Compatible Mixture of Bacterial Antagonists Developed to Protect Potato Tubers from Soft Rot Caused by *Pectobacterium* spp. and *Dickeya* spp.

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Abstract

Possibilities to protect potato tubers from rotting caused by Soft Rot Pectobacteriaceae (SRP) under disease favoring conditions were investigated using compatible mixtures of bacterial antagonists and tested with a newly developed stepwise efficacy-based screening protocol. Twenty-two bacterial antagonists were evaluated against a combination of five *Pectobacterium* and *Dickeya* strains representing species and subspecies most often associated with potato soft rot in Europe. To enable potential synergistic activity, the antagonists were initially tested against the combination of pathogens in 15 random mixtures containing up to 5 antagonists each. Three mixtures (M2, M4, and M14) out of 15 tested reduced tuber tissue maceration due to soft rot. The individual antagonists derived from M2, M4, and M14 mixtures were tested on potato slices and whole tuber injection assays. These five strains (*S. plymuthica* strain

A294, *E. amnigenus* strain A167, *R. aquatilis* strain H145, *S. rubidaea* strain H440, and *S. rubidaea* strain H469) were combined to develop a tailored biological control mixture against potato soft rot. The new mixture, designated the Great Five (GF), was tested on seed potato tubers vacuum infiltrated with antagonists and subsequently with the combination of five SRP pathogens. In these experiments, the GF mixture provided stable protection of inoculated potato tubers, reducing soft rot by 46% ($P = 0.0016$) under high disease pressure conditions. The A294, A167, H145, H440, and H469 antagonists were characterized for features important for viable commercial applications including growth at different temperatures, resistance to antibiotics, and potential toxicity toward *Caenorhabditis elegans*. The implications for control of soft rot caused by SRP with the use of the GF mixture of antagonists are discussed.

Soft rot caused by pectinolytic Soft Rot Pectobacteriaceae (SRP) (formerly pectinolytic *Erwinia* spp.) (Adeolu et al. 2016), namely *Pectobacterium* spp. and *Dickeya* spp., is an important potato disease resulting in economic losses in (seed) tuber production worldwide (Pérombelon 2002). Under disease favorable conditions,

Pectobacterium spp. and *Dickeya* spp. can cause a variety of symptoms on potato including pre-emergence decay of seed tubers, stem rot (blackleg) of field-grown plants, and soft rot of progeny tubers in storage (Pérombelon 2002). In Europe, soft rot disease results in high economic losses, mainly due to declassification and rejection of seed lots (Toth et al. 2011).

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The mixture of bacterial isolates described herein, for protection of potato tubers and ornamental plants against soft rot caused by pectinolytic *Pectobacterium* spp. and *Dickeya* spp., is the object of the patent application P.423806, which has been filed with the Polish Patent Office by University of Gdansk, Poland with inventors Robert Czajkowski, Dorota M. Krzyzanowska, Tomasz Maciag, Joanna Siwinska, and Sylwia Jafra.

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It is well established that latently infected potato (seed) tubers are the primary source of SRP (Pérombelon 1974; Pérombelon and Lowe 1975). The latent inoculum within tubers is transferred both between fields and between growing seasons (Charkowski 2006). Consequently, production of pathogen-free seed lots is considered as the most important strategy in controlling the spread of *Pectobacterium* spp. and *Dickeya* spp. in the potato ecosystem (Czajkowski et al. 2011). Pathogen-free seed tubers have been successfully produced from axenic planting material (Gopal et al. 1998). However, the use of initially clean, pathogen-free seed is of little protection as tuber contamination usually occurs in the following stages of potato seed multiplication (Charkowski, 2015; van der Wolf et al. 2017). Contamination can occur in the field during plant growth, at harvest and seed grading, as well as in storage and transit. The pathogens can also be transmitted via air, water, and by animals (mostly insects) entering the potato fields (Toth et al. 2003, 2011). The contamination may be internal, in the plant vascular system, in tuber lenticels and on the periderm, as well as in wounds incurred during handling. These niches are known to support long-term survival of the soft rot bacteria (van der Wolf and De Boer 2007). It is believed that wounds and cracks on the surface of potato tubers in particular, are easily invaded by the pathogens during handling and postharvest, and hence play an important role in the dissemination of SRP from a few rotting tubers to numerous neighboring healthy ones (Pérombelon 2002; van Vuurde and De Vries 1994).

Management of soft rot in potato tubers is difficult due to the widespread contamination, lack of resistance in commercial potato cultivars, and the absence of effective disease control agents (Czajkowski et al. 2011). Current integrative management strategies include the use of certified *Pectobacterium* spp. and *Dickeya* spp.-free seed tubers, hygienic measures to avoid introduction and dissemination of

the bacteria, and avoidance of tuber wounding and oxygen depletion as a result of tubers becoming wet, which could further impair tuber resistance. Unfortunately, the use of integrative management has not yet led to an acceptable reduction of soft rot incidences in (seed) tubers (Czajkowski et al. 2011; Pérombelon 1992).

Biological control based on the use of antagonistic bacteria is potentially a promising alternative to or a complementation of the integrative management strategy currently employed in potato production. In addition to their antibiotic potential, antagonistic bacteria occupying the same niche may prevent colonization of (seed) tubers by *Pectobacterium* spp. and *Dickeya* spp. and consequently the development of soft rot symptoms. Some of these antagonistic bacteria are endophytes able to colonize plants systemically (Lodewyckx et al. 2002). Their potential to colonize the inoculated plant internally could provide control against pectinolytic bacteria in locations inaccessible to traditional chemical and physical control measures (e.g., vascular tissue) (Czajkowski et al. 2012b).

One of the major factors limiting the efficiency of microbial-based biocontrol agents in potato is that usually a single biocontrol agent (microbial strain) is used for both tuber and foliage diseases, moreover selected against a narrow spectrum of pathogenic strains (Diallo et al. 2011). Furthermore, most attempts do not venture beyond in vitro laboratory assays for antagonism, small-scale pathogenicity assays on tuber fragments, or tests involving culture tube-raised potato plants (Kaur and Mukerji 1999; Mota et al. 2017; Pal and McSpadden Gardner 2006). Only a few studies have included trials on a large scale near commercial situations of plant growth and storage conditions (Czajkowski et al. 2012a, b). Similarly, no large-scale experiments have been performed so far to assess the effect of mixtures containing a combination of different antagonistic bacteria to be used against *Pectobacterium* spp. and *Dickeya* spp. in potato. It has been reported in the case of other crops and their pathogens that compositions containing several biological control agents with different modes of action have superior performance over individual agents in suppressing disease symptoms (Raupach and Kloepper 1998; Stockwell et al. 2011). It can be hypothesized, therefore, that in the case of control of potato soft rot, a mixture of antagonistic bacteria would provide better protection than can be obtained with an individual biocontrol agent.

The purpose of this study was to develop a mixture of bacterial antagonists to be applied to pathogen-free (seed) potato tubers to protect them against soft rot caused by a mixture of *Pectobacterium* spp. and *Dickeya* spp. In Europe, at least five SRP species have been identified to cause potato soft rot, often present as mixed inocula (van der Wolf et al. 2017). It is well accepted that single antagonists often are unable to protect plants against multiple SRP strains due to their narrow range of antagonistic activity (Baker 1987; Czajkowski et al. 2011; Köhl et al. 2011). It was therefore crucial to develop a mixture of antagonists expressing synergistic effect and active against multiple SRP pathogens. Likewise, we aimed also to characterize the resulting mixture and its components for features important for the development of a biological control agent for large-scale commercial applications.

Materials and Methods

Bacterial strains, plant material and media. Bacterial strains used in this study are listed in Table 1. All strains were routinely grown on Tryptone Soya Agar (TSA, Oxoid) or in Tryptone Soya Broth (TSB, Oxoid) at 28°C, the latter with shaking (200 rpm), for 24–48 h. For long-term storage, bacterial strains were kept in 40% glycerol (vol/vol) at –80°C. Certified, SRP-free potato tubers cv. Irga susceptible to *Pectobacterium* spp. and *Dickeya* spp. (Salaman 2014) were used in all experiments in which bacterial strains were tested on plant material. They were purchased from Pomorsko-Mazurska Hodowla Ziemniaka (Pomeranian-Masurian Potato Breeding) (<http://www.pmhz.pl/>) (Szyldak, Poland).

Development of mixtures containing bacterial strains with antagonistic potential against SRP pathogens. Twenty-two bacterial strains previously studied in our laboratory showing antagonistic activity against *Pectobacterium* spp. and/or *Dickeya* spp. (Table 1)

were randomly distributed to 15 mixtures each containing up to 5 antagonistic strains (Supplementary Table S1). The mixtures included strains expressing different modes of action against at least one strain of the target pathogens (*Pectobacterium* spp. and *Dickeya* spp.) (results not shown). The resulting mixtures (named: M1–M15) were subsequently tested against a combination (named: P) of five SRP strains (three pectinolytic *Pectobacterium* spp.: *P. atrosepticum* strain SCRI 1043 (Bell et al. 2004), *P. carotovorum* subsp. *carotovorum* strain Ecc71 (Willis et al. 1987), and *P. parmentieri* strain SCC3193 (Pirhonen et al. 1991) and two *Dickeya* spp.: *D. solani* strain IPO2222 (van der Wolf et al. 2014) and *D. dianthicola* strain CFBP 1200 (IPO1741) (Samson et al. 2005)). These SRP strains belong to species and subspecies most-commonly causing soft rot and blackleg diseases on potato in Europe. Although infections by multiple SRP strains may be rare in nature, these experiments were designed to simulate the worst-case scenario, in which potato tubers were infected with all five SRP strains (*P. atrosepticum* strain SCRI 1043, *P. carotovorum* subsp. *carotovorum* strain Ecc71, *P. parmentieri* strain SCC3193, *D. solani* strain IPO2222, *D. dianthicola* strain IPO1741) at high inoculum and incubated under disease-favorable conditions (high humidity, high temperature) as advised earlier (Charkowski 2015; Czajkowski et al. 2012a). Each mixture of antagonists contained an equal ratio of individual strains, with 10^8 CFU (colony forming units) ml^{-1} per strain. This amounted a total of $n \times 10^8$ CFU ml^{-1} , where n is the number of antagonistic strains in a given mixture. The suspension of five *Pectobacterium* spp. and *Dickeya* spp. pathogens contained in total 10^6 CFU ml^{-1} (2×10^5 CFU ml^{-1} of each pathogenic strain). All bacterial mixtures were prepared in running tap water directly before use.

Evaluation of mixtures containing bacterial antagonists against SRP on vacuum-infiltrated potato tubers. Inoculation of potato tubers with antagonistic and pathogenic bacterial strains was done using a vacuum infiltration method as previously described (Czajkowski et al. 2012a). Briefly, antagonistic bacterial strains were grown separately on TSA plates at 28°C for 24 h. Cells were harvested from agar plates and suspended in tap water to obtain cell density of ca. 10^8 CFU ml^{-1} (ca. 3 McF) of each strain. For this purpose, suspensions of individual antagonists with turbidity of 15 McF (measured for 10x diluted suspensions: 1.5 McF) were mixed in an equal ratio. Pathogenic strains viz. *P. atrosepticum* strain SCRI 1043, *P. carotovorum* subsp. *carotovorum* strain Ecc71, *P. parmentieri* strain SCC3193, *D. solani* strain IPO2222, and *D. dianthicola* strain CFBP 1200 (IPO1741) were grown separately on TSA and collected under the same conditions as described above, but the final density of the suspension, containing equal ratio of each strain, was adjusted to a total of 10^6 CFU ml^{-1} (0.03 McF). Certified seed potato tubers cv. Irga (Pomorsko-Mazurska Hodowla Ziemniaka, Poland) were washed under running tap water, surface-sterilized for 20 min in 5% commercial bleach solution in water, washed 3 times in running tap water, and dried in air. The tubers were then immersed in the antagonist suspension and vacuum infiltrated for 10 min at –80 Bar in a desiccator followed by 10 min incubation in the same suspension at atmospheric pressure to allow the bacteria to penetrate the lenticels and wounds of tubers. For the control treatments, potato tubers were vacuum infiltrated with tap water. Tubers were dried overnight and the next day they were vacuum infiltrated, under the same conditions as described above, with a combination of pectinolytic bacteria or with tap water (negative control). Inoculated potato tubers were placed in humid boxes (85 to 90% relative humidity), 10 tubers of the same treatment per box. Samples were incubated at 28°C for 5 days for soft rot symptom development. In each experimental run, 30 tubers (3 boxes, each containing 10 tubers) were used to test a single combination. The final experiments with a tailored mixture of antagonists were performed in 4 biological replicates ($n = 120$ tubers). The symptoms on each tuber were assessed using a six-rank disease severity scale developed in this study: *rank 0* – no symptoms observed on the analyzed tuber, *rank 1* – rotting symptoms localized only superficially (at the periderm) and overall on less than 25% of tuber surface, *rank 2* – symptoms observed in the rank 1 but present on 25 to 50% of tuber surface, *rank 3* – symptoms observed in the

rank 2 but additionally the tuber periderm is detached from the tuber internal tissues and rotting occupies between 50 and 90% of the tuber, rank 4 – symptoms observed in the rank 3 but overall the rotting occupies more than 90% of the tuber surface and/or reaching the tuber internal tissue (core), rank 5 – whole tuber macerated (Fig. 1A). The correlation of disease severity ranks in the established scale with the average potato tuber weight loss due to soft rot was evaluated in a separate experiment and prior to other tests (Fig. 1B).

Evaluation of biocontrol potential of individual antagonistic strains derived from selected random mixtures. The mixtures of antagonists that showed the best protection effect against soft rot in the initial experiment served as the sources of individual antagonistic strains for the follow-up assays. The selected strains were individually retested for their protective activity against a combination of five *Pectobacterium* spp. and *Dickeya* spp. For this, a potato slice assay was used as well as whole tuber injection assay, performed as previously described (Czajkowski et al. 2010, 2017; Maher and Kelman 1983).

Density dependence of the control of pectinolytic bacteria by a mixture of antagonistic strains on vacuum-infiltrated potato tubers. The influence of cell density on the biocontrol potential of the mixture of bacterial antagonists was evaluated on vacuum-infiltrated tubers using a similar experimental setup as described above. Potato tubers ($n = 30$ per treatment) were vacuum infiltrated

with mixtures containing different densities of the antagonists (10^7 , 10^8 , and 5×10^8 CFU ml $^{-1}$) and a mixture of five *Pectobacterium* spp. and *Dickeya* spp. (10^6 CFU ml $^{-1}$) and incubated under the same conditions as described above. The experiment was independently repeated once with the same setup. Disease symptoms were evaluated using the six-rank disease severity scale.

Sequencing of 16S rDNA gene to assign antagonistic strains to bacterial species and allocation of species to risk groups. Isolation of genomic DNA and sequencing of the 16S rDNA gene (fragments $\geq 1,400$ bp) were outsourced to BaseClear B. V. (The Netherlands). The strains were classified to the species level based on a BLAST search (Altschul et al. 1990) against the GenBank database (<https://www.ncbi.nlm.nih.gov/>) as previously described (Czajkowski et al. 2012a). The ATTC (American Type Culture Collection, www.attc.org) database was used to classify antagonistic strains into risk categories on the basis of their ability to cause infections in humans, animals, and plants.

Growth of antagonistic bacterial strains at different temperatures. The growth of each antagonistic strain was tested in liquid medium (TSB) over a range of four temperatures: 28, 37, 40, and 42°C and on solid medium (TSA) over a range of six temperatures: 7, 10, 28, 37, 40, and 42°C. The growth in liquid medium was assessed every hour for the total time of 16 h as previously described (Czajkowski et al. 2017). To monitor the growth of antagonistic strains on solid

Table 1. List of antagonistic isolates used in this study with reported antagonistic potential against members of Soft Rot *Pectobacteriaceae* (SRP). Antagonistic isolates developed into the final GF mixture (A294, H145, A167, H440, and H469) are marked in bold.

No.	Genus/ species	Strain	Characteristic	Biocontrol effect against	Source	Reference
1	<i>Bacillus cereus sensu lato</i>	P368	AHL inactivation, biosurfactant and siderophore production	<i>Pectobacterium</i> spp. and <i>Dickeya</i> spp. on potato	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
2	<i>Bacillus cereus sensu lato</i>	P369	AHL inactivation	<i>Pectobacterium</i> and <i>Dickeya</i> spp. on potato tubers	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
3	<i>Bacillus subtilis</i>	MB73/2	Antibiosis against SRP, biosurfactant production	<i>Dickeya</i> sp. on potato tubers	Rhizosphere of meadow weeds (Zulawy region, Poland)	(Krzyzanowska et al. 2012b)
4	<i>Bacillus subtilis</i>	P48	Antibiosis against SRP, biosurfactant and siderophore production	<i>Pectobacterium</i> and <i>Dickeya</i> spp. on potato tubers	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012a)
5	<i>Bacillus subtilis</i>	MB5	Antibiosis against SRP, biosurfactant production	<i>Dickeya</i> sp. on potato tubers	Rhizosphere of meadow weeds (Zulawy region, Poland)	(M. Obuchowski, unpublished)
6	<i>Bacillus subtilis</i>	MB5/1	Antibiosis against SRP	n.d. ^a	Rhizosphere of meadow weeds (Zulawy region, Poland)	(M. Obuchowski, unpublished)
7	<i>Bacillus subtilis</i>	MB8212	Antibiosis against SRP, biosurfactant production	n.d.	Rhizosphere of meadow weeds (Zulawy area, Poland)	(M. Obuchowski, unpublished)
8	<i>Bacillus subtilis</i>	MB41	Antibiosis against SRP, biosurfactant production	n.d.	Rhizosphere of meadow weeds (Zulawy area, Poland)	(M. Obuchowski, unpublished)
9	<i>Bacillus thuringiensis</i>	A98	AHL inactivation, biosurfactant production	<i>P. parmentieri</i> strain SCC3193 and <i>P. carotovorum</i> subsp. <i>carotovorum</i> Ecc71 on potato tubers	Potato rhizosphere (The Netherlands)	(S. Jafra, unpublished)
10	<i>Bacillus weihenstephanensis</i>	P10	AHL inactivation, biosurfactant production	<i>Pectobacterium</i> and <i>Dickeya</i> spp. on potato	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
11	<i>Delftia acidovorans</i>	A207	AHL inactivation	<i>P. parmentieri</i> strain SCC3193 and <i>P. carotovorum</i> subsp. <i>carotovorum</i> Ecc71 on potato tubers	Potato rhizosphere (The Netherlands)	(Jafra et al. 2006)
12	<i>Enterobacter amnigenus</i>	A167	AHL inactivation	<i>P. parmentieri</i> strain SCC3193 and <i>P. carotovorum</i> subsp. <i>carotovorum</i> Ecc71 on potato tubers	Potato rhizosphere (The Netherlands)	(Jafra et al. 2006)

(Continued on next page)

^a n.d. = not determined.

medium, 2- μ l aliquots of 50 times diluted in TSB overnight bacterial cultures grown in TSB were placed on the surface of TSA plates and incubated for a total period of 120 h. The growth of each strain was investigated every 24 h and rated using the following index: ‘-’ – no visible growth, ‘+/-’ – slow visible growth (small colonies), ‘+’ – visible regular growth. The growth of each strain was analyzed in two replicates and the entire experiment was repeated once with the same setup.

Antibiotic susceptibility of selected antagonistic strains. The antibiotic susceptibility of selected antagonistic strains was determined by a disc diffusion method as previously described (Bauer et al. 1966). The antibiotic discs (all from BD BBL - Sensi-Disc antimicrobial test discs) used in this study were: fusidic acid (10 μ g), oxacillin (1 μ g), rifampicin (5 μ g), aztreonam (30 μ g), chloramphenicol (30 μ g), imipenem (10 μ g), ciprofloxacin (5 μ g), linezolid (10 μ g), doxycycline (30 μ g), tigecycline (15 μ g), streptomycin (300 μ g), Synercid (4.5 μ g quinupristin + 10.5 μ g dalbapristin), gentamicin (10 μ g), ampicillin (10 μ g), clindamycin (2 μ g), fosfomycin (200 μ g), colistin (10 μ g), ceftazimide (10 μ g), piperacillin+tazobactam (30 μ g + 6 μ g), ampicillin+sulbactam (10 μ g + 10 μ g), and vancomycin (5 μ g).

***Caenorhabditis elegans* survival assay.** Liquid killing assay (Kirienko et al. 2014) was employed to assess the putative pathogenicity of the selected antagonistic strains to *C. elegans*. The wild-type Bristol N2 strain of *C. elegans* obtained from the *Caenorhabditis*

Genetic Center (CGC) was cultured on Nematode Growth Medium (NGM) plates with the lawn of *Escherichia coli* strain OP50 and at 25°C according to the protocol described previously (Stiernagle 2006). For the killing assay, nematodes and their eggs were harvested from the NGM plates by washing the media surface with distilled water and collecting the liquid. The resulting suspension was treated with 5% bleaching solution to isolate eggs and to synchronize culture for further growth. After 48 h, when all nematodes achieved L4 larval stage, fluorodeoxyuridine (final concentration 50 μ M) (Sigma) was added to liquid killing medium (LKM) to prevent the reproduction of nematodes. Cocultures of *C. elegans* and bacterial strains were performed in 48-well plates (Falcon). 100 μ l aliquots of *C. elegans* culture containing approx. 30 nematodes each were placed per well and then supplemented with 100 μ l of bacterial inoculum (0.5 McF, ca. 10^7 CFU ml⁻¹) in LKM. Plates were incubated for 3 days at 25°C, and the number of living nematodes was determined as described earlier (Stiernagle 2006) daily using MZ10F stereomicroscope (Leica). *Pseudomonas aeruginosa* strain PA14, with known killing potential to *C. elegans* (Tan et al. 1999), was used as a positive control. For negative control, the nematodes were grown in a medium supplemented with *E. coli* OP50. The experiment was done on three biological replicates, each containing three technical repetitions.

Statistical analyses. All statistical analyses concerning potato tuber protection assays were conducted with R version 3.3.2 (R Core

Table 1. (Continued from previous page)

No.	Genus/ species	Strain	Characteristic	Biocontrol effect against	Source	Reference
13	<i>Erwinia persicinus</i> / <i>Serratia rubidaea</i>	H452	AHL inactivation, siderophore production	<i>D. solani</i> strain IPO2019 on hyacinth bulbs	Hyacinth bulb cv. Aiolas, scale leaves (The Netherlands)	(Jafra et al. 2009)
14	<i>Ochrobactrum</i> sp.	A44	AHL inactivation	<i>P. parmentieri</i> strain SCC3193 and <i>P. carotovorum</i> subsp. <i>carotovorum</i> Ecc71 on potato tubers	Potato rhizosphere (The Netherlands)	(Jafra et al. 2006)
15	<i>Pseudomonas donghuensis</i>	P482	Antibiosis against SRP, biosurfactant and siderophore production	<i>Pectobacterium</i> sp. on potato and <i>D. solani</i> on chicory	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
16	<i>Pseudomonas</i> sp.	P486	Antibiosis against SRP, biosurfactant and siderophore production	<i>Pectobacterium</i> spp. and <i>Dickeya</i> spp. on potato	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
17	<i>Pseudomonas</i> sp.	P103	Antibiosis against SRP, biosurfactant and siderophore production	<i>Pectobacterium</i> spp. and <i>Dickeya</i> spp. on potato	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
18	<i>Rahnella aquatilis</i>	H145	Antibiosis against SRP, siderophore production	<i>D. solani</i> strain IPO2019 on hyacinth bulbs	Hyacinth bulb cv. Delft Blauw, basal plate (The Netherlands)	(Jafra et al. 2009)
19	<i>Rhodococcus erythropolis</i>	A185	AHL inactivation	<i>P. parmentieri</i> strain SCC3193 and <i>P. carotovorum</i> subsp. <i>carotovorum</i> Ecc71 on potato tubers	Potato rhizosphere (The Netherlands)	(Jafra et al. 2006)
20	<i>Serratia plymuthica</i>	A294	Antibiosis against SRP, biosurfactant and siderophore production	<i>P. parmentieri</i> strain SCC3193 and <i>P. carotovorum</i> subsp. <i>carotovorum</i> Ecc71 on potato tubers	Potato rhizosphere (The Netherlands)	(Jafra et al. 2006)
21	<i>Serratia rubidaea</i>	H440	AHL inactivation, biosurfactant and siderophore production	<i>D. solani</i> strain IPO2019 on hyacinth bulbs	Hyacinth bulb cv. Aiolas, scale leaves (The Netherlands)	(Jafra et al. 2009)
22	<i>Serratia rubidaea</i>	H469	Antibiosis against SRP, siderophore production	<i>D. solani</i> strain IPO2019 on hyacinth bulbs	Hyacinth bulb cv. Aiolas, scale leaves (The Netherlands)	(Jafra et al. 2009)

Team 2016; <https://www.R-project.org/>) using the RStudio (RStudio Team 2016; <https://www.rstudio.com/>) and the PCMR package (Pohlert 2014). The normality of data were assessed by Shapiro-Wilk test (Shapiro and Wilk 1965). Nonparametric Kruskal–Wallis test (Kruskal and Wallis 1952) was used to determine the differences between samples. For analyzing the differences between specific sample pairs, the Dunn's post hoc test was applied (Dunn 1964). Sample sizes and *P* values for each analysis are indicated in the respective figure captions.

For the *C. elegans* liquid killing assay, the survival rate, calculated over the whole experimental time, was statistically analyzed using ANOVA (analysis of variance). Results were considered to be significant at *P* < 0.05, and pair-wise differences were obtained using the *t* test.

Results

Development of the six-rank disease severity scale to assess soft rotting of potato tubers artificially inoculated with SRP. Two hundred and ten certified pathogen-free seed tubers cv. Irga were inoculated with a mixture of five pectinolytic bacteria by vacuum infiltration and kept in humid boxes, 10 tubers per box (*n* = 21), under disease favorable conditions. At the end of the experiment, each tuber was assigned a particular rank depending on the extent of soft rot symptoms (Fig. 1A). These data were compared with the average tuber weight loss (by removing rotted tissue) per box (21 boxes, 10 tubers each). The correlation coefficient (*R*²) of disease severity ranks in the established six-rank disease severity scale with the average potato tuber weight loss due to soft rot was 0.9343 (Fig. 1B).

Preliminary evaluation of mixtures containing antagonistic bacteria for the protection effect on potato tubers against SRP.

In the preliminary evaluation, 15 mixtures each containing up to 5 randomly selected antagonistic strains were tested for their protective effect on potato tubers vacuum infiltrated with a mixture of three soft rot *Pectobacterium* spp. (*P. atrosepticum*, *P. carotovorum* subsp. *carotovorum*, *P. parmentieri*) and two *Dickeya* spp. (*D. solani* and *D. dianthicola*) (five pathogens in total) under disease favorable conditions (28°C and 85–90% relative humidity). Three of the tested mixtures, designated M2 (containing strains: MB73/2, A207, A44, H145, and H469), M4 (containing strains: P368, P486, A167, and H440), and M14 (containing strains: A207, P103, A294, H145, and H469), reduced tuber maceration in comparison with the positive control (potato tubers inoculated with soft rot pathogens alone). Antagonistic bacterial strains present in the M2, M4, and M14 mixtures have been selected for the follow-up experiments. No protective effect was observed in the case of the 12 other tested mixtures (data not shown).

Selection of individual antagonistic strains from preliminary random mixtures and their evaluation in potato slice assay and tuber injection assay. The assay was performed to determine which individual antagonistic strains, occurring randomly in the mixtures M2, M4, M14 and selected as described above, are the most active against a mixture of SRP pathogens under disease favorable conditions. The selected mixtures contained, in total, 11 different strains: P368, MB73/2, A207, A44, P103, P486, A294, A167, H145, H440, and H469. Both the mixtures and their 11 singled-out components

Fig. 1. Assessment of soft rot development in potato tubers. (A) Six-rank disease severity scale developed for the evaluation of soft rot symptoms on vacuum infiltrated potato tubers. 0 – no symptoms, 1 – rotting localized only superficially (at the periderm) and on less than 25% of tuber surface, 2 – symptoms present on 25 to 50% of tuber surface, 3 – symptoms present on 50 to 90% of the tuber, with additional detachment of the periderm from the core, 4 – symptoms present on >90% of the tuber surface and/or reaching the tuber core, 5 – whole tuber macerated. The figure shows representative photos of soft rot affected tubers graded in the adopted scale. (B) Correlation of disease severity ranks in the established six-rank disease severity scale with the average potato tuber weight loss due to soft rot. Correlation was calculated for certified potato tubers cv. Irga inoculated with a mixture of pectolytic pathogens alone. Analyzed data points (*n* = 21) correspond to average rank vs. average tuber weight loss obtained for 21 boxes, 10 potatoes each.

were tested, in a potato slice assay, for the ability to reduce tuber tissue decay caused by a mixture of five SRP pathogens. Rotting symptoms were reduced by 100% in comparison with control (tuber slices inoculated with pathogens alone) for all three mixtures M2, M4, and M14 and for five of the 11 individual strains *viz.* A294, A167, H145, H440, and H469 (Fig. 2). These five effective antagonists, as well as the newly composed mixture designated GF (*the Great Five*) and containing the equal ratios of each of the five antagonists (A294, A167, H145, H440, and H469), were further tested for the protection effect against the mixture of five *Pectobacterium* spp. and *Dickeya* spp. strains in a tuber injection assay. In this experimental setup, the individual strains as well as their mixture (GF) were able to reduce tuber tissue maceration by at least 50% in comparison with the control inoculated with pathogens alone (Fig. 3).

Evaluation of the developed GF mixture for protection effect against SRP in vacuum-infiltrated potato tubers. The replicated experiments on certified pathogen-free potato tubers cv. Irga ($n = 120$) vacuum-infiltrated with antagonistic strains and subsequently, the day after, with soft rot pathogens, were conducted to assess the protection effect of the newly developed GF mixture against a combination of five soft rot pathogens. Under disease-conducive

Fig. 2. Protective efficacy of mixtures M2, M4, and M14 and their singled-out components against a blend of SRP pathogens on potato tuber slices. PC – positive control slices inoculated with SRPs alone; M2, M4, and M14 – tubers co-inoculated with the respective mixtures and SRPs; NC – negative control. In the box plot, boxes determine the inter-quartile range (Q1–Q3), broad lines indicate median values, the X's are average values, whiskers indicate extreme values within 1.5 times distance from the inter-quartile range, and single data points are outliers. Each bar was created for 18 values normalized to PC. Values significantly different from PC ($P < 0.05$) are marked with an asterisk.

Fig. 3. Reduction of tuber weight loss to soft rot by the application of antagonists in a whole tuber injection assay. PC – positive control: tubers inoculated with a mixture of SRP pathogens alone; A294, A167, H145, H440, H469 – tubers co-inoculated with the respective antagonists (single strains) and the mixture of SRPs; GF – tubers inoculated with the GF mixture of antagonists and the SRPs; NC – negative control. Data were normalized to the average for positive control. Results from two independent experiments were pooled for analysis ($n = 20$). In the plot, boxes determine the inter-quartile range (Q1–Q3), broad lines indicate median values, the X's are average values, whiskers indicate extreme values within 1.5 times distance from the inter-quartile range, and single data points are outliers. Values indicated by asterisks are significantly different from PC: * $P < 0.05$; ** $P < 0.01$.

conditions (temperature of 28°C, 85–90% relative humidity, 5 days of incubation), the GF mixture significantly reduced the severity of soft rot symptoms when compared with tubers inoculated with pathogens alone: the average rank in the symptom severity scale was reduced by 46%, and average disease incidence was reduced by 56%. The overall disease incidence for 120 tubers inoculated with pathogens alone was 38% (Fig. 4).

Density effect of the GF mixture on tuber rotting caused by pectinolytic bacteria. The effect of the inoculum density of the GF mixture of antagonistic bacteria on its ability to protect potato tuber tissue against soft rot caused by pectinolytic bacteria when co-inoculated *via* vacuum infiltration on potato tubers was tested. Maceration of tuber tissue by pectinolytic bacteria was inhibited by GF at the density of 10^8 CFU ml⁻¹ and 5×10^8 CFU ml⁻¹, but no significant protection was observed for 10^7 CFU ml⁻¹ (data not shown).

Allocation of antagonists comprising the GF mixture to species and risk categories. The five selected antagonists were assigned to particular species based on their 16S rDNA gene sequences (>1,400 bp). The classification of strains is as follows: A294 – *Serratia plymuthica*, A167 – *Enterobacter amnigenus*, H145 – *Rahnella aquatilis*, H440 – *Serratia rubidaea*, and H469 – *Serratia rubidaea*. Sequence identity between the 16S rDNA genes of the studied strains and the reference sequences of the respective species available in the GenBank database was 99 to 100% (data not shown). All five selected antagonists are conceded as GRAS (Generally Recognized As Safe) according to the American Food and Drug Administration (FDA). They all belong to the risk category 1 according to ATCC; they are neither known to consistently cause disease in healthy individuals nor do they pose risk for animals, plants, and the environment.

Growth of selected antagonists at different temperatures. In replicated experiments, growth of A294, A167, H145, H440, and H469 strains was determined at 28, 37, 40, and 42°C in liquid medium over a period of 16 h and at 7, 10, 28, 37, 40, and 42°C on a solid medium for 120 h. In liquid medium, all five strains grew at all tested temperatures, reaching, after 16 h, an average log CFU between 7.5 and 9.2 (Supplementary Fig. S1). On the solid medium, after 5 days of incubation, all five strains showed growth at 7, 10, 28, and 37°C. Additionally, strains A167, H440, and H469 grew at 40°C, and strains H440 and H469 grew at 42°C (Supplementary Table S3).

Fig. 4. Efficacy of the GF mixture of antagonists in suppressing soft rot symptoms caused by a blend of SRP pathogens on vacuum-infiltrated potato tubers. Box plot shows the disease severity ranks obtained for tubers infiltrated with: PC – tubers infiltrated with SRP pathogens alone (positive control); GF – tubers infiltrated subsequently with the GF mixture of antagonistic strains and the SRP pathogens; NC – tubers infiltrated with water (negative control). Values shown in the graph were derived from 4 independent experiments, 30 tubers each ($n = 120$). Confidence level for difference between the positive control and the GF treatment is given in the graph.

Antibiotic susceptibility profile of the selected antagonistic strains. Strains A294, A167, H145, H440, and H469 were evaluated for their susceptibility to 21 commercially available antibiotics. All strains were susceptible to: chloramphenicol doxycycline, colistin, piperacillin + tazobactam, aztreonam, imipenem, ciprofloxacin, tige-cycline, gentamicin, ceftazidime, fosfomicin, rifampin, ampicillin + sulbactam, and streptomycin and were resistant to: oxacillin, dalfo-pristin + quinupristin (Synercid), clindamycin, linezolid, vancomycin, and fusidic acid. Strain H145 was resistant to ampicillin, unlike strains A294, A167, H440, and H469. In total, strains A294, A167, H440, and H469 showed susceptibility to 15 antibiotics, and strain H145 was susceptible to 14 of the 21 antibiotics tested (Supplementary Table S2).

Influence of the selected antagonists on the survival of *Caenorhabditis elegans*. At the end point of the liquid killing assay (3 days), the average survival rate of *C. elegans* cultivated on *E. coli* OP50 as a food source (negative control) was 91%. At the same time, no viable nematodes were found in the presence of *P. aeruginosa* PA14, known for its killing potential to the nematode (positive control). The survival rate for *C. elegans* treated with either A294, A167, H145, H440, or H469 ranged from 58 to 91%. Among the tested strains, A294 and H440 ensured the lowest average survival rates (58 and 65%, respectively). Nematode survival rates obtained for treatments containing A167, H145, and H469 amounted to 88, 90, and 81%, respectively, and were not significantly statistically different from the negative control (OP50) (Supplementary Fig. S2).

Discussion

This study was conducted to develop and evaluate a mixture of antagonistic bacterial strains for protection of potato seed tubers against SRP. Numerous bacterial antagonists against different *Pectobacterium* and *Dickeya* species have been isolated and characterized previously to control these bacteria on potato and other crops (Diallo et al. 2011). However, this study, to our knowledge, is the first dealing with a stepwise screening to develop a mixture of antagonists to be used against a spectrum of different *Pectobacterium* spp. and *Dickeya* spp. applied simultaneously, and under high disease pressure (Boyd 1972). This approach was taken to simulate a worst-case scenario in which a susceptible host under adverse environmental conditions is challenged simultaneously by several SRP pathogens present in high numbers. Therefore, we postulated that if the mixture of antagonists is able to provide protection against SRP under the proposed experimental conditions, it will be also able to control the bacteria under natural conditions less favorable to disease development (Pérombelon and Lowe 1975).

To date, no biological control measures exist to protect potato tubers against SRP bacteria. The use of compatible mixtures of antagonistic bacteria has, however, many advantages over applications containing single biological control agents (Rechcigl 2017). Application of such artificial communities may provide better protection of the plant thanks to a broader range of pathogen-suppressive mechanisms, more efficient colonization of the host, and higher persistence in the plant environment, i.e., due to higher adaptation to changes throughout the growing season. Mixtures are also more likely to be effective against a wider range of pathogens at the time (Siddiqui and Shaukat 2002; Stockwell et al. 2010).

Although for the initial screening we used 22 antagonists well-characterized in our previous studies, distributed randomly in 15 mixtures of 4 to 5 strains each, only three of these mixtures: M2, M4, and M14 protected potato tubers from rotting caused by a combination of five *Pectobacterium* spp. and *Dickeya* spp. This can be partly explained by the fact that the individual antagonists were selected initially against a limited number of SRP strains (Jafra et al. 2006, 2009; Krzyzanowska et al. 2012a, b). The biocontrol potential of some of them was reported to be highly dependent on the species of soft rot pathogen (Krzyzanowska et al. 2012b). Similarly, it cannot be excluded that some of the antagonists also inhibited other members of the mixture. The majority of strains used in this study originate from the rhizospheres of diverse plants (Table 1). These niches are highly competitive, and it is widely accepted now that in such

complex environments microorganisms most often express competitive phenotypes (Foster and Bell 2012), which could affect development of new biocontrol mixtures (Jetiyanon and Kloepper 2002).

The final outcome of this study, the GF mixture of five effective antagonists, provided stable and significantly high level of seed tuber protection against all tested SRP under disease favorable conditions. The bacteria present in this mixture possess different mechanisms by which they can control SRP on potato tubers. This includes direct antagonism *via* production of antibiotic compounds and biosurfactants (strains A294, H145, H469), inactivation of signal molecules regulating expression of virulence factors in SRP (so-called quorum quenching) (strains A167, H440), and production of siderophores chelating iron ions in environment (strains H145, A294, H440, H469) (Table 1). These features have all been reported to play a role in biological control of phytopathogens including potato-infecting SRP (Baker 1987; Shoda 2000). Mixtures of bacterial antagonists can be applied as stand-alone plant protection measures, as demonstrated in this study, making them of interest in organic crop production. It is equally possible to apply these agents together with conventional treatments to reduce the use of chemicals and/or to improve their efficiency in agricultural applications (Ferron and Deguine 2005).

We consider that *S. plymuthica* strain A294, *E. amnigenus* strain A167, *R. aquatilis* strain H145, *S. rubidaea* strain H440, and *S. rubidaea* strain H469 are potentially good candidates for developing a commercial protection product for potato tubers for several reasons. In our assays, each of the strains alone provided a significant protection of tubers against rotting caused by SRP under disease promoting conditions and in different setups. Furthermore, in former studies, three of the strains comprising the GF mixture, namely H145, H440, and H469, were shown to have a protective effect against soft rot of hyacinth bulbs (Jafra et al. 2009). This implies that the developed mixture is likely to be applicable on other soft rot-affected agricultural plants and ornamentals. Likewise, the five strains are all classified into risk category 1 according to ATCC and recognized as GRAS according to FDA, meaning that they are not expected to pose risks for humans and/or the environment. As well, they are susceptible to the commercially available antibiotics. Members of these species have been already used in (commercial) biological control applications on different crops including strawberry, cucumber, oil-seed rape, potato, grape, tomato, apple, and others (Calvo et al. 2007; Chen et al. 2007; De Vleeschauwer and Höfte 2003; El-Hendawy et al. 2005). Furthermore, in experiments in which *C. elegans* was grown on A294, H145, A167, H440, or H469, all investigated strains showed significantly less pathogenicity toward nematodes compared with well-known nematode-killing *P. aeruginosa* strain PA14. In all cases, nematodes treated with the antagonistic bacteria survived till the end of experiment. *C. elegans* killing assay is a well-accepted model system to study bacterial pathogenicity toward eukaryotic organisms. This model is also recommended as a first test to assess if a particular isolate can be considered as biological control agent to be used in agriculture (Zachow et al. 2009).

We propose to use the developed screening system as a method of choice for a more reliable selection of antagonistic mixtures. Briefly, once the initial individual antagonists of choice have been selected, several random mixtures containing up to five antagonists each are prepared and tested in assays with pathogens under disease favorable conditions. Some of the tested mixtures would provide better protection than others, and these should be selected for further studies. In the next step, the individual antagonists from the selected mixtures are then tested alone using either a simpler laboratory assay or the same assay as the one used for the initial screening. The antagonists providing the best protection should be selected and finally mixed together to create a new working composition which is evaluated under disease favorable conditions (Supplementary Fig. S3). This step-wise selection of antagonists' mixture could be applied commonly and work not only on potato but also on other crop systems.

In conclusion, although the results obtained in this study are promising for biocontrol of soft rot caused by a mixture of SRP and under conditions promoting disease development, there is still considerable work to be done to achieve a viable commercial application. Points

that require further examination are: formulation of a bacterial preparation, optimization of application procedures, assessing the longevity of the applied mixture on (seed) potato tubers during storage and in transit, as well as the effectiveness of the mixture when applied on a wide range of potato cultivars and under different storage conditions. Finally, elucidation of the molecular basis of interactions between the five antagonistic strains in the mixture will be of interest, not only to contribute to fundamental knowledge, but also to explore the use of this combination in other pathogen–host systems.

Author Contributions

Conceptualization: DK, TM, RC; data curation: DK, TM; formal analysis: DK, SJ, RC; funding acquisition: RC, MK; investigation: DK, TM, JS, MK; methodology: DK, TM, MK, JS; project administration: DK, RC; resources: RC, MK; supervision, validation: SJ, RC; visualization: DK, TM, MK, RC; writing ± original draft: DK, RC; writing ± review and editing: DK, SJ, RC

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