; no separation mesh) within 40 s by means of a Henkovac Compact Eco Mini table vacuum-sealer (HFE Vacuum Systems, Den Bosch, The Netherlands). Putting crushed stem segments under vacuum, in contrast to putting intact stem segments under vacuum, appeared to go with suction of plant sap, loose seals and eventual leakage of the bag. Therefore, hammering was done after, not before, vacuum sealing, which resulted in visible release—if present—of tiny air bubbles from stem segments soaked in stem sap. Per experiment, two types of experimental objects were prepared: objects containing five 2-cm stem segments derived from five different Cs-colonised plants ('objects 1'), and objects containing five 2-cm stem segments derived from five different non-infected plants ('objects 2'), inoculated by injecting 10 μl of circa 1×10^{10} cfu/mL of a freshly prepared Cs-suspension into the stem segments immediately before preparation of the objects. These encapsulated groups of stem segments served as experimental objects and were subjected to the various temperature treatments immediately after preparation. To determine the effect of the various temperature treatments on the culturability of Cs, the extracts were plated (100 μl per plate) undiluted, and 10 times and 100 times diluted on semi-selective YGM-agar. A plated dilution series (10^0 to 10^6 times diluted; 100 μl per plate) of freshly prepared Cs-suspension (circa 5×10^8 cfu/mL) served as control. After twelve days, the plates were visually inspected for the presence of Cs or other colonies.

Bioassay with Eggplants

The extracts obtained in experiment 3 from temperature treated potato stem and tuber material, and the extracts obtained in experiments 4-6 from temperature treated experimental objects 1 were directly plated on a semi-selective medium, but also injected in 3-4-week-old eggplants (cultivar Black Beauty) to enrich Cs in this susceptible host prior to detection. To get rid of soil and plant particles, the freshly prepared potato stem and tuber extracts from experiment 3 were supplemented with Ringer's solution up to a volume of 40 mL, centrifuged at low speed (300 g for 10 min at 8 °C), and after transfer

to a new tube concentrated by means of centrifugation (10,000 g for 10 min at 8 °C) and subsequent resuspension in 1 mL of Ringer's solution. These suspensions and the freshly prepared extracts obtained in experiments 4-6 were immediately administered to the eggplants by injection into the stem beneath and directly above the cotyledons (ca. 10-20 µl per plant). The bioassay was performed in accordance with directive 2006/56/EC (Anonymous 2006b). Positive control plants were injected with Cs suspensions (10^6, 10^7 and 10^9 cfu/mL) freshly prepared from Cs cultures on YGM-agar. Ten plants were inoculated per sample. Plant culture conditions were 21 °C and 16 h daylight. Circa five weeks after inoculation, the plants were inspected for visible symptoms and samples were taken for Cs detection by means of plating on YGM-agar containing streptomycin, and subsequent colony PCR. Plant sampling was done by taking a 5-cm-long stem piece 1 cm above the inoculation point from each plant using disinfected pruning scissors. For each group of ten plants, two composite samples were prepared by combining the stem segments of five plants. These composite samples were disinfected for 1 min in 70% ethanol, washed twice with sterile water, and subsequently extracted according to the procedure described in 2006/56/EC (Anonymous 2006b). The extracts were plated undiluted and 100 times diluted (100 µl per plate) on YGM-agar containing streptomycin (experiment 3) or streptomycin and cycloheximid (200 mg/l) (experiment 4-6). After 6 days of incubation at 20 °C, the plates were visually inspected and colony PCR was performed.

Statistical Analysis

Numerical data were recorded and analysed in Microsoft Excel 2010, and when appropriate in the 19th edition of the statistical program Genstat (VSN International, Rothamsted UK). Changes of Cs and Cm populations during incubation at room temperature were tested using a General Analysis of Variance (ANOVA) and Duncan's Multiple Range Test.

Results

Generation of Cs-Colonised Potato Stem and Tuber Material

The intended experiments 1, 2 and 3 required potato stem and tuber material that had been colonised by the streptomycin resistant Cs strain. This Cs-colonised plant material was produced by administering Cs-inoculum to pre-sprouted seed tubers of two cultivars (cv. Kondor and cv. Bintje) and subsequently to the stems of the plantlets that arose from these tubers. Five mock-inoculated plants were produced by administering Ringer's buffer in the same manner. About 3.5 months after planting, the main stems and the daughter tubers of the full-grown plants were harvested. Only 16 of the 59 inoculated plants had visible symptoms of ring rot in their above ground parts, i.e. interveinal spaces of the leaves became light green to pale yellow, and as the disease progressed, leaves became necrotic, starting at the margins or in interveinal tissue. To enable selection of infected stems and daughter tubers that were envisaged to be the test objects for the temperature treatments, the lower part (1-2 cm) of each main plant stem, and the stem end and the bud end of one of the daughter tubers of each plant were

analysed by means of a TaqMan assay. Analysis with a TaqMan assay showed that the main stems of all inoculated plants were infected with Cs (Table 1). A low Cs incidence was found in the daughter tubers: 41% of the plants showed Cs-positive stem ends and 5% showed Cs-positive bud ends on the analysed tubers (Table 1). In agreement with this, more tuber stem ends than bud ends appeared to be soft and discoloured. This may be explained by the fact that the bacterium enters the daughter tuber from the mother tuber via the stolon. The mock-inoculated control groups of Kondor and Bintje unexpectedly produced one Cs-positive sample (Table 1); apparently, cross contamination had occurred between these groups and the infected groups, which were grown together in the same greenhouse compartment.

To serve the follow-up experiments 4, 5 and 6, Cs-colonised potato stem material was produced by administering inoculum to 30 pre-sprouted seed tubers of cv. Bintje and subsequently administering Cs-inoculum to the stems of the plantlets growing from these tubers. Twenty plants were mock-inoculated by administering Ringer's solution in the same manner. Three months after planting, none of the 50, full-grown plants showed visible symptoms of ring rot. To enable selection of the Cs-positive stems that were envisaged to be the test objects for the temperature treatments, the lower part (20 cm) of two main plant stems per plant were harvested and analysed by means of TaqMan PCR. Stems of all 30 inoculated plants and none of the 20 mock-inoculated plants appeared to be Cs-positive. The average Ct-value of the positive stem samples was 24.4 which corresponded with a Cs density of 5×10^7 cfu per cm of stem, as calculated from data obtained with control stem extracts spiked with Cs.

Survival of Suspended Cs in Heated Water

The temperature treatments that are required to eradicate Cs inside the natural environment of the potato plant material were assumed to be at least what is required for sterilisation of aqueous suspensions of Cs. In order to establish whether exposure to the relevant composting temperatures kills the pathogen, suspensions of the streptomycin resistant Cs strain, freshly prepared from mucoid biofilm cultures grown on agar medium, were incubated in 5-fold in water baths at 70 °C for 1 h, and at 60 °C for 6

Table 1 Disease (ring rot) incidence and infection incidence of shoots and daughter tubers (stem- and bud end) as determined by a TaqMan assay at 3.5 months after inoculation of the mother tuber with *Clavibacter sepedonicus* which was followed six weeks later by a stem inoculation of young plantlets. Mock-inoculated plants served as negative controls

Cultivar	Group	N	Visible symptoms	Positive (Ct ≤ 35)		
				Shoot	Tuber	
				Lower stem	Stem end	Bud end
Kondor	Inoculated	9	2	9	5	1
	Mock-inoculated	2	0	1	0	0
Bintje	Inoculated	50	14	50	19	2
	Mock-inoculated	3	0	0	1	0

h. Control suspensions were exposed to room temperature for 1 h and 6 h (n=4). Survival for Cm was determined at the same time in the same water baths using suspensions of a rifampicin resistant strain of Cm. The experiment was performed twice. Immediately after the temperature treatments, samples were taken and plated on a semi-selective agar medium. No significant increase or decrease of cfu was found after exposure to room temperature between 1 h and 6 h for both Cs and Cm (t-value=-1.938, df=8.55, P=0.086). In contrast, none of the Cs and Cm suspensions in Ringer's solution produced any colonies after exposure to 70 °C for 1 h and to 60 °C for 6 h. It was therefore decided to investigate the survival of Cs in potato material by exposing tuber and stem segments for 0-6 h to temperatures in the range of 55 °C to 70 °C.

Heat Transfer in Potato Tuber Material

Cs colonises the xylem vessels of its hosts, and therefore, the bacterium can be located in the vascular ring located ca. 0.5 cm beneath the tuber surface. Pilot experiments with 1-cm-thick tuber disks (circa 2 cm i.d.) showed that it takes about 5 min and 10 min to bring the core temperature of the disks to equilibrium in water baths of 55 °C and 70 °C, respectively. These experiments also showed that it takes circa 30 min and more than 60 min for the inner tissue core of small potatoes (60-70 g) to arrive at a temperature equilibrium in water baths of 55 °C and 70 °C, respectively. To prevent a temperature drop caused by the initial low temperature of the tuber segments in experiment 1, 2 and 3, the start temperatures of the preheated incubation bottles were circa 5 °C above the target temperatures.

Effect of Temperature Treatments on the Culturability of Cs Present Inside Potato Stem and Tuber Tissue (Experiments 1, 2 and 3)

Potato stem and tuber tissue colonised by the streptomycin resistant Cs strain were exposed to water at 55 °C (for 1 h, 2 h and 6 h), at 60 °C (for 1 h, 2 h and 6 h), at 70 °C (for 10 min, 1 h and 2 h) and at room temperature (23 °C) for 6 h. In order to determine the effect of the temperature treatments on culturability of Cs, immediately after the incubations, extracts were prepared from the potato tissues and plated undiluted on semi-selective YGM-agar. After circa one week, plates were then visually inspected for the presence of Cs. The identity of colonies that showed a Cs-appearance was corroborated by means of a colony TaqMan assay. Table 2 presents an overview of the results.

Being dense cultures of monoclonal species, bacterial colonies present high copy numbers of their specific DNA, and consequently lead to relatively low Ct-values in TaqMan-assays. Our colony-PCR procedure performed on colonies cultured from positive controls invariably resulted in Ct-values below 23 with sigmoidal curves and high ΔRn values of 1.75. Applying this criterion, the results indicate that all elevated temperature treatments deprived Cs present in infected tuber material from their culturability on semi-selective YGM-agar (Table 2). In contrast, Cs present in the extracts prepared from the tubers exposed to room temperature could easily be detected on semi-selective YGM-agar (Table 2). Cs grown inside stem segments also appeared vulnerable to all elevated temperature treatments applied; culturable Cs could only be recovered from stem segments that had been exposed for 6 h at 60 °C in experiment 3

Table 2 Culturability of Cs extracted from Cs-infected potato tuber and stem segments after exposure to the indicated temperature treatment

Treatment		Tubers segments[1]			Stem segments[2]		
T (°C)	Time (min)	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
70	10	-[3]	-	-	-	-	-
70	60	-	-	-	-	-	-
70	120	-	-	-	-	-	-
60	60	-	-	-	-	-	-
60	120	-	-	-	-	-	-
60	360	-	-	-	-	-	+
55	60	-	-	-	-	-	-
55	120	-	-	-	-	-	-
55	360	-	-	-	-	-	-
RT	360	+	+	+	+	+	+

[1] Per treatment, 4 segments of infected tubers were used supplemented with segments of non-infected tuber material up to a total of 20 grams in a 50-mL tube

[2] Per treatment, five 2-cm basal stem ends were used supplemented with infected stem material up to a total of 3.5 grams in a 50-mL tube

[3] -: no Cs colonies detected after plating on solid nutrient plates; all colonies tested by means of TaqMan-assay were Cs-negative (Ct-values between 28.5 and 40), and often showed a colony-appearance different from that of Cs. +: colonies with a typical Cs appearance were detected after plating on solid nutrient plates; Cs-identity corroborated by means of TaqMan-assay (Ct-values between 14.7 and 22.6)

(Table 2). The control treatments on stem material kept at room temperature all were Cs positive (Table 2).

Eggplant Bioassay (Experiment 3)

To establish whether viable Cs cells did survive inside the hot water treated tuber and stem tissue in experiment 3, freshly prepared tissue extracts were inoculated in young eggplants. All positive control plants developed symptomatic necrosis at some of the leaf edges and chlorotic spots between leaf veins. All plates inoculated with stem extracts of positive control plants became covered with Cs-resembling colonies, which were positive in the colony-TaqMan assay. Eggplants inoculated with water tested negative. Extracts of Cs-infected potato tuber and stem tissue which had been exposed to room temperature (23 °C) led to some symptomatic eggplants (four out of ten plants exhibited necrotic and three out of ten plants chlorotic leaf spots); Ct-values obtained after colony-PCR were between 20.5 and 22.6 (Table 3). However, none of the extracts prepared from Cs-infected potato tissue that had been exposed to elevated temperatures resulted in symptomatic eggplants. Furthermore, none of the composite eggplant stem samples prepared from plants inoculated with tuber extracts exposed to elevated temperatures generated Cs colonies on the semi-selective YGM-agar, as confirmed by colony-PCR (Table 3). The same was true for extracts prepared from potato stem tissue exposed to elevated temperatures, except for the extract derived from stem tissue exposed to 60 °C for 6 h (Table 3). One corresponding composite eggplant sample

generated multiple tiny white colonies, which tested Cs-positive (Ct-values between 20.8 and 22.6; Table 3). This means that between one and five eggplants had been infected with this particular potato stem extract, thus confirming that Cs in the original stem tissue had survived the temperature treatment. This single Cs-positive sample in experiment 3 contrasts with the corresponding Cs-negative samples in experiments 1 and 2. It also contrasts with the observation that Cs did not survive the treatments that involved lower temperatures and shorter periods of exposure (Table 3). Apparently, in some niches of intact stem material, Cs can survive at temperatures that generally are lethal.

Effect of 60 °C on the Culturability of Cs in Crushed and Deaerated Potato Stem Tissue (Experiments 4, 5 and 6)

The rare case observed in experiment 3 of Cs survival in intact stem tissue at 60 °C for 6 h suggested that occasionally Cs inside stem tissue might be protected against heat, for example by trapped air. Therefore, experiments 4, 5 and 6 were performed with Cs-colonised potato stem tissue that had been crushed and deaerated (experimental objects 1), simulating macerated and deaerated stem tissue in the compost heap. In addition, temperature treatments were tested on non-infected stem tissue that had been crushed, deaerated, and artificially inoculated (spiked) with a freshly prepared Cs-suspension (experimental objects 2), thus partly imitating the experimental objects used by Steinmöller et al. (2013). They exposed encapsulated potato plant material spiked with freshly prepared Cs suspension to elevated temperatures by burying the filled capsules in compost heaps. The intact potato tuber and stem segments in experiments 1, 2 and 3 were directly exposed to large volumes of warm water. In experiments 4, 5 and 6, the crushed and deaerated plant tissue was encapsulated in vacuum sealed extraction bags, which were exposed to warm water. Objects 1 were exposed to 60 °C for 0, 1, 2 and 6 h, whereas objects 2 were exposed to 60 °C for 0 and 6 h.

Immediately after application of the treatments, diluted and undiluted extracts of objects 1 and 2 were plated on YGM-medium. The colonies grown from freshly prepared Cs suspension (Cs positive controls) had a white, slimy, shiny, smooth and convex appearance, which is characteristic for Cs on YGM-medium. Two different colony TaqMan assays (Schaad protocol; Nytor protocol) applied to the Cs controls both typically produced low Ct-values ranging between 13 and 20. As expected, the extracts from the experimental objects 1 and 2 that had been exposed to 60 °C for 0 h and to room temperature for 6 h, exhibited abundant Cs-like cultures on the plates with Ct-values between 14.3 and 19.7, similar to the positive controls. No Cs colonies and no colonies resembling Cs were found on the plate cultures of extracts from experimental objects 1 and 2 that had been exposed to 60 °C for 1, 2 and 6 h. Some of those plates presented atypical colonies, mostly spreader-like, similar to *Bacillus* colonies. Every single atypical colony was analysed individually. TaqMan PCR applied to these colonies invariably resulted in relatively high Ct-values, ranging between 27.6 and >40. Table 4 presents for all treated experimental objects whether Cs could be detected in their extracts after plate culturing on YGM-medium. In conclusion, exposure of the encapsulated, crushed and deaerated Cs-colonised potato stem tissue to 60 °C for 1, 2 or 6 h, led to complete loss of culturability of Cs.

Table 3 The number of symptomatic and infected eggplants at 5 weeks after inoculation with potato tuber and stem extracts inoculated with *Clavabacter sepedonicus* or mock-inoculated that had been exposed to various temperature treatments in experiment 3

Treatment		Tuber segments Exp 3[1]				Stem segments Exp 3[2]			
T (°C)	Time (min)	Nr of symptomatic plants	Plant extract[3]	Number of Cs-resembling colonies	Ct-value	Number of symptomatic plants	Plant extract	Number of Cs-resembling colonies	Ct-value
70	10	0	A	0		0	A	0	
			B	1	>40[4]		B	3	>40
70	60	0	A	1	>40	0	A	0	
			B	0			B	1	>40
70	120	0	A	0		0	A	1	>40
			B	3	>40		B	0	
60	60	0	A	6	>40	0	A	0	
			B	0			B	0	
60	120	0	A	0		0	A	0	
			B	1	>40		B	2	>40
60	360	0	A	0		0	A	0	
			B	1	>40		B	>>	20.8-21.8
55	60	0	A	0		0	A	0	
			B	0			B	>>	>40
55	120	0	A	0		0	A	0	
			B	16	>40		B	4	>40
55	360	0	A	0		0	A	1	>40
			B	5	>40		B	0	
RT[6]	360	4	A	>>[5]	nd[7]	3	A	>>	20.5
			B	>>	nd		B	>>	21.2

[1] Per treatment, 4 segments of infected tubers were used supplemented with segments of non-infected tuber material up to a total of 20 grams in a 50-mL tube

[2] Per treatment, five 2-cm basal stem ends were used supplemented with infected stem material up to a total of 3.5 gram in a 50-mL tube

[3] Composite stem extracts A and B of five plants per group of eggplants were dilution plated on a semi-selective medium. The colonies resembling *C. sepedonicus* were counted and analysed by colony-PCR

[4] Analyses resulting in no Ct-value after 40 cycles are indicated with '>40'

[5] >>, many hundreds

[6] RT, room temperature

[7] nd, not determined

Eggplant Bioassay: Virulence of Cs from Crushed and Deaerated Potato Stem Extracts (Experiments 4, 5 and 6)

Immediately after finishing the temperature treatments, the extracts collected from objects 1 were administered to young eggplants. Table 5 lists the disease incidence.

Table 4 Summary of the screening for the presence of *Clavibacter sepedonicus* (Cs) in potato stem objects 1 and 2, which had been exposed to the various temperature treatments (experiment 4, 5 and 6)

Treatment of potato stem segments[1]		Exp. object	Experiment 4 Colony identification			Experiment 5 Colony identification			Experiment 6 Colony identification		
T (°C)	Time (min)		Cs-like?[2]	TaqMan positive?[3]		Cs-like?	TaqMan positive?		Cs-like?	TaqMan positive?	
				A	B		A	B		A	B
60	0	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
60	60	1	No	No	No	No	No	No	No	No	No
60	120	1	No	No	No	No	No	No	No	No	No
60	360	1	No	No	No	No	No	No	No	No	No
RT[4]	360	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
60	0	2	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
60	60	2	No	No	No	No	No	No	No	No	No

[1] Per treatment, five 2-cm basal stem ends were used supplemented with infected stem material up to a total of 3.5 grams in a 50-mL tube

[2] Cs was detected by dilution plating on a semi-selective medium

[3] Colonies were analysed by TaqMan PCR for Cs, which was performed twice, i.e. according to Schaad (A) and according to Nytor protocol (B)

[4] RT, room temperature

All or almost all eggplants belonging to the positive control groups developed disease symptoms, i.e. symptomatic necrosis at leaf edges and chlorotic spots between leaf veins, except the plants that had been inoculated with the lowest inoculum density (10^2 cfu/mL). The negative controls were without symptoms. None of the eggplants inoculated with extracts from objects 1 that had been exposed to 60 °C for 1, 2 or 6 h, exhibited any disease symptoms. In contrast, most eggplants inoculated with extracts from objects 1 that had been immediately prepared after 0 h of incubation, or after exposure to room temperature for 6 h, clearly showed disease symptoms.

All plates inoculated with stem extracts of positive control plants, including extracts from the symptomless plants inoculated with 10^2 cfu/mL, developed abundant growth of Cs-resembling colonies. Colony TaqMan PCR resulted in relatively low Ct-values typically ranging between 17 and 22.7. Extracts from the negative control plants did not produce any colonies. Stem extracts of eggplants inoculated with extracts from objects 1 that had been exposed to 60 °C for 0 h, or to room temperature for 6 h, gave abundant growth of Cs-resembling colonies. Colony TaqMan PCR presented low Ct-values (< 23). In contrast, stem extracts of eggplants inoculated with extracts from objects 1, which had been exposed to 60 °C for 1, 2 or 6 h, did not generate any Cs-resembling colonies on the plates. Some of these plates developed atypical colonies. None of these colonies proved to be Cs, as judged from colony TaqMan PCR (Ct>40). Table 5 summarises the effects of the various temperature treatments on Cs present in experimental objects 1 with respect to their virulence towards eggplants. In conclusion, exposure of the encapsulated, crushed and deaerated Cs-colonised potato stem tissue to 60 °C for 1, 2 or 6 h, led to complete loss of virulence of Cs.

Table 5 Disease and infection incidences of eggplants at four weeks after inoculation with extracts of potato stems infected or challenged with *Clavibacter sepedonicus* (Cs) and exposed to various temperature treatments (experiments 4, 5 and 6)

Treatment		Experiment 4			Experiment 5			Experiment 6		
T	(°C)	Time (min)	Nr of	symptomatic plants	Plant extract[1]	Cs	TaqMan positive?[2]	Nr of symptomatic plants	Plant extract	Cs TaqMan positive?
Nr of		symptomatic plants	Plant extract	Cs TaqMan positive?						
60	0	9	A	Yes	10	A	Yes	10	A	Yes
			B	nd[3]		B	Yes		B	Yes
60	60	0	A	No	0	A	No	0	A	No
			B	No		B	No		B	No
60	120	0	A	No	0	A	No	0	A	No
			B	No		B	No		B	No
60	360	0	A	No	0	A	No	0	A	No
			B	No		B	No		B	No
RT[4]	360	10	A	Yes	10	A	Yes	10	A	Yes
			B	Yes		B	Yes		B	Yes

[1] Composite extracts A and B of 5 plants each were plated on semi-selective medium

[2] Colonies were analysed by TaqMan PCR for Cs

[3] nd, not determined

[4] RT, room temperature

Discussion

The experimental objects of this study, i.e. Cs-infected potato stems and tubers, were produced by plants grown from seed potatoes which were inoculated with Cs by means of injection. Although the inoculation thus was artificial, it is resonable to assume that the colonisation of the potato plants followed the natural infection process, and that Cs was present in its protective multicellular biofilm inside the xylem vessels (Marques et al. 2003). Therefore, the colonised stem and tuber segments used in this study are considered to be representative for the practical situation during composting and pasteurisation of intact, naturally infected potato material.

Exposure of suspended Cs and Cm to warm water showed that Cs and Cm cells that are not enclosed by their natural organic matrix lose their culturability completely after exposure to 70 °C for 1 h and to 60 °C for 6 h. It was therefore decided to investigate the survival of Cs in potato material by exposing tuber and stem segments for 0-6 h to temperatures in the relevant range of 55 °C to 70 °C. Obviously, the efficiency by which heat is transferred from the environment to the plant tissue highly determines the disinfection efficiency. In order to minimise this factor, the experimental set-up was designed to ensure that the elevated temperatures applied to the tuber, and stem segments were constant from the beginning to the end throughout the set period of heat exposure. Pilot experiments showed that heat was readily transferred from the aqueous environment to the centre of the tuber segments. It is therefore likely that variation in Cs viability found in replicate experiments should be attributed to variation of microconditions inside its organic potato matrices.

Here, we presented three repeated experiments with nine different heat treatments applied to Cs-infected intact potato stem and intact tuber tissue (experiments 1, 2 and 3). Only in one occasion culturable Cs could be recovered, i.e. from the extract of intact potato stem tissue which had been exposed to 60 °C for 6 h in experiment 3. In extracts of this tissue, viable Cs was found by dilution-plating on a semi-selective medium and after inoculation of eggplants. All other extracts from plant tissue exposed to elevated temperatures did not show any culturable Cs. The observation that extracts from tissue exposed for a shorter period of time than 6 h, or to a lower temperature than 60 °C were devoid of viable Cs indicates that the heat susceptibility of Cs inside potato tissue shows variation, possibly due to variation in organic matrices including biofilms or chemical composition inside the same type of potato tissue, or due to trapped air that locally blocked heat transfer. Obviously, some intact stem segments presented more favourable conditions for Cs than other segments.

Experiments 4, 5 and 6 involved encapsulated colonised potato stem tissue, hammered and deaerated, warranting the absence of trapped air bubbles between Cs-colonised niches and the surrounding liquid, and securing optimal heat transfer. The positive controls showed that Cs biofilm in colonised stem tissue kept for at least 6 h encapsulated under vacuum at room temperature did maintain its culturability and its virulence to eggplants. Exposed to water at 60 °C for 1, 2 or 6 h, the same Cs objects completely lost their culturability and their virulence to eggplants. Cs administered as suspension to stem tissue, and thus lacking the biofilm protection, completely lost its culturability and virulence after deaeration and subsequent exposure to water at 60 °C for 1 h.

These results contradict the hypothesis that the developmental status of Cs or the natural environment of Cs inside potato plants, constituted by their self-produced biofilm attached to the walls of xylem vessels, provide a significant protection to the pathogen against exposure to temperatures that are generated by the exothermic process of composting. Even exposure at 55 °C for only 1 h did completely destroy the viability of Cs inside colonised potato tuber and stem tissue. However, the single sample showing survival at 60 °C for 6 h indicated that intact stem material incidentally may provide niches in which the pathogen can escape disinfection. Possibly, these niches contain trapped air or other protective layers that block the efficacy of the heat transfer. Disintegration and maceration of the plant material during composting may help to minimise this problem.

Our results contrast with the observations of Steinmöller et al. (2013), who reported a remarkable heat resistance of the bacterium for 6 d at maximum temperatures at 70 °C, 13 d at 55 °C and 90 min pasteurisation at 70 °C. Their experimental objects were capsules containing garden-mould colonised by Cs, artificially inoculated from freshly prepared suspensions of Cs colonies harvested from plate cultures. They suggested that organic substances of the mould might explain the extreme heat resistance observed in their study. Secor et al. (1988) reported that bacteria could survive hot water treatment longer if protected by organic substances. Podolak et al. (2010) argued that heat resistance of *Salmonella* in low-moisture foods can be caused by physical and chemical food compositions. Possibly, the microbiome of the compost heap may contribute to physico-chemical conditions that provide protection to Cs against elevated temperatures.

Our results clearly show that Cs inside its natural environment of potato tissue is relatively vulnerable to composting temperatures. However, considering the results of Steinmöller et al. (2013), which were obtained with artificially inoculated experimental objects, it remains to be answered whether the composting process is able to kill Cs inside naturally colonised potato tissue as efficiently as the water baths in our experimental set-up.

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Data Availability All data underlying presented results are available on request

Declarations

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Conflict of Interest All authors herewith declare that they have no conflict of interest. This study does not contain studies with human participants or animals performed by any of the authors.

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