Biological control of potato soft rot caused by *Dickeya solani* and the survival of bacterial antagonists under cold storage conditions

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Abstract

Dickeya and *Pectobacterium* are responsible for causing blackleg of plants and soft rot of tubers in storage and in the field giving rise to losses in seed potato production. In an attempt to improve potato health, biocontrol activity of known and putative antagonists was screened using *in vitro* and *in planta* assays, followed by analysis of their persistence at various storage temperatures. Most antagonists had low survival on potato tuber surfaces at 4°C. The population dynamics of the best low-temperature tolerant strain and also the most efficient antagonist, *Serratia plymuthica* A30, along with *Dickeya solani* as target pathogen, was

studied with TaqMan real-time PCR throughout the storage period. Tubers of three potato cultivars were treated in the autumn with the antagonist and then inoculated with *D. solani*. Although the cell densities of both strains decreased during the storage period in inoculated tubers, the pathogen population was always lower in the presence of the antagonist. The treated tubers were planted in the field the following growing season to evaluate the efficiency of the bacterial antagonist for controlling disease incidence. A potato endophyte *S. plymuthica* A30 protected potato plants by reducing blackleg development on average by 58.5% and transmission to tuber progeny as latent infection by 47%−75%. These results suggest that treatment of potato tubers with biocontrol agents after harvest can reduce the severity of soft rot disease during storage and affect the transmission of soft rot bacteria from mother tubers to progeny tubers during field cultivation.

Introduction

Bacterial soft rot, caused by pectinolytic *Pectobacterium* and *Dickeya* species (soft rot *Pectobacteriaceae*, SRP), is one of the major diseases of potato (*Solanum tuberosum* L.) and remains a concern of the seed potato production sector in many potato-growing regions of the world (Czajkowski *et al*., 2015; Pritchard *et al*., 2016). In Western and Northern Europe, *D. solani* outbreak was identified in 2004, since then the presence of *D. solani* isolates has been reported in a number of countries with significant economic and yield losses due to the easy spread of *D. solani* through seed potato trade and survival under the temperate climate (Laurila *et al*., 2008; Potrykus *et al.,* 2016). *D. solani* is considered to be more aggressive than *D. dianthicola* or *Pectobacterium* species with lower inoculum level as infection threshold (Toth *et al*., 2011). The most distinctive feature of the pathogenicity of *D. solani* and other soft rot bacteria is the production of diverse plant cell wall degrading enzymes (PCWDEs), including pectinases, cellulases and protease, which results in rotting of the

tissue and release of nutrients for the bacterial growth (Pritchard *et al*., 2016). During harvest, storage or transit, rotting of tubers is promoted by wounding, poor ventilation and high humidity. The contamination of seed tubers can result in the non-emergence, blackleg or wilting during the growing season, and in the cases of severe infection, rotting of the progeny tubers in the soil (Czajkowski *et al*., 2011). Soft rot bacteria can greatly affect potato tuber quality and market value. The main losses are almost exclusively linked to downgrading and rejection of potatoes during seed potato certification, where the limit for rejection is from 0 to 0.5% rotted tubers in storage (Directive 2014/20/UE and 2014/21/UE). Although it is difficult to determine the full extent of postharvest losses due to soft rot bacteria, conservative estimates indicate that losses probably reach hundreds of millions of euro (Degefu *et al*., 2013).

Currently, the control of soft rot pathogens relies on integrated pest management, including meristem culture in the production of certified seed, rigorous inspections and seed testing schemes, good sanitation during harvest and grading of tubers and the requirement for farmers to use certified seed. Other methods, such as tuber pasteurization, chemotherapy and thermotherapy have been found to be effective in experimental settings, but are rarely used in practice (Toth *et al*., 2011; Czajkowski *et al*., 2011). Despite the introduction of certified seed tubers and good hygienic practices, the problem continues in the potato industry, in part, due to spreading of the pathogens from rotten tubers to the rest of the yield during harvest and seed grading (Pérombelon, 2002). Some rotten tubers present in the harvest can contaminate a large number of healthy tubers, on which the bacteria can invade tuber lenticels and wounds, and survive latently for a long time until environmental conditions, including free water, oxygen availability and temperature, become suitable for disease development (Elphinstone & Pérombelon, 1986; Czajkowski *et al*., 2011). These latently infected potato

tubers are the most important source of disease inoculum due to the seed-borne nature of the pathogen. By increasing the population of antagonistic microbes on seed tuber, it might be possible to inhibit multiplication and disease transmission thereby reducing the incidence of soft rot and possible infection of the progeny tubers, the seed for the next generation. Seed treatments with beneficial microorganisms, including antagonistic bacteria that occupy the spermosphere and rhizosphere of the host, represent a suitable method for biocontrol and provide a unique opportunity for protection against seed- and soil-borne pathogens (O'Callaghan, 2016).

The main biocontrol strategies that have been tried against soft rot bacteria comprise the use of (i) bacterial antagonists affecting pathogen population via antibiosis, competition for nutrients and induction of plant systemic resistance (ii) natural predators such as bacteriophages (iii) quorum-quenching bacteria with the ability to inactivate quorum-sensing signal molecules produced by pathogens, and (iv) natural compounds such as plant defence elicitors, volatile compounds and essential oils (Czajkowski *et al*., 2011; Diallo *et al*., 2011). Several publications describe promising results for biocontrol of soft rot bacteria in potato with bacterial competitors or growth promoting rhizobacteria from the *Pseudomonas*, *Bacillus*, *Serratia*, *Lactobacillus*, *Lactococcus* genera and quorum-quenching bacteria belonging to the *Delftia*, *Ochrobactrum*, and *Rhodococcus* genera showing reduction of in soft rot incidence up to 50% (Jafra *et al*., 2006; Diallo *et al*., 2011; Czajkowski *et al*., 2012a). However, bacterial biocontrol agents (BCAs) have been most often evaluated in the greenhouse and rarely in the field, mainly because the uncontrollable environmental conditions, such as temperature and humidity, in the field do not always support the growth or the action of the BCA against the target pathogen thus leading to low efficiency of the control and inconsistent success (Czajkowski *et al*., 2011; Diallo *et al.*, 2011). Biological

control during crop storage would have significant advantages compared to the field, because the environmental conditions in the storage rooms are rigorously controlled, which may increase the likelihood of having efficient control. Because of the low storage temperatures, also the BCAs need to be cold-tolerant, but finding such strains may be challenging. Polar ecosystems may harbour antagonistic bacteria that have strategies for coping with cold environment, but their use in control of pathogens under low storage temperature has thus far been limited (Silva *et al*., 2018).

Since survival and strong colonization are two of the most important traits involved in the mechanism of action of BCAs and may influence the efficacy and consistency in storage treatments, it is necessary to develop selective quantitative monitoring methods that show a high level of specificity and sensitivity for tracking the presence of BCAs in the plant environment (Van Elsas *et al*., 1998). In contrast to classical techniques based on selective growth media, followed by biochemical, physiological assays and serological methods, molecular detection methods such as polymerase chain reaction (PCR) technique allows study of the pathogenic and antagonistic bacterial population in complex environmental samples directly, omitting laborious and slow cultivation procedures. Real-time PCR combines the advantages of the conventional PCR with the higher sensitivity of detection and quantification of specific DNA targets at each cycle. It is more rapid, consistent and reproducible with potential for multiplexing and it is well adapted to high throughput analysis (Postollec *et al*., 2011). Several authors have developed TaqMan real-time PCR assays for generic and specific detection of different BCAs directly from the biological material (López-Mondéjar *et al*., 2010; Czajkowski & van der Wolf, 2012c, Rotolo *et al*., 2016). Meanwhile, recent comparisons of soft rot *Pectobacterium* and *Dickeya* detection and identification assays outline the use of TaqMan real-time PCR assays in discrimination and characterization

of environmental samples with special emphasis on potato, mainly in seed certification programmes (Czajkowski *et al*., 2015). These identification and detection procedures are potentially useful for investigating the ecological behaviour and epidemiology of soft rot pathogens. This knowledge can help to develop effective and consistent biological control strategies. To date, two real-time PCR assays for detection and differentiation of *D. solani* exist, one is a species specific TaqMan assay developed by Pritchard *et al*. (2013) by exploitation of a set of whole genome sequences of various *Dickeya* species and the second is a TaqMan assay for specific detection of *D. solani* based on the *FliC* sequences (Van Vaerenbergh *et al*., 2012).

The objective of this study was to investigate the possibility of controlling soft rot disease of potato tubers in cold storage conditions using bacterial antagonists originating from different collections and sources. Furthermore, the population dynamics of the latent bacterial pathogen and the best antagonist were studied on the potato surface using quantitative TaqMan assays to understand their survival during storage at low temperature and the following summer in the field.

Materials and Methods

Bacterial strains, media, growth conditions and plant material

Dickeya solani strain Ds0432-1, used throughout this study, was isolated in Finland from diseased potato stem (Laurila *et al*., 2008). The other *D. solani* strains included type strain IPO 2222 isolated in the Netherlands, IPO 3337 (RNS 08.23.3.1.A) and IPO 3337 (RNS 07.7.3.B) in France and IPO 2276 (IFB 0099) in Poland, all isolated from diseased potato. In addition, *D. solani* IPO 2019 isolated from a diseased hyacinth bulb in the Netherlands was included. These isolates were distributed during European-wide collaboration on *D. solani*

taxonomy (van der Wolf *et al*., 2014b) and stored in the culture collection of the Department of Agricultural Sciences. *Pectobacterium carotovorum subsp. carotovorum* strain SCC1 (Pirhonen & Palva, 1988) and *P. parmentieri* strain SCC3193 (Pirhonen *et al*., 1991), used as a target strain for testing antagonistic activity of bacterial isolates were provided from same collection. *P. atrosepticum* strain SCRI1043 was a generous gift from Dr. Ian Toth at the James Hutton Institute. Stock cultures of the bacteria were maintained deep-frozen (−80°C) in 30% glycerol. Suspensions were prepared by streaking the stock cultures grown for 48 h at 28°C onto Luria-Bertani (LB) Agar (Merck). One colony was sub-cultured in LB broth by overnight on a rotary shaker (200 rpm) at 28°C. Bacterial cells were collected by centrifugation (5000×*g* for 10 min) and washed two times in sterile Ringer solution (pH 6.9 ± 0.1 Merck) to remove media and any extra-cellular components released by the bacteria. The bacteria were then diluted in Ringer's buffer to a final concentration of approximately 10^6 CFU ml^{-1} (determined by plating), unless otherwise stated. Potato tubers of cultivars Van Gogh, Bintje and Timo used in the experiments were provided by Finnish Seed Potato Centre Ltd. and tested negative for *Dickeya* and *Pectobacterium*.

Collection of candidate bacterial antagonists

Bacterial antagonists were isolated from rotted potato tubers and from potato rhizosphere microflora by the dilution agar plating method. The samples were plated on R2A medium (Merck), Tryptic soya agar (TSA) (Sigma-Aldrich), King's B medium and LB agar plates supplemented with 200 µg ml^{-1} cycloheximide (Sigma-Aldrich) to prevent fungal growth (Jafra *et al*., 2006; Czajkowski *et al*., 2012a). Briefly, one gram (fresh soil or rotting potato tissue) of each sample was mixed with 10 ml of a 0.25 strength Ringer's buffer (Merck) containing dithiothreitol (final concentration 0.075 %) as antioxidant, and 100 µl of the appropriate dilutions was spread onto culture media and incubated at 28ºC for 24−48h. In the

end, a total of 150 bacterial isolates representing most dominant colony types were picked and sub-cultured twice to ensure purity and then maintained in 30% sterilized glycerol at -80°C. In addition, a collection of 53 candidate antagonists belonging to different bacterial species were provided from collection of different laboratories (Reiter *et al*., 2002; Czajkowski *et al*., 2012b; Krzyzanowska *et al*., 2012). *Serratia plymuthica* A30 was obtained from Dr. Jan van der Wolf, who has the IP rights for its use and thus this strain is not freely available. The other antagonists were a generous gift by Prof. Sylwia Jafra at the University of Gdańsk and Dr. Angela Sessitsch at AIT Austrian Institute of Technology, or were isolated in the group of Dr. Minna Pirhonen. Also endophytic bacteria isolated from three arctic plant species, *Oxyria digyna*, *Diapensia lapponica* and *Juncus trifidus*, growing in the low Arctic areas were included in the screening (Nissinen *et al*., 2012). For preparation of inocula for the experiments, candidate antagonistic bacteria were grown overnight in liquid LB medium on a rotary shaker (200 rpm) at 28ºC and bacterial cells were harvested by centrifugation at 5000×*g* for 10 min. The pellets were re-suspended in sterile Ringer solution and brought to a concentration of 10^9–10^{10} CFU ml^{-1} as determined by plating.

Screening for antagonist activity against soft rot pathogens

Three screening steps were implemented to assess the relative inhibition efficacy of the different isolates to select the best candidate isolates (potential BCAs). Firstly, all collected isolates were tested for antibiosis (*in vitro* inhibition growth ability) against the four soft rot species in an overlay plate assay. For this, one drop (10 µl) of well-grown overnight culture of the antagonist to be tested was spotted on the solidified agar surface of TSA medium that were previously covered with 20 ml of soft top agar (LB supplemented with 0.7% (w/v) agar) containing 200 µl of each pathogen cell suspension and pre-warmed to 45–50ºC. Plates were incubated at 28°C for 48 h. Development of inhibition zones around the bacterial inoculation

points indicated antibiotic or bacteriocin production by the tested isolates. Secondly, the isolates were tested for siderophore production as an ability to chelate iron ions using chrome azurol S-agar plates (Schwyn & Neilands, 1987). The diameter of the orange halo around the colonies was measured. Thirdly, all selected isolates were tested for their ability to reduce tissue maceration on potato tubers as previously described by Jafra *et al*. (2006) with some modifications. Whole potato tubers were used instead of potato slices to mimic postharvest treatment. Potato tubers (cv. Van Gogh) were surface sterilized using 5 % sodium hypochlorite for 10 min and then rinsed twice in sterile water. After air-drying for two hours, two wells (5 mm diameter × 8–10 mm depth) were drilled with a cork borer at the equatorial site of each tuber. Each wound site was inoculated with 50 µl aliquots of the antagonist suspension, and after 2 h of incubation at ambient temperature (22ºC), with 50 µl of the pathogen suspension or water. The control (pathogen only) tubers were inoculated with 50 µl of distilled water instead of the antagonist before the pathogen inoculation. For negative controls, each hole was inoculated with 100 µl of sterile water. To prevent suberization, the bored tissue was put back in the cylindrical wound after 2 mm pieces had been cut off to compensate for volume of the inoculum. Wounds were covered with Vaseline to avoid desiccation. All the inoculated tubers were located in dark moist polyethylene boxes and incubated at 22ºC for 72 h. Three replications of five potato tubers were used for each tested strain and the experiment was independently repeated. Results from two experiments were averaged. After incubation, the diameter of the maceration zone around each well was measured and soft rot reduction was determined. Strains able to reduce rotting of potato tuber tissue by at least 50% of the control were selected for further study. The bacterial antagonists that provoked symptoms on potato tuber were excluded (data not shown).

Effect of temperature on viability and colonization of antagonists on stored tubers

The isolates that exhibited the highest degree of soft rot suppression in the potato tuber maceration assay against at least two of the pathogens were evaluated for their ability to colonize the tuber surface at various temperatures. For this, spontaneous rifampicin-resistant mutants (Rif^+) were produced under antibiotic pressure by spread plating wild-type liquid cultures in log growth phase onto LB agar containing rifampicin at 100 µg ml^{-1} concentration. All these mutants were rescreened for antagonistic activity by potato tuber assay as described above to make sure their efficiency was not altered (data not shown). Potato tubers were coated with the Rif^+ antagonist isolates by incubating them with bacterial suspensions (ca. of 10^9–10^{10} CFU ml^{-1}) or distilled water as control for 30 min and then they were stored at three different storage temperatures (20°C, 15°C and 4°C). The 15°C treatment was representative of the temperature maintained during potato wound curing while the 4°C and 20°C treatments simulated the temperature normally used during cold storage and handling areas of tuber packinghouses, respectively. There were three replicates of five potatoes per isolate and bacterial enumerations were performed 30 days post-inoculation (dpi). After incubation, five grams of peel (2–3 mm depth) from each of the 5 tubers were transferred into extraction bags (Bioreba) and homogenized with 10 ml of 0.25 Ringer's solution. Serial dilutions of the resulting suspensions were plated on LB agar supplemented with cycloheximide (200 µg ml^{-1}) and rifampicin (100 µg ml^{-1}). Following 48 h of incubation at 28°C, bacterial colonies were counted and the mean value of colony forming units per gram of peel (CFU g^{-1}) was determined. The experiment was conducted twice.

Effect of tuber bacterization with antagonists on attenuation of soft rot development

The seven most promising candidates with the higher levels of colonization and growth in the previous experiments were selected to evaluate their efficacy to protect wounded potatoes against soft rot bacteria in storage conditions. Potato tubers (cv. Van Gogh) were surface sterilized, weighted and stabbed 20 times midway between the apical and stem ends with a multipoint inoculator (2 mm diameter needles) to a depth of ca. 2 mm. One set of potato tubers were dipped in suspensions of antagonistic bacteria for 30 min and after 2 h drying in a flow cabinet, inoculated with *D. solani* by spraying inoculum suspensions with an atomizer (0.1 ml/tuber). A second set of potato tubers, first dipped in sterile water instead of the antagonist and then sprayed with the pathogen, served as a control. A third set of tubers treated with water only was kept as negative control. Inoculated tubers were air-dried and kept in air tight plastic storage containers lined with filter paper soaked with sterile distilled water to maintain high humidity. Containers were placed in a randomized order within a storage room at 15°C. Visual observations were made after 1, 5, 15 and 30 dpi. Each sample replication consisted of 20 tubers selected to have a total weight of approx. 2.5 kg, and there were 5 replications for each treatment and time point. The experiment was independently repeated. Data on soft rot disease development were recorded in two ways: i) Disease severity was estimated using the percentage weight loss after rotting tissues were removed according to the formula described by Abd-El-Khair and Karima (2007). For each replicate, the rotted tissue of the tubers was removed, and the healthy parts of all 20 tubers were weighed together. The change between the initial weight before treatment and the weight after discarding the infected tissues was divided by the initial weight and multiplied by 100. ii) The soft rot disease severity was assessed based on visual assessment of maceration area of individual tubers using a scale of 0 to 5, where 0 = no rot, for negative control to 5 = complete rot, as described by Colyer and Mount (1984). Results from both experiments were

averaged. In method (i), the efficacy of antagonists to suppress soft rot development was expressed as a decrease in disease severity based on the percentage of weight loss and was determined using the formula described by Hajhamed *et al.* (2007): *Percentage of disease reduction (PDR) = (disease severity/loss (by weight) in control - disease severity/loss (by weight) in treatment) / disease severity in control × 100).* Statistical analyses (ANOVA) were carried out using the general linear model (GLM) of the SAS statistical package, and means were separated using least significant differences (LSD) test at $P < 0.05$.

Biocontrol efficiency of *Dickeya solani* strains by *Serratia plymuthica* strain A30

The endophytic bacterium *S. plymuthica* strain A30 was selected for further experiments on the basis of inhibition of tissue maceration by soft rot bacteria and superior survival under low temperature conditions. The effects of biocontrol strain A30 to suppress potato soft rot maceration caused by several *D. solani* strains was evaluated in potato tuber assays similarly to the trial described above. Six *D. solani* strains, Ds0432-1, IPO 2222, IPO 0019, IPO 3337 (RNS 08.23.3.1.A), IPO 3337 (RNS 07.7.3.B) and IPO 2276 (IFB 0099), isolated in 4 countries, Finland, The Netherlands, France and Poland, from 2 host plants, potato and hyacinth, were included in the analyses. Quantitative determination of average weight of rotting tuber tissue (in grams) was collected after 5 and 15dpi at 15°C in a humid box.

Quantitative TaqMan real-time PCR detection of *Dickeya solani* and *Serratia plymuthica A30*

S. plymuthica populations in potato samples were quantified using a TaqMan assay based on the single-copy *luxS* gene (Czajkowski & van der Wolf, 2012c). Populations of *D. solani* were quantified using a TaqMan assay based on the primer pair SOL-Cf/SOL-Cr as described by Pritchard *et al*. (2013). Oligonucleotides that were used as primers and probes in the

assays are listed in Supplementary Table 1. All TaqMan assays were performed in singleplex format, using the LightCycler®480 Probes Master reaction mix on the LightCycler®480 instrument with 96 well optical plates (all from Roche Diagnostics GmbH). Each real-time PCR experiment included technical triplicates. A final volume of 20 µl was used per reaction that contained 10 µl of 2x LightCycler® 480 Probes Master, 0.3 µM of primer (each), 0.1 µM of probe, and 5 µl of DNA template. Cycling conditions were as follows: an initial denaturation step of 10 min at 95°C, followed by 40 cycles each of 15 s at 95°C, 1 min at 60°C and 1 s at 72°C. A cooling step at 40°C during 10 s was done to terminate the reaction. All reactions were carried out alongside a non-template control containing all reagents except DNA (external negative control) to test for cross-contaminations. Positive controls contained DNA from a reference strains (*Dickeya solani* Ds0432-1 or *Serratia plymuthica* A30), and negative controls corresponded to the PCR water control. For all samples, an additional TaqMan assay with a COX primer-probe-set (Cox-F/R,Cox-P) (Supplementary Table 1) was used in a separate well as an amplification control for confirmation of an efficient DNA extraction from plant material and recognition of eventual inhibition of the PCR. The PCR was performed in 20 µl reaction mixtures which contained 0.6 µM of each primer, 0.3 µM of the probe and 5 µl of sample DNA. Cycling conditions were similar to those described above. Threshold cycle (Ct) values for each sample were used to calculate the cell density of the target (log CFU/g) based on a standard curve prepared by supplementing samples with a serial dilution of a suspension of *D. solani* or *S. plymuthica* A30.

Preparation of standard curves for TaqMan assays

Two different standard curves to quantify the pathogen and antagonist population levels were generated using two sets of primer pairs and probes for the mathematical conversion of Ct values to bacterial cell numbers. A standard curve was obtained by using purified genomic

DNA of each bacterial strain, extracted from a ten-fold serial dilution of bacterial suspensions ranging from 10^1 to 10^7 cell ml^{-1} in Ringer's solution in triplicates. Ringer's solution without bacteria was used as a negative control. Bacterial densities were determined using dilution plating on TSA media for *S. plymuthica* and CVP for *D. solani*, followed by incubation at 28°C for 24 h. Genomic DNA from each bacterial serial dilution was extracted using the DNeasy Blood & Tissue Kit (Qiagen) for Gram-negative bacteria according to the manufacturer's instructions. Five microliters of genomic DNA was used per reaction. A second set of standard curves were made using spiked potato samples. Fifty g of potato peels were homogenized with 100 ml of a 0.25 strength Ringer's solution. Fifteen sterile plastic tubes were filled with 15 ml of the homogenate. Seven of them were inoculated with 1 ml of ten-fold serial diluted suspension of *S. plymuthica* or *D. solani*, ranging from 10^1 to 10^7 cell ml^{-1}. A non-inoculated sample was used as a negative control. DNA was extracted from each homogenate as described above. In spiked samples with *D. solani*, the homogenate were first incubated in the pectate enrichment broth prior to adding bacteria. The potato homogenates that were used in the experiments were proven to be free of target cells (Ct values> 38.7).

Statistical analysis of TaqMan assays

Real-time PCR data were attained from the LightCycler® 480 software for calculation of Ct values and standard deviations (SDs). Standard linear regression analysis was done on the log bacterial densities (log cell g^{-1} or log cell ml^{-1}) versus the average Ct values. PCR amplification efficiency (E) was calculated from the slopes of the regressions according to $E = [10^{1/(-slope)}]-1$. The Ct values of the TaqMan assays were normalized by multiplying with a normalization correlation coefficient obtained from the positive internal control reaction according to Li *et al*. (2009). The effects of storage room (Rooms 1–3), cultivar (Bintje, Timo, Van Gogh), treatments, incubation time (dpi 1–270) and replicate (15 replicates) on

CFU values for *D. solani* and *S. plymuthica* were studied statistically by using Generalized Linear Models (Proc GLM procedure available in SAS 9.3, SAS Institute Inc.). All categorical parameters (storage, cultivar, treatment, incubation time and replicate) and all their 2-, 3- and 4-way interactions were included into the saturated model. Furthermore the effects of storage room, treatments, incubation time and replicate on CFU of *D. solani* and *S. plymuthica* were studied separately for each cultivar, Bintje, Timo and Van Gogh. The statistical differences between the main effects for each cultivar were indicated using Tukey's studentized range test (HSD) at the 0.05% risk level.

Validation of the real time PCR assays of potato samples spiked with S. plymuthica and D. solani

Standard curve data points were used to determine the real-time PCR performance and detection limits. The limit of detection (LOD) was determined in 20 replicates for which independent serial dilutions in potato homogenate were prepared. LOD was calculated by the lowest cell density giving consistent positive amplification results in 95% tested replicates (Bustin *et al*., 2009). The assay variability was established by repeated testing of samples containing various densities of *S. plymuthica* and *D. solani*. The repeatability (intra-assay variation) was evaluated using DNA extracted from the spiked potato samples with dilutions of the bacterial suspensions in densities of $5.0 \times 10^1 – 5.0 \times 10^6$ cell g^{-1} for *D. solani* and $2.5 \times 10^2 – 2.5 \times 10^6$ cell g^{-1} for *S. plymuthica.* Samples were measured 8 times each, within one TaqMan assay. The reproducibility (inter-assay variation) was evaluated with the same samples as for the repeatability but in eight independent real-time PCR experiments performed on different days. For each real-time PCR run (*S. plymuthica* and *D. solani* real-time PCR assays), each dilution point was tested in duplicate and the mean standard curves were used for quantity estimation. Potato homogenate without added bacteria served in all

experiments as negative controls. For both repeatability and reproducibility the coefficients of variation (CV) for the Ct values were experimentally determined.

Effect of potato macerate and control of inhibition

The effect of plant material on the DNA amplification of the target organisms was tested with spiked samples containing known amount of bacterial targets (10^6 cells g^{-1}) added to the potato homogenates of three cultivars. DNA was extracted from each homogenate as previously described in three replicates and used for the both TaqMan PCR assays. The Ct values were analysed and the CVs were calculated from the Ct values. In addition, the Ct values for the non-spiked and spiked potato extract samples were compared to determine the Ct cycle cut-off value. In our approach, the range of cut off values was empirically determined as described by Chandelier *et al*. (2006). Non-inoculated homogenate peel samples were initially cultured in enrichment media and found to be free of both bacteria, thus potato samples were validated as non-contaminated.

Survival and population dynamics of pathogen and antagonist during storage

The ability of strain A30 to survive on potato peel and prevent soft rot caused by *D. solani* was characterised after a post-harvest treatment of potato tubers utilizing TaqMan real-time PCR. The storage period commenced on 1st October and extended to 30th April (seven months), and the remaining tubers were planted the next growing season (15th May) in the field. Potato tubers cvs Van Gogh (medium resistant), Bintje (susceptible) and Timo (highly susceptible) were selected for the trials. Four treatments were applied: (i) tubers treated with both *S. plymuthica* and *D. solani*, (ii) tubers treated with *S. plymuthica* only, (iii) tubers treated with *D. solani* only and (iv) tubers treated with water (as control). Based on the treatments, tubers of the three potato varieties were arranged into four groups. The first and

the second groups were immersed in the antagonist suspension (one ml bacterial suspension g¯1 seed tuber) for 30 min followed by 12 h incubation at room temperature to allow bacteria to penetrate the tuber lenticels and wounds. Subsequently, the treated potato tubers were sprayed by an atomizer with a suspension of *D. solani* (i) or water (ii) to a final dosage of about 0.1 ml/tuber. The third group was first immersed in water as described above and then spray-inoculated with the pathogen suspension (iii). The fourth group was treated with water as a control. All tubers were dried in a flow cabinet overnight and the next day stored in potato paper sacks on pallets to keep the sacks off the floor. The monitoring was done 1, 3, 7, 14, 21 and 30 dpi and was continued monthly until the end of April (60, 90, 120,180, 240 and 270 dpi). The experiment was independently carried out in three storage rooms at 4–6ºC and 80% relative humidity.

During the storage period, treated tubers were sampled for DNA extraction. At each sampling time for each cultivar/treatment, three lots of 5 tubers were picked from different positions of tuber lot bags under the surface layer and separate DNA extractions were processed individually. Peel strips (2–3 mm thick) including the stolon and rose ends and a small plug of tissue from the stolon end (5–10 mm deep and wide), were removed with a disinfected hand-held potato peeler. Five gram of peel material from each sample was homogenized with 10 ml of 0.25 strength Ringer's buffer containing 0.075% dithiothreitol and pulverized using a Bio-Gen PRO200 Homogenizer with 10 mm × 115 mm saw-tooth generator probe (Model Standard universal homogenizer package, PRO Scientific, Inc.) for 60s at high speed. The blade was disinfected between each sample extraction with 70% ethanol and then rinsed in sterile water. Homogenized samples were centrifuged at 4 ˚C for 10 min at 100 ×g to remove large particles. Supernatant above the sediment was gently collected into 15 ml falcon tubes and centrifuged at 3743 ×g for 15 min at 4˚C and the bacterial pellet used either directly or

after storage at -20˚C for DNA extraction using Sbeadex maxi plant kit (LGC Genomics) in conjunction with Kingfisher Magnetic Particle processor with 96 deep well head (Thermo Scientific) according to the manufacturer's recommendations. DNA was eluted in 100 µl of elution buffer and stored at -20 ˚C. To enrich *D. solani* in plant extracts (treatments i, iii), the homogenized suspensions from tuber samples were supplemented with equal volumes of sterile Pectate Enrichment Broth (PEB) (Pérombelon & van der Wolf, 2002) and incubated at 36 ˚C for 48h with constant agitation in anaerobic conditions. Bacteria from extracts were then centrifuged at high speed and total DNA extraction and TagMan real-time PCR assay were conducted as described above.

Field experiment with treated tubers

The storage experiment was continued the following growing season to evaluate the influence of tubers treatment with *S. plymuthica* A30 on the health of progeny tubers and potato plants in the field. In the middle of May, the potato seed tubers remaining from the storage experiment were manually planted in experimental plots with a completely randomized block design. To eliminate the bias effect of the environmental conditions, plots of 100 tubers (25 tubers in four rows) per treatment were planted with three replications. Spacing of seed tubers was 30 cm within rows and 1 m between rows. During the growing season, plants were visually inspected for development of symptoms including non-emergence (pre-emergence rot of tubers), wilting and chlorosis of leaves, blackleg or browning at the stem base (blackleg symptoms), rotting of internal stem tissue and plant death. The number of plants in each plot as well as the number of plants with disease symptoms in each treatment was recorded and symptomatic stems from each plant with symptoms were tested for the presence of *D. solani* by TaqMan assay. In late August, all tubers from each plant were individually weighed, and every tuber was visually assessed for soft rot symptom and the tubers with external symptom

were counted. In addition, two subsamples of 25 asymptomatic daughter tubers were collected from asymptomatic plants in each treatment/cultivar, and tested for the presence of *D. solani* by TaqMan assay to determine the proportion of latently infected tubers. After surface-sterilization, deep tissue sample from the stolen end of each tuber was removed using a sterile scalpel and pooled into 10 ml of 0.025 Ringer's solution and then kept for 2 h at room temperature with shaking at 200 rpm. Two hundred µl of the macerates was mixed with 1800 µl of PEB medium and incubated at 28°C for 48 h to perform the enrichment, after which the bacteria were collected by centrifugation and total DNA extraction was done using a Genomic DNA purification kit (Qiagen). TaqMan PCR assay for *D. solani* was performed as describe above. In addition, the presence of *S. plymuthica* on progeny potato tubers was detected by TaqMan assay to determine the survival potential of the antagonist. Data were subjected to analysis of variance (ANOVA) using SAS software. Means were subsequently compared using GLM Procedure Tukey's Studentized range test (HSD) of values at the 0.05% risk level.

Results

Screening for antagonist activity against soft rot pathogens

At the onset of this study, 150 bacterial isolates from the potato rhizosphere and rotten potato tubers, collected from a previous experimental project, were tested for growth inhibition using *D. solani* (data not shown), and based on the results, 21 strains were chosen for further characterization in this work. In addition, 53 bacterial strains isolated from endo- and ectophytic microenvironments of various host plants were obtained from other laboratories. The most common genera among them were *Pseudomonas* (49%), *Bacillus* (11%), *Serratia* (8%), *Sphingomonas* (8%), *Paenibacillus* (5%), *Acinetobacter* (3%) and *Pedobacter* (2%).

In further characterization of the putative antagonists, the selected 74 isolates were tested for growth inhibition of four soft rot pathogens and production of siderophores. Among the tested isolates, 31 strains exhibited the capacity to inhibit the growth of at least one of the tested pathogens (diameter of inhibition zone range from 5 to 26 mm) whereas 19 isolates produced an orange/pink halo on the CAS agar plates (data not shown). The other isolates showed little or no activity. In the *in vivo* test, all isolates were tested for the ability to reduce tissue maceration in potato tuber assays caused by four soft rot bacteria, and 13 biocontrol agents protected potato tubers from the development of soft rot and suppressed the amount of tissue maceration at various levels (Table 1). Seven out of thirteen bacterial strains, *Serratia plymuthica* (A30), *Alcaligenes faecalis* (R8), *Pseudomonas fluorescence* (P301), *Flavobacterium* sp. (A14), *Pseudomonas donghuensis* (P482), *Pseudomonas* sp. (S2H1) and *Bacillus subtilis* (P48) were considered effective in reducing the soft rot caused by *Pectobacterium* spp. and *D. solani* and were characterized further.

Effect of temperature on viability and colonization of antagonists on stored tubers

To determine which of the identified antagonist strains had the ability to grow and persist at high levels on the surface of tubers during long-term storage, we carried out studies with spontaneous antibiotic resistant mutants using experimentally inoculated potato tubers at temperatures likely to occur during storage. In all experiments, controls were included to verify whether the inoculated bacteria were recovered. Control tubers inoculated with Ringer's buffer only yielded no colonies morphologically identical with the used bacteria. Initial inoculum of each of the used antagonist was approximately 8 log CFU ml^{-1} at 28°C. The tested isolates retained viability at 20°C and also showed sufficient colonization on potato peel with population level in the range of 4.1–5.8 log CFU ml^{-1} at 15ºC after 30 dpi (Table 1). In terms of the behaviour during storage at 4ºC, significant reduction in CFUs on

potato peel was evident by the strains A14, P301 and P482. The survival or growth of these isolates was limited by lower temperatures but even at 4°C they were still present at low CFU. *Bacillus subtilis* P48 and *Alcaligenes faecalis* R8 were shown to be mesophilic bacteria that could survive at 15–20°C but suffered significant mortality at 4°C, while strains A30 and S2H1 were both mesophilic and psychrotolerant as they survived between 4–20°C with almost constant CFU numbers during the 4 week storage period.

Effect of tuber bacterization on suppression of soft rotting

To evaluate the effectiveness of the antagonistic bacteria to protect wounds on potato skin against *D. solani*, as might happen after harvest and grading, experiments were conducted using superficially wounded tubers at 15°C to mimic curing temperature. The percentage of lost weight and the visual ratings of treated tubers with all tested antagonists were significantly lower than in the treatment with the pathogen alone. All the tested antagonists delayed and reduced the incidence of soft rot on the stored tubers (Table 2). Strain A30 was the most effective, reducing disease by 93.2%, while R8 and P482, the second best antagonists in this experiment, reduced disease development by 85.3% and 82.2%, respectively. The other antagonists provided 80.3–65.1% PDR on the pre-inoculated tubers after 30dpi. No symptoms were observed on the negative controls, whereas 58.5% of the tubers rotted in the positive controls.

Biocontrol efficiency of *Dickeya solani* strains by *Serratia plymuthica* A30

In biocontrol assays with *S. plymuthica* A30 and six *D. solani* strains from 4 countries and two hosts, a substantial decrease in soft rot maceration by all *D. solani* strains was observed when the antagonist treatments were compared to the treatment with the pathogens alone (Supplementary Figure 1 A, 1 B). After 15dpi incubation, the mean weights of rotted tissue

ranged from 20 to 14 g per tuber in treatment with the *D. solani* strains alone, while in the presence *S. plymuthica* A30 weight of macerated tissue reduced to 3.8 to 1.7 g per tuber. Thus, A30 exhibited significant biocontrol ability on all tested soft rot pathogens and fulfilled an important requirement of a potential biocontrol agent.

Quantitative TaqMan real-time PCR detection of *Dickeya solani* and *Serratia plymuthica A30*

Real-time PCR amplification efficiencies and detection limits

Based on the efficient antagonist activity of *S. plymuthica* A30 against *D. solani*, survival and population level of both strains were characterized during storage period using TaqMan assays. Standard curves were generated in parallel with DNA extracted from 10-fold serial dilution of the bacterial suspension of *S. plymuthica* or *D. solani* strain in sterile water or potato tuber homogenate. The densities used were determined by plate counting. Both assays were able to detect at least 10^2 CFU per ml of bacterial suspension from their respective target species. The two standard curves exhibited a very similar linearity across six orders of magnitude from 10^2 –10^7 cell ml^{-1}, with high regression coefficient of 99.33% for *D. solani* and 98.76% for *S. plymuthica* A30 (Figure 1). The lowest dilution (10 CFU ml^{-1}) produced less consistent results, and was not used for calculation of the standard curves. However, when DNA from the bacteria spiked in potato extract were used, differences in the sensitivity were observed in both assays. The species-specific *D. solani* assay was able to detect target bacteria in spiked potato samples up to 5.0×10^2 cell g^{-1} of potato peel, which corresponds to 10 cells per reaction. The *S. plymuthica* assay was less sensitive, in spiked samples a minimum of 2.5×10^3 cell g^{-1} of potato peel, equivalent of 50 cells per reaction, could be reliably detected (Figure 1, Supplementary Table 2).

Validation of the real time PCR assays for spiked potato samples

The intra- and inter-assay variation of each TaqMan assay was determined using DNA extracted from the spiked potato samples in eight duplicates. The CV of the Ct values for the eight different intra-assay experiments ranged from 1.07 to 2.83% for *D. solani* assay and from 0.76 to 3.39% for *S. plymuthica* assay. Most variation was observed in the highest dilutions. The CV of the Ct values for the eight different inter-assay experiments, including the DNA extraction procedure was from 1.82 to 4.38% and from 1.51 to 3.72% for *D. solani* and *S. plymuthica* assays, respectively (Supplementary Table 3). The repeatability and reproducibility were high for both assay with relatively low CV% value.

Effect of potato macerate

To control for the potential inhibitory effect of the potato material on the TaqMan assay, equal concentrations of *D. solani* and *S. plymuthica* A30 cells were subjected to DNA extraction and PCR amplifications were performed in the presence of three different potato macerates. The average Ct values for both assay were independent from the extracts used (Table 3). Since the CV% was 0.78% and 1.8% for the *D. solani* and *S. plymuthica* assays, respectively, it can be concluded that the presence of potato material did not significantly influence the amplification of target DNA of both assays.

Determination of the Ct cycle cut-off value

DNA extracted from non-spiked (NS) healthy tuber homogenates of three potato cultivars were used in order to establish the Ct value above which a sample is considered negative (Ct value cut-off) (Table 3). The mean Ct value observed for the different cultivars of non-spiked plant substrates was between 37.4 and 38.7. Ct value cut-off was calculated using the equation NCt = M - 3× SD, in which M is the mean Ct value calculated from healthy plant

DNA, and SD is the corresponding standard deviation (Chandelier *et al*., 2006). Mean Ct value for non-spiked potato tuber cv. Bintje, Van Gogh and Timo was 38.06, 38.90 and 38.42, respectively. Therefore calculated NCt was close to 35.3. In our experimental conditions the Ct cycle cut-off values were classified as follows: Ct <35 was considered as positive; Ct values of 35–36 were considered as inconclusive and in that case samples were retested. Ct 38–40 denoted negative reactions.

Survival and population dynamics of pathogen and antagonist during storage

During the cold storage of potato tubers, changes in the populations of *D. solani* and *S. plymuthica* A30 were monitored using strain-specific qPCR from 1st October (at the beginning of the storage period) until April of the next year. In the statistical analysis, time of sampling and treatment were statistically most significant variables (Supplementary Table 4). After inoculation, the population of *D. solani* in tubers treated only with the pathogen was continuously decreasing to a density of about 5×10^{2} cell g^{-1} tuber from mid-October till the end of November in all potato cultivars (Figure 2). In December and January, *D. solani* was not detected because its population decreased below the detection level. The incidence of the pathogen in the stored tubers increased during the new year and become detectable again in February (cv. Timo) and March (cv. Van Gogh and Bintje) and subsequently remained stable until the end of the experiment (270 dpi). This increase in *D. solani* densities between 180 and 270 dpi coincided with the emergence of soft rot incidence on the inoculated tubers. In contrast, in tubers treated first with the antagonist A30 and then with *D. solani*, the quantified numbers of *D. solani* decreased rapidly during the first weeks of storage and did not increase significantly later in the storage period (Figure 2). When the seed tubers were treated with the antagonist A30 only, its population decreased slightly in 30 days to average 2.5×10^{3} cell g^{-1} tuber and remained at this level till 270 dpi. A similar tendency in the population dynamics

was observed in all cultivars. In potato tubers treated with the antagonist and the pathogen, population dynamics of A30 followed a similar patter as above but remained in higher cell density ($10^4 – 10^5$ log cell g^{-1} tuber) compared to tubers treated only with the antagonist. No pathogenic *D. solani* or antagonistic *Serratia plymuthica* A30 were detected on negative control (water) samples. In all experiments, no symptoms occurred in the treatments that did not include the pathogen. However, on average 9.7% (SD 2.4, $P \leq 0.05$) of the tubers inoculated with *D. solani* rotted during the storage period, whereas only 2.0% (SD 0.9, $P \leq 0.05$) of the tubers inoculated with the pathogen and the antagonist showed rotting, suggesting that the antagonist treatment was able to reduce but not eliminate the rotting of the tubers during storage.

The COX probe-primer set targeting the potato cytochrome oxidase gene yielded stable Ct values of around 21.72 ± 0.19 for the DNA extracts from all the stored potato samples, indicating an overall high quality of DNA extraction. An inhibition of the internal control amplification was observed in only 1% of the examined samples, which were consequently removed from the quantification study.

Field experiment with treated tubers

To study the performance of the seed tubers treated with the antagonist and the pathogen, the remaining tubers from the storage treatment were planted in the field and their condition followed throughout the summer. The average percentage of symptomatic plants showing blackleg or internal discoloration and hollowing of stem tissue was determined at midseason (45 days post tuber planting). Severity and incidence of blackleg and soft rot disease were generally greater in all three cultivars in plots of tubers which had been inoculated with *D. solani* compared to plots that had received treatment with antagonist bacteria (Figure 3a).

There was no significant difference between cultivars Timo and Bintje in terms of the average percentage of symptomatic plants. In both cultivars, approximately 48% of plants inoculated with *Dickeya* expressed blackleg symptoms. Cultivar Van Gogh showed less disease incidence (29%) and more tolerance compared to other cultivars. The antagonist reduced significantly the percentage of symptomatic plants in all cultivars, resulting in 12, 19 and 21% diseased plants in cultivar Van Gogh, Bintje and Timo, respectively. The number of surviving plants was significantly greater after inoculation of the plants with *S. plymuthica* A30 along with *D. solani*, when compared to the *D. solani* treated control, although numbers were still lower than for the non-inoculated control. *Dickeya* was present in the water treated plants (negative control) at a very low level. It is possible that the seed tubers had a latent *D. solani* infection, the bacteria spread from plant to plant during summer or the field soil was contaminated by *D. solani* from previous years.

PCR detection of *D. solani* was performed on asymptomatic tubers harvested from asymptomatic plants which were cultivated in the presence and absence of the biocontrol strains (Figure 3b, c, d). Seed tubers treated with the biocontrol strains led to reduced pathogen propagation in the tuber progeny. Strain A30 acted differentially as potential biocontrol agent in cultivars with different susceptibility. The proportion of asymptomatic tuber infection (latent infection) was greater in the more susceptible cultivars Bintje and Timo compared to cultivar Van Gogh. A similar reduction trend was observed in the average percentage of latently infected and symptomatic tubers in all three cultivars.

Discussion

Soft rot and blackleg of potatoes are problems that are difficult to control and cause significant economic losses both in seed and ware potato production. Biocontrol has been suggested as a control method for soft rot bacteria, however, it has been most often evaluated

in the control of the disease in the greenhouse and rarely in the field or storage (Czajkowski *et al*., 2011). The present work was carried out to develop methods for biocontrol of soft rot during low temperature storage, which makes it important to select cold-tolerant BCAs. Therefore, possible antagonists were obtained from various sources, including endophytic bacteria isolated from arctic plant species, to be able to identify cold-tolerant antagonists.

The putative antagonist strains in the collection were evaluated for their ability to inhibit the growth of four bacterial strains, representing the four most common species present in Finland, and as a result, many promising strains with broad inhibition ability were identified. In the next phase of screening, whole tuber assays were used to identify the strains that were most adept in suppressing soft rot development in potato tubers. Thirteen bacterial antagonists among the collection had a significant effect on all four soft rot bacteria. Although *in vitro* antagonism does not necessarily correlate with efficacy on a plant assay, seven bacteria in the genera *Serratia*, *Alcaligenes*, *Pseudomonas*, *Flavobacterium* and *Bacillus* showed a good correlation between Petri dish growth inhibition assay and the efficacy to reduce soft rot, especially *D. solani* and *P. carotovorum*. Among them, *Serratia*, *Pseudomonas*, and *Bacillus* have been previously suggested to be efficient antagonists against soft rot bacteria (Czajkowski *et al*., 2011). Potato tubers are stored at low temperature and thus any microbial antagonist used to control postharvest soft rot should have the ability to survive under cold storage conditions. When a simple *in vivo* test was carried out to select a number of promising isolates able to colonize and persist at high level on tubers in low temperature, the results narrowed down the isolates to two strains, *Pseudomonas* sp. isolated from arctic plants and *Serratia plymuthica* A30 isolated from rotten potato tubers. The ability to survive at low temperatures has been reported also for other strains of *Serratia plymuthica.* Abuamsha *et al*. (2014) reported that bio-priming oilseed rape seed by *Serratia plymuthica*

HRO-C48 against blackleg caused by *Phoma lingam* showed that bacterial concentration in seeds stored at 4°C was higher than that in seeds stored at 20°C. In the present study, the last experiment was conducted *in planta* using wounded seed tubers inoculated with *D. solani* under curing conditions with 15°C and high humidity. The pre-treatment of tubers with antagonists successfully and significantly reduced the percentage soft rot disease of potatoes during 30 days in storage. *S. plymuthica* A30 showed the highest percentage of soft rot disease reduction between all tested bacterial antagonists. Previous studies have identified *S. plymuthica* as an efficient antagonist with the ability for internal colonization of root and stem tissues and plant stress mediating abilities via induced systemic disease resistance (Gould *et al*., 2008; Czajkowski *et al*., 2012b; Abuamsha *et al*., 2014). In the current research, the endophytic antagonist *S. plymuthica* A30 showed efficient inhibition of soft rot pathogens, including several *D. solani* isolates, and it was also identified as the most promising isolate for cold storage biocontrol of potato tubers due to its superior adaptability to cold temperature.

The population dynamics of *S. plymuthica,* along with its target pathogen *D. solani* were evaluated over time on stored potatoes with real-time PCR. Standard curves were used to evaluate the sensitivity and intra- and inter- assay variabilities, and to enable a reliable quantification of both real-time PCR assays in potato samples. With a quantitative detection limit of 10^2 cell ml^{-1}, efficiency of 99%, and a linear range of six orders of magnitude, the *D. solani* and *S. plymuthica* PCR assays allowed a precise quantification of DNA amounts extracted from pure culture preparations. The established *D. solani* and *S. plymuthica* PCR assays resulted in a relatively high TaqMan sensitivity in complex samples in agreement with those reported earlier (Czajkowski & van der Wolf, 2012c; Pritchard *et al*., 2013). In spiked tuber samples, down to 5×10^2 bacterial cells of *D. solani* and up to 2×10^3 bacterial cells of *S.*

plymuthica A30 per g of tuber peel samples could be detected. However, the *D. solani* assay detected lower cell densities than the *S. plymuthica* assay. The lower detection limit might be attributable in part to the enrichment step prior to the real-time PCR. During incubation of potato extract in PEB medium, the *Dickeya* population increased on average by a factor of 1000 (van der Wolf, 2014a). As the *D. solani* population on potatoes at cold storage is very low, $10^0 – 10^2$ cell ml^{-1}, an enrichment step may be added prior to the real-time PCR. This is particularly important due to latent survival of D. *solani* in infected seed tubers. The intra- and inter-assay coefficients of variation of the Ct values from DNA of spiked samples of both assays did not differ significantly and were relatively low but tended to increase with decreasing concentration of target DNA. These low CV values in intra- and inter-assay variability showed a good level of repeatability and reproducibility, also including DNA extraction procedure. This supports the hypothesis that the Ct value variations were independent of pipetting errors in setting up the PCR assay but may be caused by inhibitors from the potato samples.

In our study, the population dynamics of *D. solani* and the biocontrol strain *S. plymuthica* in the storage experiment indicated that the cell density of the pathogen reduced faster on stored potatoes previously treated with the antagonist, when compared to untreated potato tubers, suggesting that the treatment affected the contamination level of the tubers. Furthermore, soft rot symptoms were reduced in the treated tubers. In all three cultivars, the antagonist levels were higher in the presence of the pathogen, which could be a result of better epiphytic colonization and survival or due to nutrients released by the pathogen. Although the antagonist population gradually reduced overtime, this reduction did not result in an increase in the concentration of soft rot bacteria. By contrast, *D. solani* populations decreased significantly in the presence of the antagonist from 10^5 cell g^{-1} at 1 dpi to an average of 10^2

cell g^{-1} or less at 14 or 21 dpi depending on the cultivar. Although the pathogen cell density declined initially in all cultivars, it increased again in February. In two of the cultivars, no increase in the later stage was observed in the antagonist-treated tubers, and even in the third one (cv. Bintje) the increase was delayed, suggesting that the antagonist treatment was still effective during the spring several months after the treatment.

Biological control of bacterial soft rot is most logically directed toward suppressing the development of the disease due to contact with contaminated equipment or rotten tubers during harvest or grading. Seed lots for which a high risk for soft rot would be expected based on field surveys would not be used as seed tubers under any situations (Directive 2014/20/UE and 2014/21/UE). However, evidence for biocontrol activity is easier to establish in small field experiments when a high incidence of disease occurs in positive control treatments than when the disease incidence is low. Therefore, tubers with a high inoculum level were used in the field trial. Field results illustrated that potato tubers treated with the antagonist in the autumn stayed healthy during storage, and the treatment even protected the potato plants the following summer. Plants grown from tubers treated by *S. plymuthica* A30 had less disease symptoms in the plant stand and lower soft rot incidence in harvested daughter tubers. In addition, a decrease in latent infections was observed in all cultivars in harvested daughter tubers in plants grown from tubers treated with antagonists. No *S. plymuthica* was evident in the harvested daughter tubers in the real-time PCR, suggesting that the population level of the antagonist declined in the field to lower than the threshold of *S. plymuthica* TaqMan real time assay (data not shown). In the field, the antagonist concentration and survival may be affected by increased microbial competition and unfavourable environmental conditions (Pineda *et al*., 2013).

The purpose of the present research was to evaluate the success of biocontrol in the prevention of soft rot during cold storage of potato tubers. During this research, several promising antagonists were evaluated, and the best of them was concluded to be effective also in the field. However, the field experiment was performed only once and need to be repeated in large scale. Furthermore, the potato treatment technique that was used, dipping of the potatoes in a suspension of the antagonist, is not feasible in practice, thus new methods and new formulations for the treatment need to be developed in the future.

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Figure legends

Figure 1 Standard curves for TaqMan assay of *D. solani* and *S. plymuthica* in bacterial suspension or potato extracts. Standard curves were generated by plotting the threshold cycle values (Ct) against the log initial cell number (Log CFU) of target bacteria present in the template. Each dot represents the result of five replicate amplifications of each dilution in two separate experiments. The coefficients of determination R^2 and the slopes of the regression curve are indicated. Error bars represent standard deviation.

Figure 2 Population dynamics and survival of soft rot pathogen *D. solani* and biocontrol agent *S. plymuthica* during storage. Potato tubers of three potato cultivars Van Gogh, Bintje and Timo were inoculated either with *S. plymuthica* A30, *Dickeya solani* Ds0432-1 or inoculated with both strains in the autumn. The amount of bacteria were determined by sampling at various time points during the storage period followed by analysis by specific TaqMan assays. The experiment was independently performed in three storage rooms and the results pooled. Results from three subsamples of five potato tubers per time point were averaged.

Figure 3 Effect of seed tuber bacterization with *S. plymuthica* on disease development in potato tubers inoculated with *Dickeya solani*. Seed tubers of cultivars Van Gogh, Bintje and Timo were inoculated in the autumn either with *D. solani* Ds0432-1 alone or with the

antagonist *S. plymuthica* A30, then the tubers were stored and used as seed the following summer. The average percentage of symptomatic plants was analysed midseason (a) and number of progeny tubers (b, c, d) that were healthy or had latent or symptomatic soft rot infection were analysed after harvest. The control treatment included no pathogen nor antagonist. Values followed by identical characters are not significantly different ($P < 0.05$). Error bars indicate the standard deviation.

Table 1 Characterization of 13 bacterial isolates able to reduce tuber decay caused by *D. solani* and *Pectobacterium* spp.

Table 2 The effects of potato tuber bacterization on soft rot development.

Table 3 Comparison of Ct values obtained with the real-time PCR performed on potato tuber samples of different cultivars.

Supporting information for online publication

Supplementary Figure 1 Biocontrol assay for potato soft rot induced by different *D. solani* strains.

Supplementary Table 1 List of primers and probes used in this study.

Supplementary Table 2 Detection level of *D. solani* and *S. plymuthica* TaqMan assays in bacterial suspension or in potato tuber extract.

Supplementary Table 3 Intra- and inter assay variabilities of *D. solani* and *S. plymuthica* TaqMan assays.

Supplementary Table 4 Results of the statistical analysis performed on the long term survival of *Dickeya solani* and S*erratia plymuthica*.

Table 1 Characterization of 13 bacterial isolates able to reduce tuber decay caused by *D. solani* and *Pectobacterium* spp. Ability to inhibit the growth of soft rot pathogens in the plate assay, suppression of tuber tissue maceration and growth at different storage temperatures are presented.

Antagonistic isolates[a]	References	Inhibition zone (mm)[b]				Percent (%) inhibition of tissue maceration [c]				Cell densities (log CFU g^{-1} peel) [e]		
		Ds0432-1	SCC1	SCRI1043	SCC3193	Ds0432-1	SCC1	SCRI1043	SCC3193	20°C	15°C	4°C
Serratia plymuthica A30	Czajkowski *et al.*, 2012	++	+++	++	++	87.8 a	93.6 a	86.5 a	82.9 a	6.1 ± 0.8[d]	5.5 ± 0.6	5.1 ± 1.1
Alcaligenes faecalis R8	This study [a]	+++	++	+++	+	82.6 a	90.2 a	82.7 a	83.4 a	5.7 ± 1.1	3.7 ± 0.9	-
Acinetobacter sp. R4	This study	+++	+++	++	++	70.6 a	82.5 a	68.4 b	58.6 b	6.5 ± 1.5	4.7 ± 1.3	-
Bacillus megaterium A1	Reiter *et al.*, 2002	+	+++	++	+	55.5 b	66.6 b	65.2 b	78.8 b	nt	nt	nt
Flavobacterium sp. A14	Reiter *et al.*, 2002	+++	++	-	++	72.6 a	81.4 a	80.2 a	71.9 b	6.6 ± 0.9	4.2 ± 1.2	2.2 ± 1.5
Bacillus simplex BC2	Nissinen *et al.*, 2012	++	++	+	++	69.5 b	82.9 a	83.4 a	61.6 b	nt	nt	nt
Pedobacter sp. PD1	Nissinen *et al.* 2012	+	+	++	+++	66.6 b	63.2 b	67.3 b	73.6 b	nt	nt	nt
Pseudomonas sp. S2H1	Nissinen *et al.* 2012	++	++	+	+	79.1 a	75.5 a	58.5 b	45.4 c	5.5 ± 0.7	5.8 ± 0.8	4.6 ± 1.4
Sphingomonas faeni SF1	Nissinen *et al.* 2012	++	+	++	+	55.8 b	76.6 a	33.3 c	59.6 b	nt	nt	nt
Rhodococcus sp. RH2	Nissinen *et al.* 2012	++	++	+	+	66.5 b	63.1 b	50.2 b	52.1 b	nt	nt	nt
Bacillus subtilis P48	Krzyzanowska *et al.*, 2012	+++	++	++	+++	75.3 a	86.6 a	67.3 b	88.7 a	7.3 ± 0.2	3.5 ± 1.1	-
Pseudomonas fluorescence P301	Krzyzanowska *et al.*, 2012	+++	+++	+++	+++	80.0a	91.7 a	83.2 a	78.8 a	6.3 ± 0.5	4.6 ± 1.4	2.7 ± 1.6
Pseudomonas donghuensis P482	Krzyzanowska *et al.*, 2012	+++	+++	++	++	83.6 a	89.7 a	78.4 a	88.5 a	6.8 ± 1.3	4.1 ± 0.6	2.1 ± 1.2

[a] Bacterial isolates, recovered from rotten potato tuber tissues in this study or obtained from bacterial collections of other laboratories, producing zone of inhibition when challenged with four soft rot pathogens *D. solani* (Ds0432-1), *P. carotovorum* (SCC1), *P. atrosepticum* (SCRI1043) and *P. wasabiae* (SCC3193).

[b] Inhibition zones are mean of 9 values (three replications and three plates/replication) obtained by measuring the average diameter of the radius of the clear zone from outer edge of colony border : −, no inhibition (<1 mm); +, weak inhibition (<5 mm); ++, mild inhibition (=5 mm); and +++, strong inhibition (>10 mm). All the experiments were conducted at 28°C and repeated two times.

[c] Influence of selected isolates on tissue maceration caused by soft rot pathogens in the potato tuber assay. The maceration zone diameter was measured and normalized with respect to the control (pathogen alone) to determine soft rot reduction. Numbers in column followed by the same letter are not significantly different ($P < 0.05$).

[d] Ability of colonization and persistence of bacterial antagonists were evaluated at 20, 15 and 4°C after 30 days of incubation. The CFU per gram tuber peel ± standard error (SE) were analyzed after $\log_{10}$ transformation.

[e] Nt, not tested, - no growth.

Table 2 The effects of potato tuber bacterization on soft rot development. Potato tubers were wounded, treated with seven bacterial antagonists (with concentrations between 10^8-10^9 CFU ml^{-1}) and then inoculated with a *D. solani* (10^6 CFU mL^{-1}). Percentage of weight loss, average of visual rating and percentage of disease reduction (PDR) are shown. The inoculated tubers were incubated at 15°C temperature and data on soft rot infection was recorded within 1, 5, 15, 30 days post inoculation (dpi).

Weight loss (%) [a]					Visual rating [b]			PDR (%) [c]
Antagonist isolates	1 dpi	5 dpi	15 dpi	30 dpi	1 dpi	5 dpi	15 dpi	30 dpi
Serratia plymuthica A30	0.0a[d]	0.1a	2.2a	3.4b	0.0a	0.0a	0.3a	0.8a	93.2
Alcaligenes faecalis R8	0.0a	1.3a	2.6a	7.7c	0.0a	0.2a	0.4a	1.3b	85.3
Pseudomonas donghuensis P482	0.0a	1.8a	3.7b	10.1c	0.0a	0.3a	0.6a	1.5b	82.2
Pseudomonas sp. S2H1	0.0a	1.6a	4.5b	11.6c	0.0a	0.2a	0.7a	1.5b	80.3
Pseudomonas fluorescence P301	0.0a	1.5a	4.3b	13.5c	0.0a	0.2a	0.7a	1.6b	75.9
Flavobacterium sp. A14	0.0a	1.6a	4.6b	15.9c	0.0a	0.2a	0.8a	1.8b	69.2
Bacillus subtilis P48	0.0a	2.0a	6.3b	18.4d	0.0a	0.5a	1.2b	2.2c	65.1
Dickeya solani (control)	0.0a	9.4c	23.3d	58.5e	0.0a	1.3b	2.4c	3.8d	

[a] Loss weight (%) = amount of rotted tissue determined by weight change before and after washing off rotten tissue.

[b] Soft rot disease development expressed as visual rating (mean disease rating): 0 = no rot to 5 = complete rot, based on visual estimate of rotten tissue area.

[c] PDR (%) values were determined by comparing disease severity (based on weight loss percentage) between treatments and control (pathogen only) and data was assessed 30 days after treatment.

[d] Numbers in a column followed by the same letter are not significantly different ($P < 0.05$) by a protected LSD test.

Table 3 Comparison of Ct values obtained with the real-time PCR performed on potato tuber samples of different cultivars. Total DNA of healthy potato tubers (non-spiked = NS) and spiked samples (S) with *Dickeya solani* and *Serratia plymuthica* A30 were compared in both TaqMan assays.

	Dickeya solani TaqMan assay				*Serratia plymuthica* A30 TaqMan assay			
	10^6 cells g^{-1} (S)		0 cells g^{-1} (NS)	NCt	10^6 cells g^{-1} (S)		0 cells g^{-1} (NS)	NCt[b]
Potato cultivar	Mean Ct values ±SD[a]	CV (%)			Mean Ct values ±SD	CV (%)		
Van Gogh	20.78 ± 0.21	1.01	37.36 ±.0.77	36.05	22.33 ± 0.40	1.79	38.76 ± 1.20	35.16
Bintje	21.16 ± 0.34	1.61	38.72 ±.1.12	36.11	21.86 ± 0.17	0.78	39.07 ± 1.15	35.62
Timo	21.41 ± 0.28	1.30	38.20 ±.0.86	36.53	22.09 ± 0.35	1.58	38.64 ± 1.1	35.34
Ct cut-off				35.34				35.37
Blank PCR[c]	40		40		40		40	

[a] SD = Mean standard deviation.

[b] NCt= the Ct value above which a sample is consider as negative.

[c] Blank PCR= PCR carried out with Ultrapure water in place of DNA.