Downward Vascular Translocation of a Green Fluorescent Protein-Tagged Strain of *Dickeya* sp. (Biovar 3) from Stem and Leaf Inoculation Sites on Potato

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ABSTRACT

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Translocation of a green fluorescent protein (GFP)-tagged *Dickeya* sp. from stems or from leaves to underground parts of potato plants was studied in greenhouse experiments. Thirty days after stem inoculation, 90% of plants expressed symptoms at the stem base and 95% of plants showed browning of internal stem tissue. The GFP-tagged *Dickeya* sp. was detected by dilution plating in extracts of the stem interiors (100%), stem bases (90%), roots (80%), stolons (55%), and progeny tubers (24%). In roots, the GFP-tagged *Dickeya* sp. was found inside and between

parenchyma cells whereas, in stems and stolons, the GFP-tagged *Dickeya* sp. was found in the xylem vessels and protoxylem cells. In progeny tubers, this strain was detected in the stolon end. Thirty days after leaf inoculation, the GFP-tagged *Dickeya* sp. was detected in extracts of 75% of the leaves, 88% of the petioles, 63% of the axils, and inside 25% of the stems taken 15 cm above the ground level. UV microscopy confirmed the presence of the GFP-tagged *Dickeya* sp. inside petioles and in the main leaf veins. No blackleg or aerial stem rot and no translocation of the GFP-tagged *Dickeya* sp. to underground plant parts was observed. The implications for contamination of progeny tubers are discussed.

Additional keywords: confocal laser-scanning microscopy, *Erwinia chrysanthemi*, rep-PCR.

Dickeya spp. (syn. *Erwinia chrysanthemi* or *Pectobacterium chrysanthemi*) (37), together with *Pectobacterium* spp., are the causal organisms of blackleg, stem wet rot, and tuber soft rot diseases of potato. In Europe, *Dickeya* spp. in particular are causing increasing economic losses in seed potato production, mainly due to downgrading and rejection of seed lots (20,35).

In 2005, the former *E. chrysanthemi* species was reclassified into the genus *Dickeya*, which constitutes six different genomic species (genomo-species) inside nine biovars (37). According to this classification, potato strains in Europe isolated before 2000 almost all belonged to *Dickeya dianthicola* (previously *E. chrysanthemi* bv. *dianthicola*) (biovar 1, 7, and 9) (18,40). Since then, isolates belonging to a new genetic clade have been found frequently. This clade belongs to biovar 3 and possibly constitutes a new species (40). Strains belonging to this clade have been isolated from potato grown in Finland, Poland, the Netherlands, and Israel (11,20,40,43); the United Kingdom (J. Elphinstone, *personal communication*); France (Y. le Hingrat, *personal communication*); and Belgium (J. Van Varenbergh, *personal communication*). Increased losses in seed potato production due to *Dickeya* spp. infection may be related to the occurrence of this new clade.

Potato blackleg is predominantly a seedborne disease, and the use of pathogen-free seed lots is the best measure to control blackleg and stem rot diseases. Blackleg symptoms are nearly

always associated with the presence of rotten contaminated seed potato tubers, indicating its seedborne nature (30). An increase in tuber contamination and disease incidence within a seed lot occurs by spread of bacteria leaking from rotten tubers via soil water to daughter tubers in the field and, when soft, rotting tubers are present, due to smearing during harvest and grading (33).

Pathogen-free seed lots may become infected with blackleg- and stem-rot-causing bacteria within a few generations of growing in the field. In The Netherlands, minitubers became infected within only two field generations: 17 of 50 seed lots were found contaminated with *Dickeya* spp. when tested by enrichment polymerase chain reaction (PCR) (45). Possibly the use of contaminated machines during harvest and grading is responsible for introducing the bacteria; however, other sources of contamination cannot be excluded.

Initially clean seed may also become contaminated during cultivation. Infection of potato plants may originate from contaminated machines used for spraying crop protection agents, contaminated insects, irrigation water, rain water, aerosols, human activity during field inspections, or animals entering potato fields (8,29). In general, contamination via these sources more readily results in infection of haulms than underground plant parts. Aerial stem rot, which is frequently found in the field under wet conditions, may be the result of these introductions (33).

Progeny tubers may become colonized from infected haulm indirectly via soil or directly via translocation of bacteria inside plants. Bacteria washed off by rain from rotting stems and leaves into the soil may contaminate the progeny tubers. Despite the fact that *Dickeya* spp. cannot survive for a long time in soil, high numbers of bacteria washed from haulms and constantly reintroduced to the soil could result in tuber lenticel contamination (39).

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Bacteria in soil can also colonize roots and move via the vascular tissue of the roots into the stolons and, finally, in the progeny tubers (10).

It is unknown whether *Dickeya* spp. can be translocated from the haulm to underground plant parts (i.e., roots, stolons, and progeny tubers). Translocation of bacteria internally from aerial to underground plant parts has been described for only a few plant-associated bacteria. For example, *Pseudomonas fluorescence* injected into stems of mature maize plants could move ≈ 15 cm in stems above and below the inoculation point (14), *Erwinia amylovora* was isolated from roots after stab inoculation of apple seedlings (4), and *Xanthomonas campestris* pv. *vitians* was recovered from stem sections 2 cm below the inoculation site in stems of lettuce (2).

This study examines the ability of a strain belonging to the new biovar 3 genetic clade of *Dickeya* spp. to infect roots, stolons, and progeny tubers from inoculated potato haulm. In greenhouse experiments, the movement of the bacteria to underground parts was studied after stab inoculation of stems and inoculation by abrasion of leaves. We used a green fluorescent protein (GFP)-tagged strain for monitoring the systemic movement of bacteria inside plants using epifluorescence stereomicroscopy (ESM), confocal laser-scanning microscopy (CLSM), and dilution-plating techniques.

MATERIALS AND METHODS

Bacterial strains and media used for cultivation. In all experiments, a GFP-tagged *Dickeya* sp., strain IPO2254, was used. This strain is a derivative of the wild-type biovar 3 *Dickeya* sp. IPO2222 (43) and contains the pPROBE-AT-*gfp* plasmid (24) that confers stable production of GFP (10). Prior to use, *Dickeya* sp. IPO2254 was grown at 28°C for 24 to 48 h on tryptone soya agar (TSA) (Oxoid) or in nutrient broth (Difco Laboratories). For testing cavity formation, dilutions of isolates collected from infected plant material were plated on crystal violet pectate (CVP) (17). For pour plating, bacteria were grown in PT medium at 28°C for 24 h (34). Growth media were supplemented with ampicillin at 150 $\mu\text{g ml}^{-1}$ and cycloheximide at 200 $\mu\text{g ml}^{-1}$.

Growth of potato plants and inoculation with GFP-tagged *Dickeya* sp. Minutubers of cv. Kondor (Agrico, The Netherlands) were planted in potting soil in 5-liter plastic pots in a greenhouse and grown at a 16-and-8-h photoperiod at 26 to 28°C and $\approx 70\%$ relative humidity. Replicated experiments were conducted in June and November 2008. Inoculation of potato stems was performed 3 to 4 weeks after planting, when plants were ≈ 27 to 30 cm high and stolons were already formed. In all, 20 plants (10 per experiment) were inoculated with the GFP-tagged *Dickeya* sp. strain, and 10 plants (5 plants per experiment) were used for the water-inoculated control. Stems were inoculated 10 cm above the ground level with either 100 μl of the GFP-tagged *Dickeya* sp. in water at 10^8 CFU ml^{-1} or 100 μl of sterile demineralized water (control). A 200- μl yellow pipette tip was stabbed halfway into the stem at an angle of 45° and subsequently wrapped with parafilm to prevent drying and leakage of bacteria along the stem surface to the soil. Three stems per potato plant were inoculated. To minimize the risk of soil contamination with the GFP-tagged *Dickeya* sp. strain released from diseased (rotten) stems, the soil surface was covered with plastic film. Plants were watered from the bottom of the pots.

Inoculation of potato leaves was done at the same time and in the same greenhouse as that used for stem inoculation but with a different set of plants (cv. Kondor). For each plant, seven randomly chosen leaves were inoculated by abrasion with 1 ml of water suspension containing *Dickeya* sp. strain IPO2254 at 10^8 CFU ml^{-1} , 2.5% carborundum powder (Chemos GmbH), and 0.1% Tween 20 (Oxoid). Control plants were inoculated with

sterile water containing 2.5% carborundum powder and 0.1% Tween 20. Both the axial and abaxial leaf surfaces were gently rubbed with the suspensions for 25 s. In total, 16 plants (8 plants per experiment) were used for inoculation with the GFP-tagged *Dickeya* sp. strain and a further 8 plants (4 plants per experiment) as a water-inoculated control. Direct soil contamination with the bacteria from inoculated leaves was prevented as with the stem-inoculated plants. Plants were also watered from the bottom of the pots.

Symptom development. Plants were visually inspected weekly for symptom development. Stem-inoculated plants were assessed for wilting, black rot on the stem base, aerial stem rot, haulm desiccation, and plant death. Leaf-inoculated plants were assessed for wilting and chlorosis of leaves, wilting of primary stems, wilting of secondary stems, aerial stem rot, typical blackleg, haulm desiccation, and plant death.

Sampling of stem and leaf-inoculated plants for PT pour plating. Per plant, 2-cm-long stem fragments at the inoculation point were collected from three stems and processed as a composite sample. Similarly, 2-cm-long stem fragments at the stem base were collected from the same three stems and processed as a composite sample. Per plant, the whole root system, three stolons, and six progeny tubers were tested separately. The sampled material was washed with tap water to remove soil particles, then surfacically sterilized with 70% ethanol for 1 min, washed three times with water for 1 min, held in 1% sodium hypochloride (commercial bleach) for 4 min, and finally washed three times with water for 4 min each. Samples were weighed and a volume equivalent to twice the sample weight of 1/4 Ringer's buffer (Merck) was added. Each sample was crushed in a Universal Extraction bag (Bioreba) using a hammer. Then, 100 μl of undiluted and 10 $\times$ and 100 $\times$ diluted samples were added to wells of a 24-well plate (Greiner BioOne) with 300 μl of liquefied PT medium cooled down to 45 to 50°C supplemented with cycloheximide at 200 $\mu\text{g ml}^{-1}$ and ampicillin at 150 $\mu\text{g ml}^{-1}$. After the medium had solidified, plates were sealed with parafilm to prevent drying and incubated for 1 day at 28°C. Plates were examined for the presence of GFP-tagged *Dickeya* sp. IPO2254 colonies under 495-nm blue light using an epifluorescence microscope (Leica Wild M32 FL4) equipped with a mercury high-pressure photo-optic lamp (Leica Hg 50W/AC) and GFP plus filter at a low magnification of 10 $\times$ and 20 $\times$.

Leaf-inoculated plants were sampled 30 days post inoculation (dpi). Seven inoculated leaves, seven leaf petioles, seven axils, seven 2-cm stems segments taken 15 cm above ground level, seven 2-cm stem base segments, whole root system, three stolons, and six progeny tubers were collected per plant and pooled per sample source. Plant parts were sterilized and processed for pour plating similarly as described for stem-inoculated plants.

Microscopic observations. Sample preparation. From every stem-inoculated plant, four roots at least 5 cm long, two 1-cm stem base segments, two 2-cm stem segments taken at the inoculation point, three stolons, and six progeny tubers were randomly selected.

From every leaf-inoculated plant, two leaves, four leaf petioles, four leaf axils, two stem base segments of 1 cm, and two stem samples of 2 cm were randomly selected. Stem segments, stolons, and stolon ends of progeny tubers were transverse sectioned into 0.1-cm-thick pieces. All plant parts were washed and sterilized before microscopic observations as described for pour plating.

ESM. All plant samples were embedded in PT medium in petri dishes. The PT medium was liquefied by heating, cooled down to 45 to 50°C, and supplemented with cycloheximide at 200 $\mu\text{g ml}^{-1}$ and ampicillin at 150 $\mu\text{g ml}^{-1}$ before embedding. After the medium had solidified, plates were sealed with parafilm to prevent drying and incubated for 1 day at 28°C. Samples were examined for the presence of a GFP-tagged *Dickeya* sp. under 495-nm blue light using an epifluorescence stereomicroscope

(Leica Wild M32 FL4) equipped with a mercury high-pressure photo-optic lamp (Leica Hg 50W/AC) and GFP plus filter.

CLSM. Samples for the confocal laser-scanning microscope (Leica DM5500Q) were prepared in the same way as for the epifluorescence microscope. Samples were counterstained just before microscopic observations. For this, plant parts were washed from the agar and incubated for 0.5 to 1 min in a solution of propidium iodide (PI) (Invitrogen) at $20 \mu\text{g ml}^{-1}$. Samples were

washed briefly with demineralized water and inspected under the microscope. For excitation of GFP and PI, a 485-nm blue laser was used. For GFP, a 505-nm emission filter was used; and, for PI, a 620-nm emission filter was used. Photographs were taken with a Leica Digital System (Leica) combined with a CSLM microscope using 10 $\times$ and 63 $\times$ water-immersion objectives.

Isolation of the GFP-tagged bacteria from infected plant material. GFP-tagged bacteria were isolated from plant parts

Fig. 1. Symptoms inside stems and stolons 30 days after stem inoculation. **A**, Sections of stems taken around the inoculation point (inoculation point marked with an arrow). Browning and blackening of the vascular- and pith tissue of stems observed above and below the inoculation point. **B**, Trans-sections of stems and stolons. Inoculated plants showing necroses (discoloration) of pith tissue. In both stems and stolons of inoculated plants, a necrosis of vascular tissue was found, resulting in light- and dark-brown lesions. Trans-sections of stems were taken 5 cm below the inoculation point. Control plants (water inoculated) were free of disease symptoms. Water-inoculated control plants showed only browning of pith tissue at the inoculation side.

harboring GFP-tagged bacteria 30 dpi. For this, two stem samples at the inoculation point, two stem samples 10 cm below the inoculation point, two randomly chosen roots, two stolons, and two progeny tubers were collected from the stem-inoculated plants; two leaves, two axils, two petioles, and two stem base cuts were collected from leaf-inoculated plants. Collected parts were cut into small pieces and incubated in 0.5 ml of Ringer's buffer for 20 min with shaking (≈ 200 rpm), and 100 μ l of each suspension was plated onto TSA containing ampicillin at 150 μ g ml⁻¹ or on CVP plates for testing cavity formation. GFP-positive and cavity-forming colonies were collected from the plates.

Identification of GFP-tagged bacteria by a *Dickeya* spp.-specific PCR. For characterization of reisolated bacteria, a colony PCR procedure was used. Cells from GFP fluorescent or cavity-forming colonies were collected from TSA (Oxoid) using a sterile toothpick, and resuspended in 50 μ l of 5 mM NaOH. Suspensions were boiled for 5 min at 95°C and put on ice for 1 to 2 min. Then, 1 μ l of the cell lysate was used as a template in PCR specific for *Dickeya* spp. with ADE1/ADE2 primers (ADE1: 5' GATCAGA AAGCCCGCAGCCAGAT 3' and ADE2: 5' CTGTGGCCGAT CAGGATGGTTTTGTCTGTCG 3') (25). Amplified DNA was detected by electrophoresis in a 1.5% agarose gel in 0.5x Tris-borate-EDTA (TBE) buffer stained with ethidium bromide at 5 mg ml⁻¹. The expected fragment length amplified by the ADE1/ADE2 primers for *Dickeya* spp. was 420 bp.

Identification of reisolated *Dickeya* sp. by repetitive element PCR fingerprinting. Repetitive element PCR fingerprinting (rep-PCR) was done on randomly chosen reisolates of a GFP-tagged *Dickeya* sp. as described before (46) using REP1R/REP2I primers (REP1R: 5' IIIICGICGICATCIGGC 3' and REP2I: 5' ICGIC TTATCIGGCCTAC 3'). The Qiagen Genomic DNA purification Kit (Qiagen) for gram-negative bacteria was used according to the manufacturer's instructions for purification of genomic bacterial DNA. The DNA concentration was adjusted with demineralized, sterile water to a final concentration of ≈ 100 ng μ l⁻¹. Rep-PCR was performed in a total volume of 28 μ l using 6 U of *Taq*

polymerase (Roche) per reaction. Amplified DNA was analyzed by electrophoresis in a 1.5% agarose gel in 0.5x TBE buffer and stained with ethidium bromide at 5 mg ml⁻¹. The gel was run for 6 to 7 h at 90 to 95 V and at room temperature. A 1-kb ladder (BioRad) was used as a size marker.

Statistical analysis. Bacterial count data were analyzed using a generalized linear model (23) implemented within the statistical software package GenStat (27). Before applying the model, we estimated expected counts for samples that were recorded as uncountable due to high densities of cavity-forming bacteria (10). Briefly, the value 10⁶ CFU g⁻¹ was taken as a likely cut-off level for the censored observations and bacterial density estimation was

TABLE 1. Plant samples analyzed by dilution plating for the presence of green fluorescent protein-tagged *Dickeya* sp. IPO2254 30 days after stem or leaf inoculation

Plant part	No. tested	No. positive	Positive (%)
Stem inoculation			
Inoculation point ^a	20	20	100
Stem base ^a	20	18	90
Roots ^b	20	16	80
Stolons ^c	60	33	55
Progeny tubers ^d	120	32	24
Leaf inoculation			
Leaves ^e	16	12	75
Petioles ^e	16	14	88
Axils ^e	16	10	63
Stems ^e	16	4	25
Stem bases ^e	16	0	0
Roots ^b	16	0	0
Stolons ^c	48	0	0
Progeny tubers ^d	96	0	0

^a Composite sample of three stem parts analyzed per plant.

^b Total root system analyzed per plant.

^c Three stolons individually analyzed per plant.

^d Six progeny tubers individually analyzed per plant.

^e Composite sample of seven plant parts analyzed per plant.

Fig. 2. Symptom expression on leaves inoculated with *Dickeya* sp. IPO2254 by abrasion with carborundum powder. Inoculated leaves are indicated with black arrows. After 30 days, maceration, rotting, and necrosis of leaf tissue were observed in plants inoculated with *Dickeya* sp. IPO2254, whereas leaves of water-inoculated control plants showed only chlorosis and slight necrosis due to mechanical damage.

based on normality assumptions (38). Count data were modeled using a standard Poisson regression method (7). In real count data, the underlying assumption of equality of mean and variance was rarely met. Most count data had variance greater than the mean or were Poisson overdispersed. The negative binomial distribution was the natural choice to model that overdispersed count data (16). Type of tissue effects were considered to be significant at $P \leq 0.05$, and pairwise differences were obtained using the t test.

RESULTS

Symptom development. Fifteen days after stem inoculation, the first symptoms started to develop in inoculated plants. At 30 dpi, all stem-inoculated plants showed wilting and chlorosis of leaves. In 90% of plants, aerial stem rot, a typical blackening of the stem base, or both were observed. Trans-sections of stems of symptomatic plants showed degradation of vascular tissue and pith above and below the inoculation point, resulting in a hollowing of stems and browning of the internal stem tissue of 95% of tested plants (Fig. 1).

In leaf-inoculated plants, the first symptoms appeared at 7 dpi; all inoculated leaves showed chlorosis and wilting. In the fol-

lowing 2 weeks, these symptoms were also found frequently in adjacent leaves (Fig. 2). We did not observe symptoms on or in stems during the period of 30 days (data not shown).

Dilution plating of stem-inoculated plants. At 30 dpi, plant material was disinfected, extracted, and pour plated in PT to determine the percentage of infected plant tissues and to quantify populations of *Dickeya* sp. IPO2254. GFP-tagged cells were found at the point of inoculation of all plants. GFP-tagged cells were found in 90% of the stem bases, inside 80% of the roots, in 55% of the stolons, and in 24% of progeny tubers (Table 1). We did not detect GFP-tagged *Dickeya* spp. in any water-inoculated control plants.

At the inoculation point, the highest estimated densities of 10^6 to 10^7 CFU g^{-1} for the GFP-tagged *Dickeya* sp. were found (Fig. 3A). Densities at the stem base were still relatively high (10^4 CFU g^{-1}) but relatively low (<100 CFU g^{-1}) in roots, stolons, and progeny tubers. Bacterial densities in stolons and progeny tubers varied largely per sample.

In 10% of stem-inoculated plants, no systemic colonization of GFP-tagged *Dickeya* sp. IPO2254 was found. In those plants, disease symptoms (rotting and browning of the tissues) and presence of bacteria were restricted to the inoculation site.

Fig. 3. Densities of *Dickeya* sp. IPO2254 (in log CFU + 1 g^{-1} of plant tissue) in **A**, stem-inoculated and **B**, leaf-inoculated plants 30 days postinoculation as determined by pour plating. Predicted values are averages from **A**, 20 and **B**, 16 individual plants from two independent experiments. Values followed by identical characters are not significantly different ($P = 0.05$).

Dilution plating of leaf-inoculation plants. Leaf-inoculated plant samples were also examined 30 dpi by pour-dilution plating (Fig. 3B). The densities of GFP-tagged *Dickeya* spp. found in inoculated leaves were generally low. The estimated density in the inoculated leaves was 1,000 CFU g⁻¹; in leaf petioles and axils, 100 CFU g⁻¹; and, in stems, only 10 CFU g⁻¹ (Fig. 3B). On average, the GFP-tagged *Dickeya* sp. was found in 75% of the inoculated leaves, 88% of the leaf petioles, 63% in the axils, and 25% of stems directly attached to the axils of inoculated leaves (Table 1). We did not detect GFP-tagged *Dickeya* spp. in stem segments collected at ground level (stem base) or inside roots, stolons, and progeny tubers. In all, 70% of the leaf-inoculated plants exhibited disease symptoms at the inoculation point. Only low populations of GFP-tagged *Dickeya* spp. were observed in axils of leaves (≈ 14 CFU g⁻¹) and main stems (≈ 1.5 CFU g⁻¹)

(Fig. 3B). However, in 25% of the tested plants, GFP-tagged bacteria were isolated from stem fragments taken near the inoculated compound leaves but not from the stem bases or underground part of the plants (roots, stolons, and progeny tubers). The densities of GFP-tagged *Dickeya* spp. in inoculated leaves, leaf petioles, and axils were not statistically different (Fig. 3B).

Microscopic observations of infected plant tissues. To determine in which plant tissue *Dickeya* sp. IPO2254 was present, different plant parts were analyzed with an epifluorescence stereomicroscope at low magnifications (2.5 $\times$ to 10 $\times$) and with a confocal laser-scanning microscope at a magnification of 640 $\times$ to 1,000 $\times$.

ESM. In stem-inoculated plants, a GFP signal was found in the vascular tissue of stems, stem bases, and stolons. In progeny tubers, the signal was observed in the vascular ring of the stolon end. In roots, the signal was detected in pith tissue (Fig. 4).

Fig. 4. Colonization of potato stems, stolons, roots, and progeny tubers with green fluorescent protein (GFP)-tagged *Dickeya* sp. IPO 2254 at 30 days post stem inoculation analyzed with epifluorescence stereomicroscopy. Plant parts embedded in PT agar for 24 h at 28°C were screened for the presence of a GFP signal. The GFP signal was found in vascular tissue of stems, stolons, and the stolon end of progeny tubers and in pith tissue of roots (white arrows). Control = water-inoculated plants.

In leaf-inoculated plants, a GFP signal was detected in the main vein of the leaves and inside petioles (Fig. 5) but not inside the stem basis (data not shown). We did not detect GFP-tagged *Dickeya* spp. in any water-inoculated control plant.

CLSM. Detailed studies with CLSM on the localization of GFP-tagged *Dickeya* spp. showed that, 30 days post stem inoculation, bacteria were mainly present inside xylem vessels and between protoxylem cells of stems, stem bases, and stolons. In roots of stem-inoculated plants, GFP-tagged bacteria were found inside parenchyma cells of pith tissue both inter- and intracellularly. In progeny tubers, GFP-tagged bacteria were found inside xylem and between xylem vessels of the stolon ends (Fig. 6). We did not do the CLSM studies on material from leaf-inoculated plants.

Characterization of GFP-expressing bacteria from infected plant tissue. Plant samples with a typical GFP signal observed under the epifluorescence microscope 30 dpi were collected, extracted, and plated on TSA or CVP. From stem-inoculated plants, 10 isolates were selected (2 from inoculation points, 2 from stems 10 cm below the inoculation point, 2 from roots, 2 from stolons, and 2 from progeny tubers). From leaf-inoculated plants, eight isolates were selected (two from the inoculated leaves, two from petioles, two from the axils, and two from stems). All isolates produced typical cavities on CVP, were green fluorescent in ESM, and were positive in a *Dickeya* sp.-specific PCR, showing the expected 420-bp PCR product (data not shown). Rep-PCR analyses showed that all fingerprints of isolates from stem- and leaf-inoculated plants were identical to strain *Dickeya* sp. IPO2254 and the parental wild-type strain *Dickeya* sp. IPO2222 used for GFP tagging (data not shown).

DISCUSSION

This study showed that inoculation of *Dickeya* sp. biovar 3 into the vascular system of stems can result in downward translocation and colonization of underground plant parts, including roots, stolons, and progeny tubers. Stem inoculation resulted in typical

blackleg symptoms in the majority of infected plants. It was already known that *Dickeya* spp. could move upward in the vascular system inside plant tissue and cause systemic colonization of plants (32), as shown for stab-inoculated root cuttings of *Chrysanthemum morifolium* (28,31) and naturally infected tomato plants (1). The downward movement of this bacterium, however, has not been described before.

The downward translocation of *Dickeya* spp. inside potato plants via xylem vessels is in agreement with our previous observations in which, after soil inoculation, a GFP-tagged *Dickeya* sp. was recovered from the xylem of symptomless stems (10). There are also reports of other plant-pathogenic bacteria that use xylem vessels to systemically colonize plant tissue. *X. campestris* pv. *viti*ans is able to move inside xylem vessels in stab-inoculated lettuce plants (2) and *Pseudomonas syringae* pv. *lachrymans* is able to colonize cucumber seedlings via the xylem vessels (19).

In theory, there are three mechanisms by which bacteria are transported downward in xylem vessels: via degradation and embolism of xylem vessels followed by colonization of the xylem elements (26), via reverse water transport in xylem during dark periods (42), and upstream via pilus-driven twitching motility (5,22). Degradation of xylem vessels leads to creation of large horizontal and vertical spaces filled with rotten tissue and bacterial slime that can be easily colonized. In general, degraded plant tissue near the infection point allows bacteria to move only short distances in infected tissue. The movement of *X. campestris* pv. *viti*ans in lettuce stems, for example, was a result of mechanical damage of stems and embolism of the xylem (2), and the bacteria were only detected up to 2 cm below the inoculation point.

Water movement in xylem vessels drags the bacteria rapidly to distant plant parts. Movement of sap in xylem vessels is dependent upon evaporation of water from the surface of mesophyll cells (transpiration) in leaves, thereby pulling water up from the soil via the root system. Therefore, the directional movement of water is usually from roots to leaves (36). A reverse (downward)

Fig. 5. Colonization of potato leaves by green fluorescent protein (GFP)-tagged *Dickeya* sp. IPO 2254 at 30 days post leaf inoculation analyzed with epifluorescence stereomicroscopy. Plant parts were sterilized and embedded in PT agar for 24 h. Control = water-inoculated plants. GFP signal was found inside main veins and inside petioles.

water movement in xylem from leaves to roots only occurs when xylem sap is subjected to negative hydraulic pressure as a result of low water uptake from roots and reduced leaf evaporation. This is consistent with the cohesion-tension theory (42,44). *E. amylovora*, for example, was recovered from the roots of apple seedlings after stem inoculation due to negative hydraulic pressure of the plant sap in xylem vessels (4).

Upstream migration in xylem vessels via twitching motility has been described for the nonmotile plant pathogen *Xylella fastidiosa* in grapevine (22). There is strong evidence that *Ralstonia solanacearum* can also move in xylem vessels of tomato plants via twitching motility (21). With *D. dadanti*, however, twitching motility could not be detected (12). Therefore we consider it unlikely that the *Dickeya* sp. biovar 3 strain can move via this form of migration.

Leaf-inoculated plants only harbored low *Dickeya* sp. populations in stems 30 dpi, indicating that the risk for translocation from infected leaves to progeny tubers is small. Downward vascular translocation from infected leaves may be limited to the low densities of *Dickeya* spp. present in leaf tissue but also by the stem–leaf junction physiology. Many vessels end in these junctions, and those nearby contain intervessel pit membranes (13). These membranes are part of the defense mechanism of the plant to protect vascular tissue from microorganisms that can infect leaves (9).

The risks of systemic infections after contamination of leaves will depend not only on the efficiency of translocation of bacteria but also in the ability of *Dickeya* spp. to survive the phyllosphere. The phyllosphere is recognized as a harsh environment in which, most of the time, bacteria are subjected to nutrient limitation,

Fig. 6. Colonization of xylem vessels of potato stems, stolons, parenchyma cells of roots, and the stolon end containing xylem elements of progeny tubers by green fluorescent protein-tagged *Dickeya* sp. IPO2254 at 30 days post stem inoculation analyzed with confocal laser-scanning microscopy. Plant parts were embedded in PT agar for 24 h prior to analyses. Plant cells were counterstained with the red-fluorescent dye propidium iodide. Bacteria were found inside xylem vessels in stems and stolons, inside and between parenchyma cells in roots, and inside xylem cells of the vascular ring of the stolon end in progeny tubers (white arrows).

desiccation, direct UV, and visible radiation stress (3,6,15,41). It has been reported that *Dickeya* spp. are sensitive to drought and direct UV radiation (41); therefore, they are not expected to survive for long periods on the leaf surface.

Our results suggest that stem infection during cultivation practices can result in infected progeny due to the internal movement of *Dickeya* spp. to underground plant parts. Infection of stems resulting in aerial stem rot under wet conditions occurs via the use of contaminated machines, pest insects, or humans and animals (8,33).

The risks of systemic colonization of potato plants from aerial stem infections will depend on various factors such as potato cultivar, *Dickeya* sp., initial inoculum, air temperature, and humidity. The conditions in our experiments were highly favorable for disease development. Tubers of cv. Kondor were used, which are highly susceptible to *Dickeya* spp. (H. Velvis, *personal communication*). High bacterial densities of a strain belonging to a new genetic clade of *Dickeya* sp. biovar 3, which appears to be highly virulent (*unpublished results*), were used. Also, the high temperature and high relative humidity in the greenhouse favored disease development. Field studies are required to further determine, in practice, the risks of infection of progeny tubers via haulm infections.

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