Ecology and control of Dickeya spp. in potato

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Chapter 6

Characterization of bacterial isolates from rotting potato tuber tissue showing antagonism to *Dickeya* sp. biovar 3 in vitro and *in planta*

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ABSTRACT

Possibilities for biocontrol of biovar 3 *Dickeya* sp. in potato were investigated, using bacteria from rotting potato tissue isolated by dilution plating on non-selective agar media. In a plate assay, 649 isolates were screened for antibiosis against *Dickeya* sp. IPO2222 and for the production of siderophores. Forty one strains (6.4%) produced antibiotics and 112 strains (17.3%) produced siderophores. A selection of 41 antibiotic-producing strains and 41 siderophore-producing strains were tested in a potato slice assay for control of the *Dickeya* sp. Strains able to reduce rotting of potato tuber tissue by at least 50% of the control were selected. Strains were characterized by 16S rDNA analysis as *Bacillus*, *Pseudomonas*, *Rhodococcus*, *Serratia*, *Obesumbacterium* and *Lysinibacillus* genera. Twenty three isolates belonging to different species and genera; 13 producing antibiotics and 10 producing siderophores, were further characterized by testing for quorum quenching, motility, biosurfactant production, growth at low (4.0) and high (10.0) pH, growth at 10 °C under aerobic and anaerobic conditions and for auxin production. In replicated greenhouse experiments, 4 selected antagonists on the base of the *in vitro* tests, were tested *in planta* using wounded or intact mini tubers of cv. Kondor subsequently-inoculated by vacuum infiltration with an antagonist and a GFP-tagged biovar 3 *Dickeya* sp. strain. A potato endophyte A30, characterized as *S. plymuthica* protected potato plants by reducing blackleg development by 100% and colonization of stems by *Dickeya* sp. by 97%. The potential use of *S. plymuthica* strain A30 for the bio-control of *Dickeya* sp. is discussed.

INTRODUCTION

Soft rot and blackleg diseases of potato caused by pectinolytic bacteria belonging to *Pectobacterium* and *Dickeya* spp. are a continuous threat to (seed) potato production worldwide. Potato plants and tubers are affected by the presence of pectinolytic bacterial pathogens in virtually all phases of tuber production including storage. In Western and Northern Europe, in particular *Dickeya* spp. cause increasing economic losses in seed potato production, generally due to declassification and rejection of seed lots (Netherlands General Inspection Service for Agricultural Seeds and Seed Potatoes, personal communication). *Dickeya* spp. cause pre-emergence seed piece decay, blackleg and aerial stem rot, and soft rot of progeny tubers (Perombelon, 2002). Increased losses in seed potato production in

Europe resulting from *Dickeya* spp. infection are related to the recent occurrence and spread of a new *Dickeya* sp. genetic clade belonging to biovar 3 which probably constitutes a new *Dickeya* species (Tsror et al., 2008).

Potato (seed) tubers are the primary source of soft rot and blackleg inoculum (Perombelon, 1974). Production of pathogen-free seed tuber lots or eradication of the existing bacteria in seed lots is consequently of major interest. Selection for blackleg and soft rot resistant potato cultivars has not resulted in seed lots completely free from *Pectobacterium* and *Dickeya* spp. bacteria. Soft rot and blackleg control therefore, is presently based on an integrated strategy which includes the use of pathogen-free initial propagation material and practices which avoid the wounding of tubers and plants, the occurrence of oxygen depletion by free water on tuber surface and smearing and dissemination of the pathogens within or between seed lots by mechanical equipment (Perombelon, 2002).

Simultaneously, the use of physical (e. g. hot water treatment) and chemical disinfection procedures for controlling blackleg and soft rot have resulted in a reduction, but not in an elimination of the bacterial inoculum (Perombelon & Salmond, 1995). During chemical and/or physical sterilization bacteria present on or in the periderm may be destroyed, but not those present inside the tubers. It has already been reported that the highest densities of pectinolytic *Pectobacterium* and *Dickeya* spp. are located in the vascular system at the stolon end of tubers (Czajkowski et al., 2009). Inoculum present inside tuber tissue will not be affected by superficial sterilization procedures.

Bacterial antagonists can be considered as an alternative to chemical and physical control strategies to reduce *Dickeya* spp. populations in plant vascular tissue and in tubers during storage. Successful, commercially available bio-control agents were, however only developed for a limited number of bacterial phytopathogens. *Agrobacterium radiobacter* K84 was developed to control of *A. tumefaciens* the causative agent of grown gall (Vicedo et al., 1993). A product base on *Erwinia herbicola* (*Pantoea agglomerans*) successfully controlled *E. amylovora*, responsible for fire blight in apple and pear (Stockwell et al., 1998).

Several publications describe promising results for biocontrol of bacterial pathogens in potato. For *Clavibacter michiganensis* subsp. *sepedonicus*, the causal agent of bacterial ring rot, in field experiments, reasonable levels of control have been obtained with *Arthrobacter histidinolovorans* and *Serratia* sp. in field experiments. Both strains reduced tuber infections by 35-70% and infection in plants by 60-80% (Gamard & De Boer, 1995). Wilt symptoms caused by *Ralstonia solanacearum* could be reduced by up to 83 and 73% using *Bacillus subtilis* and *Pseudomonas fluorescens* strains respectively (Aliye et al., 2008). *Streptomyces*

scabies, causing common scab, was antagonized by a *Bacillus* sp. strain in pot assay experiments and reduced infections in potato tubers by up to 35% (Han et al., 2005). Fluorescent *Pseudomonas* spp., applied to tubers, reduced populations of blackleg and soft rot bacteria on potato roots and inside progeny tubers. They were also effective in controlling postharvest soft rot on potato (Kloepper, 1983). Finally, *Bacillus subtilis* producing antibiotics active against a broad spectrum of bacteria, showed a high level of control towards *Pectobacterium* spp. in *vitro* and on potato tubers (Sharga & Lyon, 1998). None of the bacterial bio-control agents, however have been used in commercial application.

Only limited attempts have been made for the biocontrol of *Dickeya* spp. Various Gram-positive and Gram-negative bacteria isolated from different hosts and environments producing antibiotics against *Dickeya* spp. or siderophores competing with *Dickeya* spp. for iron have been characterized (Kastelein et al., 1999, Jafra et al., 2006b). Bacteria isolated from the potato tuber surface (Kastelein et al., 1999) or the potato rhizosphere (Jafra et al., 2006b) proved useful in the protection of tuber tissue from *P. atrosepticum* and *Dickeya* spp. However, to date, these bacteria have not been tested for the control of *Dickeya* spp. in potato plants.

The purpose of this study was to select and characterize bacterial biocontrol agents against the recently described genetic clade of *Dickeya* sp. biovar 3 in potato. Bacteria were isolated from rotten potato tuber tissue to obtain isolates able to compete with *Dickeya* spp. in this harsh environment. Selection of antagonists involved the antibiotic production, the ability to compete for iron with the pathogen by the production of siderophores, the production of (antibacterial) surfactants and the ability to interfere with quorum sensing and the ability to grow under different environmental conditions. The selection also included greenhouse experiments with treated tubers.

MATERIALS AND METHODS

Bacterial strains and media used for cultivation

Bacterial strains isolated and used in this study are listed in Table 1. Tryptic soya agar (TSA) (Oxoid, Badhovoevendorp, The Netherlands), King's B agar (Fluka, Zwijndrecht, The Netherlands), nutrient agar (NA) (Difco, Breda, The Netherlands) and R2A (Difco) agar media supplemented with 200 µg ml^{-1} of cycloheximide (Sigma) to prevent fungal growth were used to isolate bacteria from rotting tuber tissue. NA or TSA and/or nutrient broth (NB) (Difco) were used for

bacteria maintenance. Bacteria were grown on or in media for 24 - 48 h at 28 °C. Growth of bacteria in liquid media was done by shaking at 200 rpm during incubation. Biovar 3 type strain, *Dickeya* sp. IPO2222 (Tsror et al., 2008) was grown on TSA or in NB and *Dickeya* sp. IPO2254 pPROBE-AT-*gfp* (Czajkowski et al., 2010a) was grown on TSA or in NB supplemented with 150 µg ml^{-1} of ampicillin.

Rotting of potato tubers and isolation of potato endophytes

Dickeya spp.-free minitubers of cv. Kondor, and seed tubers of cv. Arcade, Agria, Konsul and Kondor were used to isolate bacteria from rotting potato tissue. To isolate bacterial endophytes indigenously present in potato tubers, tubers were sterilized by two times 10 min incubation in 70% ethanol followed by washing in running tap water. Surface-sterilized tubers were firstly mechanically wounded and subsequently enclosed in Saran Wrap (foil) and kept for 5 to 10 days at 28 °C. Isolation of bacteria able to colonize rotting tuber tissue from soil was done using tubers buried either in loamy sand, a rich soil collected in the region of Wageningen, NL (S1) (Garbeva et al., 2003), or in potting compost (S2) (basis potgrond nr. 4, Hortmea group, The Netherlands) and kept for 5 to 10 days under low-oxygen conditions obtained by immersion of pots in water to 80% its capacity and 90% relative humidity at 28 °C in closed humid boxes. Rotting tubers in soil were washed with tap water before processing. Approximately 20 g of the rotting potato tissue, collected if possible from the inside of each tuber, was resuspended in 50 ml of a quarter strength (1/4) Ringer's buffer (Merck) supplemented with 0.02% diethyldithiocarbamic acid (DIECA) (Acros Organics) as an antioxidant. Rotting tuber suspensions were incubated at room temperature for 1 h with shaking (200 rpm). Serial dilutions of the rotting tissue extracts were made in a Ringer's buffer and 100 µl of 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated on King's B (Fluka), R2A (Difco), TSA (Oxoid) and NA (Difco) plates supplemented with 200 µg ml^{-1} cycloheximide (Sigma, Zwijndrecht, The Netherlands). Plates were incubated at 28 °C for 24 – 48 h. Single bacterial colonies of different colony morphologies were collected from plates inoculated with material from different potato tubers and different treatments for further analyses. Bacteria were grown to pure cultures on TSA or NA and stored at -80 °C in NB/glycerol (60%/40%) stocks.

Pre-screening of candidate antagonists against Dickeya sp. *IPO2222*

Pure cultures of collected strains were tested for the ability to produce antibiotics against *Dickeya* sp. IPO2222. or siderophores.

The availability of iron ions is crucial during plant infection. In an iron poor environment the virulence of *Dickeya* spp. is seriously reduced (Expert, 1999). The presence of bacteria able to produce strong siderophores and sequester iron from the environment will theoretically reduce disease development by *Dickeya* sp..

In each case, two independent replications were made for each tested strain and the experiment was independently repeated one time. Results from both experiments were averaged.

Production of antibiotics

Production of antibiotics was tested in an overlay assay with *Dickeya* sp. IPO2222 as indicator strain. 200 µl of an overnight culture of *Dickeya* sp. (ca. 10^9 cfu ml^{-1}) in NB was mixed with 20 ml of soft top agar (NB supplemented with 0.7% agar) at 45-50 °C and poured onto square (15 x 15 cm) TSA plates. Once the agar had solidified, 2.5 µl of an overnight culture of the antagonist grown in NB (ca. 10^9 cfu ml^{-1}) was spotted on the surface of the agar plate. Plates were incubated for 24 – 48 h at 28 °C. Isolates inhibiting growth of *Dickeya* sp. IPO2222 and causing a clear 'halo' around their colonies were collected for further analyses.

Siderophore production assay

A CAS plate assay was used to evaluate siderophore production by test isolates (Schwyn & Neilands, 1997). 2.5 µl of overnight isolate culture in NB (approx. 10^9 cfu ml^{-1}) was spotted on the surface of the CAS agar plate. Plates were incubated for 24 – 48 h at 28 °C. Isolates producing an orange/pink halo around colonies, indicating siderophore production, were collected for further analyses.

16S rDNA sequence analyses

To identify the bacterial isolates, a 16S rDNA fragment between 968 and 1401 bp (numbering based on the *Escherichia coli* genome) was sequenced with primers

F968 (5'AACGCGAAGAACCTTAC 3') and R1401 (5'CGGTGTGTACAAGGCCCGGGAACG3') in both directions. A colony PCR procedure was used to amplify DNA. Individual colonies were collected from TSA plates using a sterile toothpick and suspended in 50 µl of 5 mM NaOH (Sigma). Suspensions were boiled at 95 °C for 5 min and kept at 4 °C. One µl of the cell lysate was used per PCR reaction. PCR products were purified with the PCR purification kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Ijssel, The Netherlands). DNA sequences were compared with available sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) using nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) for nucleotides (blastn) alignments accessed from the website (http://www.ncbi.nlm.nih.gov/BLAST/).

Determination of species in risk groups

The DSMZ (German Collection of Micro-organisms and Cell Cultures) (http://www.dsmz.de/) data base was used to classify bacterial isolates into risk categories on the basis of their ability to cause disease in humans (WHO – World Health Organization http://www.who.int/en/).

Screening for antagonism in a potato slice assay

A potato slice assay was used to evaluate the ability of selected isolates to reduce potato tissue maceration by *Dickeya* sp. IPO2222. Bacteria were centrifuged (5 min, 6000 x g) and washed twice with 1/4 Ringer's buffer. Densities of test isolates were adjusted to ca. 10^8 cfu ml^{-1} and *Dickeya* sp. to ca. 10^6 cfu ml^{-1} with sterile water. Potato tubers of cv. Agria (Agrico, The Netherlands) were rinsed with tap water, surface-sterilized with 70% ethanol for 10 min, rinsed with tap water and dried with tissue paper. Tubers were cut into 0.7 cm transverse slices using a sterile knife. Three wells (5 x 5 x 5 mm) per slice were made using a sterile cork borer and were filled with 50 µl suspension containing 10^8 cfu ml^{-1} of test isolate and 10^6 cfu ml^{-1} of *Dickeya* sp. For each test strain, 3 potato slices derived from three different tubers were used. Negative control was 50 µl of sterile water instead of bacterial suspension. The effect of test isolates on potato tissue was measured by comparing ratio of the average diameter of rotting potato tissue around wells subsequently-inoculated with *Dickeya* sp. and an antagonist with the average diameter of rotting potato tissue around wells of the positive control (*Dickeya* sp.

only). Two replications were made for each tested strain and the experiment was independently repeated one time. Results from both experiments were averaged.

Characterization of selected isolates for features potentially involved in antagonism

Isolates showing at least 50% decrease of rotting ability of *Dickeya* sp. in the potato slice assay were screened for motility, AHLs production and degradation, auxin production with and without supplementation of L-tryptophan, biosurfactant production, growth at pH 4.0 and pH 10.0 and at 10 °C in anaerobic and aerobic conditions and for pectinolytic enzyme production.

Motility is an important feature in active colonization of plant surfaces (e. g. roots, leaves), migration in soil and in establishment of high bacterial populations on plants (Andersen et al., 2003). Motile antagonistic bacteria will have an advantage in competition with motile *Dickeya* spp. in the plant and soil environment.

AHL based quorum sensing mechanism is involved in regulation of different physiological processes in bacteria including production of pathogenicity factors. The ability to interfere with this mechanism by degradation of signal molecules (so-called quorum quenching) proved useful in the biocontrol of plant pathogenic bacteria (Uroz et al., 2003). Due to the fact that the bioassays employed in this study for testing the AHLs degradation cannot be used if a strain produce own AHLs, firstly the AHLs production in the selected strains was tested. All the isolates able to produce own signal molecules were excluded from AHLs degradation assay.

Antagonists were tested for their ability to produce auxins. Microbial plant hormones many contribute to plant development and fitness (Leveau, 2010).

Biosurfactants are surface active substances decreasing the surface tension acting as dispersants or detergents. In the bio-control, the main mode of surfactant action is a destabilization of the cell membranes and lysis of the cells. Biosurfactants can also induced plant systemic resistance towards plant pathogens (Tran et al., 2007).

For the field application of a bio-control agent important is that the isolate can survive under different environmental conditions. Potatoes may be cultivated using most types of soil from sandy rich to clay poor that largely differ in pH and organic matter and salt content. The optimal therefore would be if the antagonist was able to persistent in different pH and temperature conditions.

Competition for the same carbon and nitrogen sources released during degradation of plant tissue may be advantageous in biological control. Although there is a risk for secondary infections and enhanced tuber decay.

For each assay, two independent replications were made for each tested strain and the experiment was independently repeated one time. The results from both experiments were averaged.

Motility assay

The motility of selected isolates was tested using a motility agar assay (NB supplemented with 0.3% agar) (S. Jafra, University of Gdansk, Gdansk, Poland, personal communication). Briefly, 2.5 µl of an overnight culture of the test isolate (10^9 cfu ml^{-1}) grown in NB and diluted 10 times in NB (ca. 10^8 cfu ml^{-1}) was spotted on the surface of the motility agar plates and incubated for 24 h at 28 °C. The colony diameter after incubation time was measured. Bacterial motility was assessed using the following indexation based on the diameter of the colony: "-"below 5 mm, "+" 6 – 15 mm, "+ +" 16 – 30 mm, "+ + +" 31 – 50 mm, "+ + + +" above 51 mm.

AHLs production

Production of AHLs was tested using two AHL reporter strains; *Chromobacterium violaceum* CVO26 and *Escherichia coli* JB534 (Andersen et al., 2001). These strains produce a purple pigment (CVO26) or GFP (JB534) in the presence of exogenous AHLs. Overnight culture of the reporter strains grown on agar plates were streaked on the surface of TSA plates in two lines at a distance of 3 cm using a sterile 1 µl inoculation loop. Thereafter, 2.5 µl of an overnight culture of the test isolate grown in NB was spotted at 0.5 cm distance from reporter strain lines. Prepared plates were incubated at 28 °C for 24-48 h and visually inspected for the presence of purple pigment (CVO26) and for GFP fluorescence (JB534). The following indexation was used: "+/+" for strains positive for both reporter strains, "+/-" for strains positive for one reporter strain, and "-/-" for strains negative for both reporter strains.

AHLs degradation

To test the ability of bacterial strains to degrade AHLs, a fast screening method was used with AHLs reporter strain *Escherichia coli* JB534. AHL degradation was studied for four synthetic signal molecules: hexonoyl-L-homoserine lactone (HHL) (Sigma, Zwijndrecht, The Netherlands), 3-oxo-hexonoyl-L-homoserine lactone

(OHHL) (Sigma), octanoyl-L-homoserine lactone (OHL) (Sigma) and 3-oxo-octanoyl-L-homoserine lactone (OOHL) (Sigma). 50 µl of 10 µg ml^{-1} of the individual AHL and 20 µl of the test isolate (10^9 cfu ml^{-1} in NB) was added to 100 µl of NB buffered to pH 6.4 with 1M MOPS (Sigma) in the well of a 96-wells microtitre plate. Plates were incubated for 4 h at 28 °C. Bacteria were killed under UV light for 30 min and wells were filled with 100 µl of a suspension of *E. coli* JB534 (10^8 cfu ml^{-1} in NB). Plates were then incubated at 37 °C for 12 h and screened for GFP fluorescence using the Fluoroscan Ascent FL (Thermo Fisher Scientific, Roskilde, Denmark) with the filter sets 495 nm (excitation) and 530 nm (emission)

Auxin production

To screen for L-tryptophan (L-Trp)-independent or L-Trp-dependent auxin production, test isolates were grown overnight in NB or in NB supplemented with 0.5% L-tryptophan (Sigma) in shaken cultures respectively. Cultures were centrifuged (8000 x g, 10 min) and 3 ml of the clear supernatant was collected per sample. Auxin production was measured as IAA (indole-3-acetic acid) equivalents: 3 ml of bacterial supernatant were mixed with 2 ml of freshly prepared Salkowski reagent (2ml of 0.5M $FeCl_3$ + 98 ml 35% $HClO_4$) A standard curve of IAA was made by preparing solutions of 1 µg ml^{-1} to 30 µg ml^{-1} IAA (Sigma) in NB. As a negative control, non- auxin producing strain *Escherichia coli* strain DH5α (Invitrogen, Breda, The Netherlands), which does not produce auxins, was used. For color development, solutions were left at room temperature for 30 min. The intensity of the color was measured spectrophotometrically at 535 nm wavelength. The following indexation was used: '-' for isolates not producing auxins, "±" auxin concentration 0.1 – 0.4 µg ml^{-1}, '+' 0.5 – 1.0 µg ml^{-1}, '++' 1.1 – 2.0 µg ml^{-1}, '+++' 2.1 – 5.0 µg ml^{-1}, '++++' 5.1 – 15.0 µg ml^{-1}, and '+++++' > 15.1 µg ml^{-1} of auxins.

Biosurfactant production

Biosurfactant production was investigated by using a modified oil spreading method (Youssef et al., 2004) (S. Jafra, University of Gdansk, Gdansk, Poland, personal communication). Test isolates were grown overnight in NB at 28 °C. Cultures were centrifuged for 20 min at 8000 x g to remove the bacteria. A 40 µl of mineral oil (BioRad, Veenendaal, The Netherlands) was spotted on the surface of the 30 ml demineralized water and 5 µl of the supernatant or sterilized water

(control) was placed on the top of the oil drop. The diameter of the circle created by oil is proportional to the biosurfactant concentration. The diameter of the circle was measured for each isolate and compared with the control. The following indexation was used: '-' no biosurfactant production, '+' diameter of the circle 1 – 6 mm, '++' – 7 – 15 mm, '+++' – 16 - 30 mm, '++++' – > 30 mm.

Growth under different conditions

- *Growth at pH 4.0 and pH 10.0*

To test the growth of isolates at low and high pH, 100 µl of a suspension of 10^8 cfu ml^{-1} of the test strain in NB was added to 20 ml of NB buffered to pH 4.0 or pH 10.0 with 1M MOPS (3-(N-morpholino) – propane-sulfonic acid) (Sigma) or to 20 ml of NB, pH 7.0 (control) (S. Atkinson, Institute of Infection, Immunity and Inflammation, Nottingham, United Kingdom, personal communication). Shaken cultures (200 rpm). were incubated for 48 h at 28 °C. The turbidity of the bacterial cultures was inspected by eye and measured spectrophotometrically at 600 nm wavelength at the start (T=0) and end of the experiment (T=48). The following indexation was used: '-' no growth (increase of OD_{600} < 0.050, '+' growth (increase of $OD_{600} \geq 2$).

- *Growth at 10 °C under anaerobic and aerobic conditions*

To test the growth of isolates at 10 °C under anaerobic and aerobic conditions, 100 µl of 10^8 cfu ml^{-1} of bacteria in NB was added to 10 ml of potato dextrose broth (PDB) (Difco) buffered to pH 7.0 with 1M MOPS (Sigma). For anaerobic incubation, 5 ml of liquid paraffin (Sigma) was added on the top of the inoculated medium. Tubes were kept at 10 °C for 3 to 5 days and inspected by eye daily for turbidity. On, day 5, turbidity of the cultures was measured spectrophotometrically at 600 nm wavelength. Two experiment was repeated one time and the results were averaged. The growth of the tested isolates was evaluated by comparing the optical density (OD_{600}) at T= 0 and T= 120 h. The indexation used was: '-' increase of OD_{600} < 0.050, '+' increase of OD_{600} between 2 and 5 times, '++' increase of OD_{600} between 5 and 10 times, '+++' increase of $OD_{600} \geq 10$ times.

Production of pectinolytic enzymes

Pectinolytic activity of the test isolates was determined on polygalacturonic minimal medium (PMM) (per L: 3 g KH_2PO_4, 7 g K_2HPO_4, 2 g $(NH_4)_2SO_4$, 4 g polygalacturonic acid (PGA), 15 g agar; pH 7.0. As a positive control *Dickeya* sp. IPO2222 was used as a positive control. 2.5 µl of the test isolate (10^9 cfu ml^{-1} in NB) was spotted on the surface of PMM. Plates were incubated for 24-48 h at 28 °C. After this time, plates were washed with 10% copper acetate solution in water and left for 15 min at room temperature for halo development. The diameter of the white halo around of the bacterial colony is proportional to the concentration of pectinolytic enzymes. The following indexation was used, based on the diameter of the halo: '-' no halo, '+' – 0.1 – 10 mm, '++' 11 – 15 mm, '+++' 16- 25 mm.

Inoculation of potato tubers and growth of potato plants

In the first greenhouse experiment, in May – June 2009, 12 selected antagonists were tested for their ability to protect potato plants from infection by *Dickeya* sp. IPO2254 and disease development. Suspensions of test isolates and *Dickeya* sp. IPO2254 were prepared in sterile demineralized water to achieve densities of 10^{11} cfu ml^{-1} (antagonist) and 10^6 cfu ml^{-1} (*Dickeya* sp. IPO2254). Certified *Dickeya* spp.-free minitubers of cv. Kondor (Dutch Plant Inspection Service for agricultural seeds and seed potatoes (NAK), Emmeloord, The Netherlands) were used. Half of the tubers were wounded by removing 0.5 cm of stolon end with a sterile knife and others left intact. Both types of minitubers were immersed in the antagonist suspension and vacuum infiltrated for 10 min at -800 mBar in a desciccator followed by 10 min incubation at atmospheric pressure to allow the bacteria to penetrate the tuber lenticels and wounds. For control, intact and wounded mini tubers were vacuum infiltrated with sterile demineralized water. Tubers were dried in a flow cabinet overnight and the next day they were vacuum infiltrated with *Dickeya* sp. IPO2254 suspension or, for the control, with sterile demineralized water and dried overnight in flow cabinet. Tubers were planted in potting compost in 5 l plastic pots and kept at a 16/8 h photoperiod, 80% relative humidity (RH) and 28 °C for 3 weeks in a greenhouse. To eliminate the bias effect of the environmental conditions, a random plot design of the pots was applied (8 blocks containing 5 pots from each individual strain). In each treatment, 20 intact and 20 wounded (tubers from which 0.5 cm of the stolon end was cut off with sterile knife) Kondor minitubers were used per test strain. In the subsequent second

greenhouse experiment in June and July 2010, the four most promising candidates from first greenhouse experiment, using the same design and under the same conditions as in the first experiment were tested.

Symptom development

Plants were visually inspected weekly for symptoms development and were evaluated for wilting, chlorosis of leaves, black rot at the stem base, aerial stem rot, haulm desiccation and whole plant death.

Sampling of potato plants derived from vacuum infiltrated tubers

Potato plants were sampled 25 days post tuber planting. Approx. 1.5 cm long stem segments taken 5 cm above ground level were collected per plant and pooled. Stems were then surfacially sterilized for 1 min. in 70% ethanol, washed once with tap water, disinfected in 1% sodium hypochloride (commercial bleach) for 3 min, washed 3 times with tap water and dried with tissue paper. Samples were weighted and 1/4 Ringer's buffer (Merck) containing 0.02% of diethyl-dithiocarbamic acid (Acros Organics) was added to twice the weight of the sample. Samples were crushed in Universal Bioreba Bags (BIOREBA AG, Reinach, Switzerland) using a hammer. 100 µl of undiluted and decimal dilutions (0, 10^{-1} and 10^{-2}), of plant extract were mixed with 300 µl of pre-warmed to 45-50 °C PT medium containing 200 µg ml^{-1} of cycloheximide and 150 µg ml^{-1} of ampicillin in the well of a 24 well plate (Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands). After the medium had solidified, plates were incubated for 24-48 h at 28 °C to allow the bacteria to grow. Wells were inspected for the presence of GFP-tagged *Dickeya* sp. IPO2254 under 495 nm blue light using an epifluorescence stereo microscope (Leica Wild M32 FL4) equipped with a mercury high pressure photo-optic lamp (Leica Hg 50W/AC) and GFP plus filter (Czajkowski et al., 2010a). GFP positive bacterial colonies were counted.

Statistical analyses

Greenhouse experiments were done according to a completely randomized block design. In the experiment with 12 selected strains 40 plants (20 with wounded and 20 with intact tubers) were used per treatment divided over 8 complete blocks containing 5 plants each. In the experiment with a sub-selection of 4 strains, 40

plants (20 with wounded and 20 with intact tubers) were used per treatment divided over 5 complete block containing 6 plants and one complete block containing 5 plants. Observations were as follows: (1) incidence of tuber sprouting or not, (2) level of emerged plants with blackleg symptoms and (3) measurements of plant height and weight. Sprouting and the incidence of blackleg symptoms were analyzed using a standard generalized linear mixed model (GLMM) with the binomial distribution and a logit link to map the discrete outcome onto the real line (Cramer, 2004). A normal distribution was assumed for plant height and weight. Effects were considered to be significant at $P \leq 0.05$ and pair-wise differences were obtained using the *t* test. Data were analyzed using the statistical software package GenStat (Payne et al., 2009).

RESULTS

Collection of candidate bacterial antagonists

In total, 649 isolates were collected from tubers belonging to different potato cultivars and from various agar media (NB, R2A, King's B and TSA): 289 isolates from tubers rotting in soil type I (S1), 143 isolates from tubers rotting in soil type II (S2) and 217 isolates from tubers rotting in plastic foil (foil). Bacteria with different colony morphologies were selected. In a first screening, all isolates were tested for antibiosis against *Dickeya* sp. IPO2222 in an overlay plate assay and for production of siderophores. Forty-one isolates (6.3 %) produced antibiotic factors against *Dickeya* sp. IPO2222 strain whereas 112 isolates (17.3 %) produced siderophores, from which 41 isolates (6.3 %) produced a large orange/pink halo on the CAS agar plates (data not shown). None of the strains produced both antibiotics and siderophores. Forty one strong siderophore producers together with the 41 strains producing antibiotics were selected for further studies.

Table. 1 Characterization of 23 bacterial isolates from rotting potato tubers able to reduce tuber decay caused by *Dickeya* on the basis of motility, AHLs production and degradation, production of auxin, siderophores and biosurfactants, antibiosis, pectinolysis and growth in a medium at pH 4.0 and 10.0 and at 10 °C, under aerobic and anaerobic conditions

Isolate[1]	origin: potato cultivar	origin: rotting type[2]	Motility[3]	AHLs production[4]: CVO26/JB534	AHLs degradation[5]: HHL	AHLs degradation[5]: OHHL	AHLs degradation[5]: OHL	AHLs degradation[5]: OOHL	Auxin[6]: -Trp	Auxin[6]: +Trp	Pectinolysis[7]	Siderophore[8]	Antibiosis[9]	Biosurfactant[10]	growth in NB[11]: at pH 4.0	growth in NB[11]: at pH 10.0	growth at 10 °C[12]: anaerobic	growth at 10 °C[12]: aerobic
A3 * (*Lysinibacillus sphaericus*)	Kondor	S1	++	-/+	nd.	nd.	nd.	nd.	+	+++	+++	-	+	+++	-	-	+++	+
	Kondor (minitubers)	S1																
A6 * (*Bacillus simplex*)	Kondor	S2	-	-/-	+	+	+	+	+	+++	-	-	+	-	-	-	+	+
A10 * (*Rhodococcus erythropolis*)	Agria	foil	++++	-/+	nd.	nd.	nd.	nd.	±	+++	+++	-	+	±	-	-	+	++
A12 * (*B. subtilis*)	Arcade	S1	-	-/-	+	+	+	+	±	+++	++	-	+	+	-	-	-	-
A13 * (*Pseudomonas brassicacearum*)	Arcade	foil	++++	-/-	+	+	+	+	±	+++	-	-	+	-	-	-	-	+
A17 (*B. subtilis*)	Konsul	S1	-	-/-	-	-	-	-	+	+++	+	-	+	+++	-	-	-	-
A19 ** (*B. simplex*)	Arcade	foil	-	-/-	+	+	+	+	+	+++	-	-	+	-	-	+	-	+
A20 (*B. simplex*)	Arcade	foil	-	-/-	-	-	-	-	+	+++	-	-	+	++	-	-	-	-
A21 (*B. subtilis*)	Arcade	foil	++	-/-	-	-	-	-	±	+++	-	-	+	++	-	-	-	-
A23 (*Serratia plymuthica*)	Arcade	foil	++++	-/+	nd.	nd.	nd.	nd.	±	+++++	-	-	+	+	-	-	++	+++
A30 ** (*S. plymuthica*)	Arcade	foil	++++	-/+	nd.	nd.	nd.	nd.	±	+++++	-	-	+	+++	-	+	++	+++
A34 (*S. plymuthica*)	Agria	foil	++++	-/+	nd.	nd.	nd.	nd.	±	+++++	-	-	+	++	-	+	++	+++
A36 (*B. subtilis*)	Agria	foil.	-	-/-	+	+	+	+	+	+++	++	-	+	+	-	-	-	+
S3 * (*P. putida*)	Arcade	S1	++++	-/+	nd.	nd.	nd.	nd.	++	+++	-	+	-	-	-	-	-	+
S9 * (*Obesumbacterium proteus*)	Konsul	S1	++++	-/+	nd.	nd.	nd.	nd.	++	+++	-	+	-	+	+	+	-	++
S10 (*P. putida*)	Konsul	S2	++++	-/+	nd.	nd.	nd.	nd.	++	+++	-	+	-	++++	-	-	-	+
S20 ** (*P. putida*)	Konsul	S1	++++	-/-	+	+	+	+	++	+++	-	+	-	+	-	-	-	+
S21 (*P. putida*)	Agria	foil	++++	-/+	nd.	nd.	nd.	nd.	++	+++	-	+	-	-	-	+	-	+++
S23 * (*P. fulva*)	Arcade	foil	++++	+/+	nd.	nd.	nd.	nd.	++	+++++	-	+	-	+++	-	+	++	+
S26 (*P. putida*)	Agria	S2	++++	-/-	+	+	+	+	+	+++++	-	+	-	+++	-	+	-	++
S31 ** (*P. fulva*)	Arcade	foil	++++	-/+	nd.	nd.	nd.	nd.	+	++	-	+	-	+++	-	+	-	+
S37 (*P. putida*)	Arcade	foil	++++	-/-	+	+	+	+	+	++	-	+	-	-	-	+	-	-
S38 (*P. putida*)			++++	-/-	+	+	+	+	++	+++	-	+	-	-	-	+	-	+

[1] Bacterial isolates from rotting potato tissue; 12 isolates were used in a first greenhouse experiment (*) and 4 isolates were used in a second (**) , only 23 isolates belonging to risk category 1(+) and protecting potato from maceration causing by *Dickeya* sp. IPO2222 in a potato slice assay were characterized in detail

[2] Potato tubers were rotting in loamy sand, a rich soil collected in the region of Wageningen, NL (S1) (Garbeva et al., 2003), or in potting compost (S2) or wrapped in plastic foil (foil). Tubers were kept for 5 to 10 days under low-oxygen conditions and 90% relative humidity at 28 °C in closed humid boxes

[3] Motility was tested in a motility agar assay: "-" diameter colony < 5 mm, "+" diameter colony 5 - 15 mm, "++" 16-30 mm, "+++" 31 - 50 mm, "++++" > 50 mm

[4] AHLs production tested in a bioassay with two indicator strains *C. violaceum* (CVO26) and *E. coli* (JB534); "-" strain does not produce AHLs, "+" strain produces AHLs

[5] AHLs degradation was determined using an *E. coli* reporter strain responding to the presence of AHLs by production of a green fluorescent protein. Degradation was determined for HHL – hexanoyl-L-homoserine lactone, OHHL – 3-oxo-hexanoyl-L-homoserine lactone, OHL – octanoyl-L-homoserine lactone and OHHL – 3-oxo-octanoyl-L-homoserine lactone. ""+" degradation of the tested signal molecule, "-" lack of the degradation, "nd." – activity not determined

[6] Auxin concentrations were spectrophotometrically determined in media supplemented with L-tryptophan (+Trp) (auxin precursor) or without (-Trp): ."-"no production of auxins, "±" concentration 0.1 – 0.4 µg ml-1, "+" 0.5 – 1.0 µg ml-1, "++" 1.1 – 2.0 µg ml-1, "+++" 2.1 – 5.0 µg ml-1, "++++" 5.1 to 15.0 µg ml-1 and "+++++" > 15.1 µg ml-1

[7] Pectinolysis was determined on a polygalacturonic minimal medium on which pectinolysis results in a white halo: '-' no halo, '+' – diameter 0.1 – 10 mm, '++' 11 – 15 mm, "+++" 16- 25 mm

[8] Siderophore production was tested in a CAS agar plate assay, in which siderophore production resulted in a pink halo "-" no halo "+" halo

[9] Antibiosis was tested *in vitro* in an overlay assay with *Dickeya* sp. IPO2222 as indicator strain; "-"no inhibition , "+" inhibition

Suppression of soft rot development on potato slices

Selected isolates (41 antibiotics and 41 siderophore producers) were tested in a potato slice assay for their ability to reduce tuber decay caused by *Dickeya* sp. IPO2222. Thirty two antibiotic producers (from 41 isolated - 78%) and 41 strong siderophore producers in CAS agar assay (from 112 isolated - 37%) were able to reduce the tuber rot to at least 50% of the control, i.e. potato slices inoculated only with *Dickeya* sp. IPO2222 (data not shown).

Identification of potential antagonists

Identification of the eighty two isolates (41 antibiotic producers and 41 siderophore producers) was achieved by partially sequencing their 16S rDNA. Alignment with

sequences deposited in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) resulted in a classification of isolates into the following genera: *Alcaliges*, *Lysinibacillus*, *Aeromonas*, *Bacillus*, *Proteus*, *Haemophilus*, *Rhodococcus*, *Pseudomonas*, *Serratia*, *Acinetobacter*, *Enterobacter*, *Obesumbacterium*, *Stenotrophomonas*, *Pantoea* and *Raoultella* (data not shown). Alignments showed similarities of the 16S rDNA sequences of test isolate and reference species in the GenBank database between 95 and 100% (data not shown). Fifty nine out of 82 tested isolates (72%) were classified as risk category 2 species (human, animal or plant pathogens) and were therefore excluded from further studies.

Characterization of features involved in antagonism

Twenty three isolates classified as risk category 1 or 1+ bacteria belonging to *Lysinibacillus sphaericus*, *Bacillus simplex*, *Rhodococcus erythropolis*, *B. subtilis*, *Pseudomonas brassicacearum*, *Serratia plymuthica*, *P. putida*, *Obesumbacterium proteus*, and *P. fulva* species, that did not pose a risk for human and animal health and were not plant pathogens, were characterized in detail (Tab. 1). Isolates were investigated for the ability to produce quorum sensing signal molecules (AHLs), biosurfactants, pectinolytic enzymes and auxins, for growth at low and high pH, for growth under anaerobic and aerobic conditions at 10 °C and for motility and AHL degradation. No situation was observed in which a particular strain reacted differently in repeated experiments. Average values of each test were calculated and distributed into categories (Tab. 1)

AHLs production. Eleven out of 23 strains (A3, A10, A23, A30, A34, S3, S9, S10, S21, S23 and S31) were able to produce AHLs in both bioassays. AHLs producers belonged to the genera *Pseudomonas* (S21, S23 and S31), *Serratia* (A23, A30 and A34) and *Obesumbacterium* species (S9). As expected, no AHLs production was observed in the Gram positive *Bacillus* spp. isolates (A17, A19, A20 andA36).

AHLs degradation. Twelve isolates that were not producing AHLs were tested for their ability to degrade 4 synthetic signal molecules (HHL, OHL, OHHL and OOHL). Nine (A6, A13, A19, A36, S20, S37 and S38) degraded all four synthetic AHLs. These strains were *Pseudomonas* and *Bacillus* species.

Biosurfactant production. Sixteen strains produced biosurfactant in the *in vitro* assay (all isolates except A6, A13, A19, S3, S21, S37 and S38). The concentration of the surfactant produced varied largely per isolate as indicated by the oil drop spread assay. The largest average diameter of the oil circle was produced by *P. fulva*, *L. sphaericus* and *B. subtilis* (A3, A17, S10, S23, S26 and S31) isolates and

the smallest by *Rhodococcus erythropolis* isolate A10. Most isolates of *P. putida* and *P. brassicacearum* were negative for biosurfactant production.

Motility. The majority of test isolates were highly motile (17 isolates out of 23), especially those identified as *Pseudomonas* and *Serratia* species. Non-motile isolates (A6, A12, A17, A19, A20 and A36) were all characterized as *Bacillus* species.

Production of pectinolytic enzymes. Five isolates (A3, A10, A12, A17 and A36) out of 23 produced recordable amounts of pectinolytic enzymes under *in vitro* conditions, characterized as *L. sphaericus*, *R. erythropolis*, *B. subtilis*, *B. subtilis* and *B. subtilis*, respectively. Isolates A3 and A10 showed the highest concentration of pectinolytic enzymes in the *in vitro* plate assay.

Auxin production. All 23 isolates produced auxins in the absence of L-tryptophan; the supplementation of L-tryptophan increased auxin production in all strains. The highest increase was observed for isolates A23, A30, A34, S23 and S26 characterized as *S. plymuthica*, *P. putida* or *P. fulva*.

Growth at low and high pH. None of the isolates, except *O. proteus* were able to grow in NB at pH 4.0. Ten out of 23 isolates showed growth at pH 10.0, namely *P. putida* and *P. fulva* species (S21, S23, S26, S31, S37 and S38), *S. plymuthica* (A30 and A34), *B. simplex* (A19) and *O. proteus* (S9).

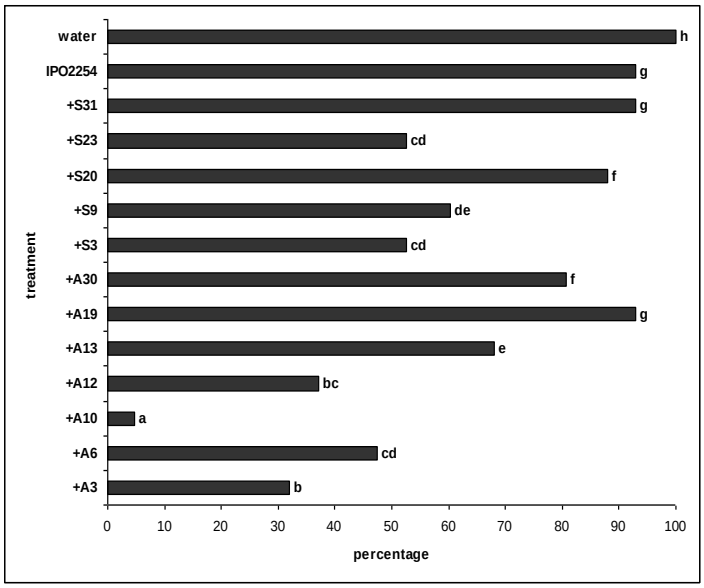
Growth under aerobic and anaerobic conditions. Under anaerobic conditions, seven strains (A3, A6, A10, A23, A30, A34 and S23) and under aerobic conditions 18 strains (all except A12, A17, A20, A21 and S37) grew at 10 $^{\circ}$C in PDB.

Greenhouse studies with vacuum-infiltrated minitubers

In the first greenhouse experiment 12 bacterial isolates (A3, A6, A10, A12, A13, A19, A30, S3, S9, S20, S23 and S31) belonging to 9 different species (*B. simplex, B. subtilis, R. erythropolis, P. brassicacearum, S. plymuthica, P. putida, P. fulva, O. proteus*, and *L. sphaericus*) were tested for protection of potato plants against biovar 3, *Dickeya* sp. IPO2254. They were selected on the basis of results in *in vitro* assays and features that may play a role in antagonism, in order to maximize variation of strains and modes of antagonistic actions. Antagonists were tested for their ability to reduce pre-emergence rot of tubers, blackleg symptoms and colonization of stems by *Dickeya* sp. IPO2254. Wounded (cut) or intact mini tubers of cv. Kondor were subsequently-inoculated subsequently with an antagonist and a GFP-tagged biovar 3 *Dickeya* sp. strain (*Dickeya* sp. IPO2254) by vacuum infiltration. Plants were screened for pre-emergence rot 15 days after planting, and weekly for symptom development. In a second greenhouse experiment, 4

antagonists (A19, A30, S20 and S31) which showed the best protection in the first greenhouse trial were used.

A



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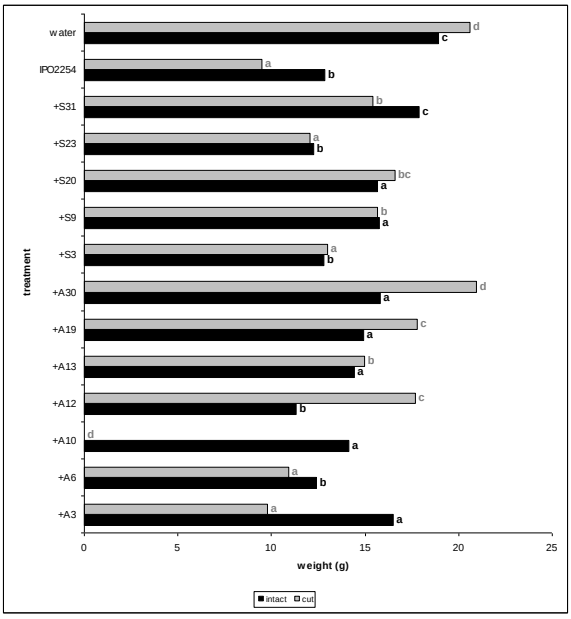


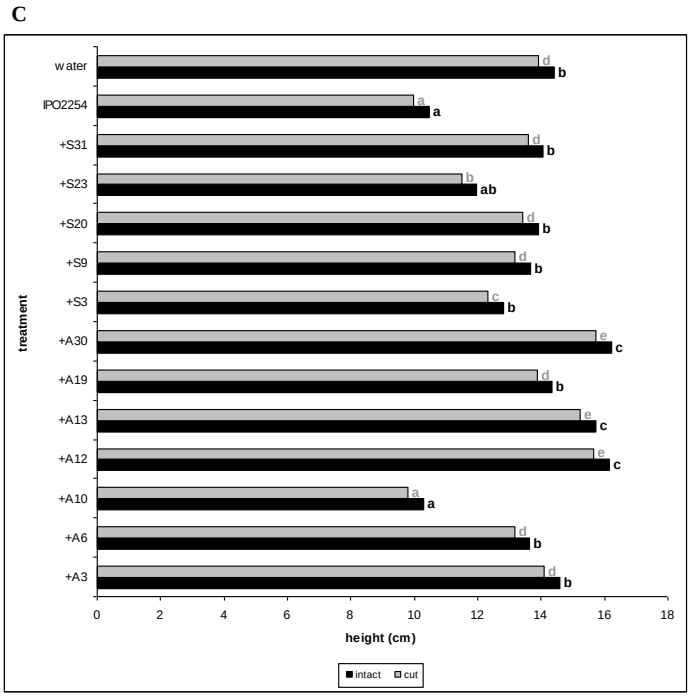


Figure 1. Effect of co-inoculation of seed tubers with *Dickeya* sp. IPO2254 and different antagonists on the percentage of sprouted tubers (a), weight (b) and maximum shoot length (c).
A) Average percentage of sprouted tubers 15 days post tuber co-inoculation. Control tubers were inoculated with water or only with *Dickeya* sp. IPO2254. No significant difference between treatments containing intact and cut tubers was observed therefore treatments were analyzed together. Values followed by identical characters are not significantly different (P=0.05)
B)Average fresh weight of shoots determined 25 days post tuber co-inoculation. Intact tubers (intact) and tubers from which stolon end part was removed (cut) were used. Control tubers were inoculated with water or only with *Dickeya* sp. IPO2254. Data from two independent experiments were analyzed together. Values followed by identical characters are not significantly different (P=0.05)
C) Average height of the highest shoot measured 25 days after tuber co-inoculation. Intact tubers (intact) and tubers from which stolon end part was removed (cut) were used. Control tubers were inoculated with water or only with *Dickeya* sp. IPO2254. Data from two independent experiments were analyzed together. Values followed by identical characters are not significantly different (P=0.05)

Non-emergence of potato plants

Non emergence of subsequently-inoculated potato tubers and controls was evaluated 15 days after planting. The percentage of tubers that generated shoots was calculated (Fig. 1A). No significant differences between treatments with intact

and cut tubers were found (data not shown), therefore treatments were analyzed together. Interestingly, inoculation with *Dickeya* IPO2254 resulted in a relative low percentage of non-emergence averaging at 7%. Equal percentages of non-emergence were found for treatments with *Pseudomonas fulva* S31 and *Bacillus simplex* A19. Co-inoculations of *Dickeya* sp. with other antagonists resulted in a higher percentage of non-emergence, from on average 19% for *S. plymuthica* A30 to a maximum of 95% for *R. erythropolis* A10.

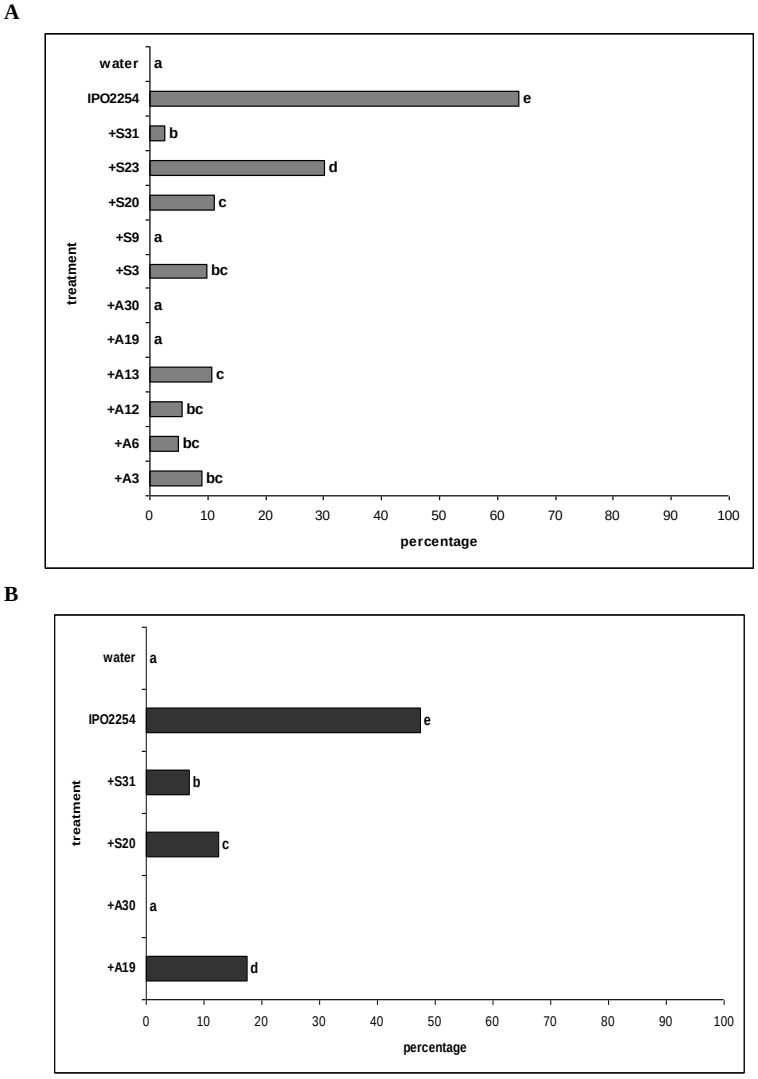
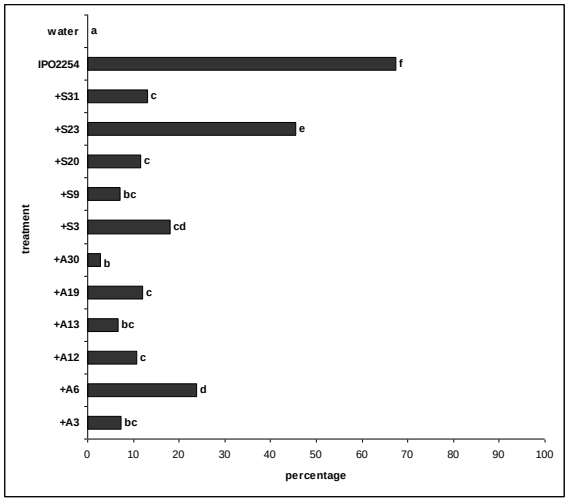


Figure 2. Effect of co-inoculation of seed tubers with *Dickeya* sp. IPO2254 and antagonists on the average percentage of symptomatic plants (showing blackleg or browning and rotting of internal stem

tissue) 25 days after incubation. In experiment 1, 12 antagonists were tested (A) and in experiment 2 a sub-selection of 4 antagonists were tested (B).
Control tubers were inoculated with water or only with *Dickeya* sp. IPO2254. No significant differences between treatments with intact and cut tubers were observed in both experiments therefore treatments were pooled before analyses. Values followed by identical characters are not significantly different (P=0.05).

A



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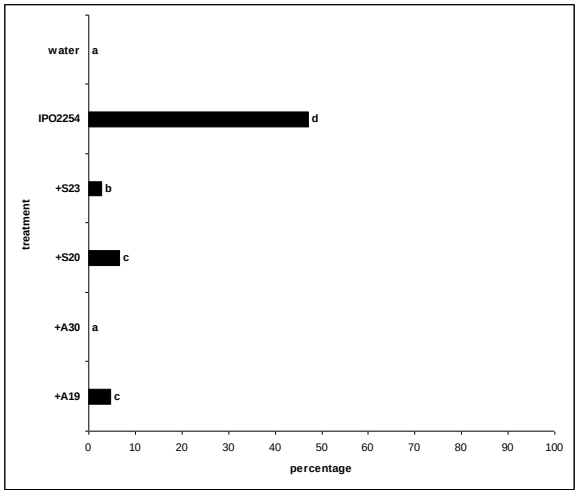

Figure 3. Effect of co-inoculation of seed tubers with *Dickeya* sp. IPO2254 and antagonists on the average percentage of plants harboring *Dickeya* sp. IPO2254, 25 days after co-inoculation. In

experiment 1, 12 antagonists were tested (A) and in experiment 2 a sub-selection of 4 antagonists were tested (B). Control tubers were inoculated with water or only with *Dickeya* sp. IPO2254.Values followed by identical characters are not significantly different (P=0.05).

Effect of antagonistic strain on the fresh weight of potato shoots

Twenty-five days after planting, the average fresh weight of potato shoots per treatment was determined (Fig. 1B). For intact tubers, fresh plant weight was significantly decreased by all treatments except for tubers subsequently-inoculated with both *Pseudomonas fulva* S31 and *Dickeya* sp. IPO2254 for which the weight of the shoots was similar to that of the water inoculated control plants. For plants raised from cut (wounded) tubers, only a treatment with *Serratia plymuthica* A30 resulted in an improvement on the adverse effect of *Dickeya* sp. on shoot weight.

Effect of antagonistic strains on the average length of the highest shoot

Twenty-five days after planting, the average length of the highest potato shoot per plant per treatment was determined for the sprouted tubers (Fig. 1C). For both intact and wounded tubers, all antagonists except *Rhodococcus erythropolis* A10, at least partially increased shoot length relative to *Dickeya* sp. Treatments with the antagonists *Bacillus subtilis* A12, *Pseudomonas brassicacearum* A13 and *Serratia plymuthica* A30 resulted in an increase in shoot height of plants derived from both cut and intact tubers relative to the control.

Effect of antagonistic strains on blackleg incidence

Twenty-five days after planting, the average percentage of symptomatic plants showing blackleg or internal discoloration and hollowing of stem tissue was determined (Fig. 2A). A significant difference between the results of intact and cut tubers was not found in any of the treatments (data not shown), therefore results of both treatments were analyzed together. Results of the treatment with joint inoculation with *R. erythropolis* A10 and *Dickeya* spp. were removed from the analysis, because of the high percentage of pre-emergence rot. All other antagonists reduced the percentage of symptomatic plants significantly. Treatment with *B. simplex* A19, *S. plymuthica* A30 and *O. proteus* S9 completely prevented symptom development. Co-inoculation of *P. fulva* S23 with *Dickeya* sp. IPO2254 resulted in 47% reduction of symptomatic plants, but for plants with the other

bacterial co-inoculations, blackleg incidence was reduced from at least 52 to 96% in all other treatments.

In a second greenhouse experiment, 4 antagonists were selected on the basis of results from the first experiment (i. e. average percentage of sprouted tubers, height and weight of shoots, reduction of blackleg symptoms and *Dickeya* sp. IPO2254 densities in stems), namely *S. plymuthica* A30, *B. simplex* A19 and *P. putida* S20 and *P. fulva* S31. A significant difference in the results between intact and cut tubers was not found in any of the treatments (data not shown), therefore data for both treatments were analyzed jointly. Approximately 47% of control plants inoculated with *Dickeya* sp. IPO2254 expressed blackleg symptoms. All four antagonists reduced the blackleg incidence significantly. Co-inoculation of potato tubers with *S. plymuthica* A30, *P. fulva* S31, *P. putida* S20 or *B. simplex* A19, resulted in 0, 7, 12 and 17% diseased plants, respectively.

Effect of antagonistic strains on Dickeya sp. *populations in stems*

Twenty-five days after planting, the percentage of plants harboring GFP-tagged *Dickeya* sp. inside stems was determined by pour-plating of stem extracts and screening for typical green fluorescent colonies (Fig. 3A). A significant difference in the results between intact and cut tubers was not found in any of the treatments (data not shown) therefore results of both treatments analyzed together. All antagonists reduced infection incidence significantly, relative to the *Dickeya* sp. control at 70 % , from 45% for *Pseudomonas fulva* S23 to 3% for *S. plymuthica* A30. *Dickeya* sp. IPO2254 was not present in any of the water inoculated plants.

In a second greenhouse experiment, as above, results of both cut and intact tubers treatments were analyzed jointly. Twenty-five days after inoculation, approximately 47% of control plants inoculated with *Dickeya* sp. IPO2254 harbored the pathogen. In contrast, the four antagonists reduced infection incidence significantly, 0, 2, 6 and 5% infected plants grown from potato tubers subsequently-inoculated with *S. plymuthica* A30, *P. fulva* S31, *P. putida* S20 or *B. simplex* A19, respectively.

Densities of *Dickeya* sp. IPO2254 in both experiments varied largely per treatment and plant screened. No statistically significant differences in results between intact and cut tubers were found (data not shown). On average in both experiments, in plants inoculated with IPO2254, 25 dpi $10^3 – 10^4$ cfu $g^{-1}$of stem tissue were present. Co-inoculation of tubers with A19, S20 and S31 antagonists resulted in a reduction of *Dickeya* sp. populations on average to 10, 100 and 100 cfu g^{-1} stem tissue, respectively (data not shown).

DISCUSSION

This study was conducted to assess the potential of antagonistic bacteria isolated from rotting potato tubers to control a distinct genetic clade of *Dickeya* sp. biovar 3, that is currently one of the dominant potato blackleg causing pathogens in Europe.

Although bacterial antagonists against *Dickeya* spp. have been isolated previously, this study is the first dealing with antagonists isolated from rotting potato tuber tissue. This approach was taken to acquire bacteria able to grow in an environment in which *Dickeya* spp. are highly active. It is generally accepted, that success of a biocontrol agent in controlling a pathogen depends on the occupation of the same niches, utilization of the same carbon and nitrogen sources or adaptation and multiplication in the same environmental conditions (Völksch & May, 2001). Therefore we speculated to find potential antagonists present in rotting potato tissue where they have to face high levels of antimicrobial metabolites and oxygen depletion rather than in healthy tuber tissue. It is known that rotting tubers contain high concentrations of different plant metabolites including ethanol, acetone, 2-butanone, acetalaldehyde, methyl acetate, ethyl acetate, propanol and butanol that show antimicrobial activity towards a variety of Gram-positive and Gram-negative bacteria (Maga, 1994). The high microbial activity in rotting tubers is also expected to result in high concentrations of antimicrobial metabolites produced by micro-organisms, including antibiotics and siderophores.

Perhaps because of the few sources from which bacteria were isolated, only a limited number of bacterial species antagonistic to *Dickeya* spp. were found in rotting tissue. The initial selection based on cultivation, and antibiotic and siderophore production resulted in bacteria belonging to only 18 genera. It was previously mentioned that potato tubers host a limited number of culturable bacterial species such as fluorescent and non-fluorescent *Pseudomonas* spp., *Bacillus* spp., *Serratia* spp. and *Xanthomonas* spp. (Sturz, 1995). To our knowledge, only very limited information is available on bacterial species present in potato rotting tissue, and no intensive study has been conducted till now.

The majority of the 82 selected bacterial strains were classified as (opportunistic) human pathogens (i.e. *Enterobacter cloace, Bacillus cereus, Alcaligens feacalis, Proteus vulgaris, Aeromonas salmonicida* and *Enterobacter cancerogenus*). Tuber decay can result in the occurrence of high densities (10^3-10^5 cfu g^{-1}) of these potential human pathogenic bacteria. They can spread over tubers

during harvesting, grading and packaging and may pose a health risk for people and animal exposed. Protection in rotting tuber tissue may result in enhanced survival periods of these pathogens. Growth of (opportunistic) human pathogens in rotting plant material has been reported for other crops. For example, it was found that vegetables (e. g. beet, broccoli, cabbage, cucumber, carrot, pepper, radish, squash and tomato) infected with pectinolytic bacteria often harbored higher densities of *Salmonella* spp. than healthy plant tissues (Wells & Butterfield, 1997). Carlin and co-workers (Carlin et al., 1995) reported an increase in the density of *Listeria monocytogenes* in decaying endive leaf tissue in comparison with healthy tissues.

The bacteria isolated from rotting potato tissue possessed different mechanisms that can interfere with *Dickeya* spp. *in planta* such as competition for iron ions by production of siderophores, antibiosis which may be partially based on the production of biosurfactants, degradation of quorum sensing signal molecules and competition for nutrients (e. g. pectinolysis). These features have been reported previously to play a role in bio-control (Uroz et al., 2003).

Selected isolates possessed various other features that potentially enhance their colonization, adaptation and survival in decaying potato tissue such as spore formation, motility and the ability to grow under anaerobic conditions. Oxygen depletion is one of the factors inducing rotting of potato tubers by pectinolytic bacteria, by attenuation of the plant defense mechanism (Perombelon & Lowe, 1975a). Antagonists that are able to grow under low oxygen conditions might have an advantage when competing with *Dickeya* spp. during tuber decay. Spore forming strains, such as *Bacillus* spp. are able to persist for long periods under harsh conditions as they are tolerant to heat and desiccation (Weller, 1988). Use of these strains may result in a longer protection of tubers compared to non spore forming strains. Motility enhances colonization of (plant) surfaces, which may be therefore an advantage in the competing with the motile *Dickeya* spp. both in plants and in soil.

The presence of high numbers of antagonistic bacterial species isolated from rotting potato tissue may explain partially the variation in symptom expression in plants homogeneously inoculated by vacuum infiltration with *Dickeya* sp. (unpublished results) and contributes to other factors affecting blackleg development (Perombelon, 2002). The presence of high numbers of antagonistic bacteria may also explain false negative results in isolations from diseased tubers or plants or detection procedures based on enrichment for *Dickeya* spp. (Degefu et al., 2009).

The strategy of selecting antagonists, based on *in vitro* assays and a tuber slice test appears to be successful in obtaining antagonists effective in the biocontrol of biovar 3 *Dickeya* sp. *in planta*. From a sub-selection of 12 strains, 10 were able to reduce the blackleg incidence by more than 50%. This is in contradiction with former studies in which *in vitro* assays had only a limited value in predicting the antagonist potential for *in planta* tests. It must, however, be noted that co-inoculation of tubers with 11 out of 12 strains and *Dickeya* sp. resulted in an increased incidence of non-emergence. In particular, co-inoculation with A3, A10 and A12, characterized as *Lysinibacillus sphaericus, R. erythropolis* and *Bacillus subtilis*, respectively, resulted in a high level of pre-emergence rot, which may be related to their ability to produce pectinolytic enzymes.

One strain, characterized as *Serratia plymuthica* (A30), provided a consistent high level of blackleg disease control in repeated greenhouse experiments, even under conditions very favorable for disease development. *S. plymuthica* strain A30 possesses different features that may play a role in antagonism of *Dickeya* sp. IPO2254; the strain produces antibiotics and surfactants, it is motile and produced auxins.

Protection was found after vacuum infiltration of intact tubers and tubers from which part of the stolon end had been mechanically removed, under warm (28 °C) and humid (80% relative humidity) conditions favorable for blackleg disease development. Strains of *S. plymuthica* have been frequently used to control fungal pathogens of plants, but not to our knowledge to control phytopathogenic bacteria (De Vleesschauwer & Hofte, 2007). Potato plants, subsequently-inoculated with *S. plymuthica* strain A30 and the pathogen, were protected against systemic colonization by the pathogen and consequently also against disease development. The tubers were vacuum- infiltrated with a high inoculum dose ($10^{10} – 10^{11}$ cfu ml^{-1}) of the antagonist to ensure relatively high densities in the tuber periderm. Results indicate the ability of *S. plymuthica* strain A30 as an antagonist to control of blackleg caused by biovar 3 *Dickeya* sp. on potato.

S. plymuthica A30 is potentially a good candidate for developing a commercial potato crop protection product. *S. plymuthica* is classified into risk group 1 according to DSMZ (German Collection of Microorganisms and Cell cultures), meaning that the species is not expected to pose a risk for humans and environment, and to date no human or animal-related pathogenicity factors for *S. plymuthica* have been described. Our strain A30 strain does not produce prodigiosin, a red pigment (Czajkowski, unpublished results), an antifungal and anti-eukaryotic compound that can be produced by *S. marcescens* (risk group 2); *S. plymuthica* strain A30 strain is also susceptible to a number of antibiotics routinely

used in medicine to treat bacterial infections in humans (Czajkowski, unpublished results); Finally, a commercial product named Rhizostar (E-nema GmbH Raisdorf, Germany) based on *S. plymuthica* HRO-C48 and active against *Rhizoctonia solani* and *Verticillum dahliae* is presently available on the market in Europe (European patent 98124694.5) indicating the possibility of using strains belonging to *S. plymuthica* species as biocontrol agents in agriculture.

To fully explore the usefulness of *S. plymuthica* strain A30 strain in practice, additional studies are required on effectiveness and consistency of control in the field, including population dynamics in the potato ecosystem, application timing, production and formulation and eco-toxicological risks. Work with deletion (knockout) mutants is now being conducted to understand the molecular basis of the antagonistic activity of *S. plymuthica* strain A30 against *Dickeya* sp. IPO2254.

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