Systemic Colonization of Potato Plants by a Soilborne, Green Fluorescent Protein-Tagged Strain of *Dickeya* sp. Biovar 3

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ABSTRACT

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Colonization of potato plants by soilborne, green fluorescent protein (GFP)-tagged *Dickeya* sp. IPO2254 was investigated by selective plating, epifluorescence stereo microscopy (ESM), and confocal laser scanning microscopy (CLSM). Replicated experiments were carried out in a greenhouse using plants with an intact root system and plants from which ca. 30% of the lateral roots was removed. One day after soil inoculation, adherence of the pathogen on the roots and the internal colonization of the plants were detected using ESM and CLSM of plant parts embedded

in an agar medium. Fifteen days post-soil inoculation, *Dickeya* sp. was found on average inside 42% of the roots, 13% of the stems, and 13% of the stolons in plants with undamaged roots. At the same time-point, in plants with damaged roots, *Dickeya* sp. was found inside 50% of the roots, 25% of the stems, and 25% of the stolons. Thirty days post-inoculation, some plants showed true blackleg symptoms. In roots, *Dickeya* sp. was detected in parenchyma cells of the cortex, both inter- and intracellularly. In stems, bacteria were found in xylem vessels and in protoxylem cells. Microscopical observations were confirmed by dilution spread-plating the plant extracts onto agar medium directly after harvest. The implications of infection from soilborne inoculum are discussed.

Additional keywords: blackleg, roots, progeny tubers, vascular tissue.

Dickeya spp. (syn. *Erwinia chrysanthemi* and/or *Pectobacterium chrysanthemi*) (38), together with *Pectobacterium atrosepticum* (formerly *E. carotovora* subsp. *atroseptica*) and *P. carotovorum* subsp. *carotovorum* (formerly *E. carotovora* subsp. *carotovora*), are the causative agents of potato blackleg worldwide (7,30,31). Until recently, the majority of *Dickeya* spp. strains found in association with potato blackleg belonged to biovars 1 or 7 (*D. dianthicola*). These strains have a relatively low maximum growth temperature compared with other *Dickeya* species and seem to be more adapted to European climate conditions (18). In the last 3 years, however, *Dickeya* spp. strains belonging to a new biovar 3 clade, probably constituting a new species, have been frequently isolated from potato tubers in Western Europe and Israel (19,40,42). The finding of this biovar 3 *Dickeya* spp. in seed potato tubers is associated with high incidences of potato blackleg in The Netherlands (Netherlands General Inspection Service for Agricultural Seeds and Seed Potatoes, *personal communication*).

In Western and Northern Europe in particular, *Dickeya* species are causing increasingly severe economic losses in potato crops. The costs in seed potato production resulting from *Dickeya* spp. caused blackleg infections are high due to rejection and declassification of seed lots (tubers) (19,33).

Potato blackleg caused by *Dickeya* and *Pectobacterium* spp. is primarily a seedborne disease. Typical blackening of the stem base (blackleg) and wilting of the potato plants are always associated with rotting of the seed (mother) tuber and spreading of the inoculum (28). Symptom expression occurs when temperature and soil moisture are high, favoring multiplication of the blackleg bacteria and rotting of the seed potato (41).

For *P. atrosepticum*, it is well-established that spreading of infections within a seed lot frequently occurs during harvest and grading, particularly if rotten tubers are present harboring high densities of inoculum (9). Dispersal of *P. atrosepticum* within a seed lot can also occur during plant cultivation. During tuber decay massive amounts of bacteria are released into the soil from where they can move via free soil water up to a distance of 10 m (12). The bacteria can be introduced into the lenticels, which are open during heavy rainfall under anaerobic condition (1,25). During harvest, a high percentage of tubers are bruised and are readily infected by contaminated equipment (11,31). Bacteria present on intact tuber peel will die off rapidly, but those in lenticels, suberized wounds, and cracks may persist until the new growing season (44). It is assumed that contamination of potato tubers with *Dickeya* species occurs similarly.

Both lenticel contamination and infection of wounds during (post) harvest activities will primarily result in infections of the potato tuber periderm (peel). It is likely that tuber infection by *Dickeya* spp. can occur in a similar way.

For both *Dickeya* spp. and *P. atrosepticum*, however, a relatively high infection incidence is found in the stolon ends (4–6). This implies that during plant growth, internal colonization of progeny tubers via the stolons occurs more frequently than assumed. Internal infections of progeny tubers may take place in two different ways. First, bacteria can be transported directly from the mother tuber via the plant vascular system into the stems, stolons and progeny tubers (13). Second, the soilborne *Dickeya* sp. may also infect potato roots, from where the bacteria may further colonize the plant, including the progeny tubers. *Dickeya* spp., if present in soil, may enter not only the roots of the infected plant but also the roots of adjacent plants by wounds caused by soilborne pathogens and pest organisms or by natural openings which arise during lateral root formation.

The aim of this work was to investigate the ability of a representative potato strain of the new biovar 3 clade of *Dickeya* sp. to

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infect roots from inoculated soil and to colonize potato plants, including progeny tubers. To enable this, a green fluorescent protein (GFP)-tagged strain was generated and evaluated for growth and virulence. In greenhouse experiments, population dynamics of the pathogen in plants with an intact (undamaged) and damaged (cut) root system was studied using epifluorescence stereo microscopy (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 strain of *Dickeya* sp. IPO2222 biovar 3 was used. Wild-type strain IPO 2222 was isolated from seed potato tubers cv. Melody in The Netherlands in 2007 (42). Bacteria were grown at 28°C for 24 to 48 h on tryptic soya agar (TSA) (Oxoid, Basingstoke, England), in nutrient broth (NB) (Difco, Le Pont de Claix, France), on crystal violet pectate (CVP) (16), or pectate enrichment broth (PEB) (32) prior to use. If required, growth media were supplemented with cycloheximide (Sigma Chemical, St. Louis) to a final concentration of 200 µg ml⁻¹ and with ampicillin (Sigma) to a final concentration of 150 µg ml⁻¹.

Generation of GFP-tagged *Dickeya* sp. strains. Plasmid pPROBE-AT-*gfp* (23) was used for generation of GFP-tagged *Dickeya* sp. IPO2254 (parental strain *Dickeya* sp. IPO2222). The plasmid carrying *gfp* gene was introduced to bacterial cells by electroporation (3). Briefly, suspensions of approximately 50 µl of *Dickeya* sp. competent cells were mixed with 0.5 µl of plasmid DNA (approximately 100 ng µl⁻¹) and electroschocked at 2.5 kV for 1 to 2 s at 4°C using a Bio-Rad Gene Pulser 200/2.0 (Bio-Rad, Hercules, CA). After electroporation, cells were resuscitated for 1 h in 500 µl of NB at 28°C with shaking. One hundred microliters of the transformed cells was plated on TSA containing ampicillin (150 µg µl⁻¹) and incubated for 48 h at 28°C for selection of positive transformants.

Maintenance of the pPROBE-AT-*gfp* plasmid in *Dickeya* sp. cells in vitro plant model. Maintenance of the plasmid carrying *gfp* gene in *Dickeya* sp. IPO2254 cells in planta was evaluated in a potato slice assay. Bacterial strains were grown overnight in NB supplemented with ampicillin (150 µg ml⁻¹) (NBa) at 28°C with a shaking rate of 200 rpm. Bacterial suspensions were diluted in Ringer's buffer (Merck, Darmstadt, Germany) to a concentration of approximately 10⁸ CFU ml⁻¹ (optical density at 600 nm [OD₆₀₀] = 0.1).

Dickeya-free minitubers of cultivar Kondor (Vitrocom, Westland, The Netherlands) were rinsed with running tap water, subsequently washed twice with 70% ethanol for 5 min, and washed twice for 1 min with demineralized water. Tubers were dried with tissue paper and cut into 0.7 cm transverse disk slices. One 5 mm deep well per slice was made using a sterile cork borer with a diameter of 5 mm. Wells were filled with 50 µl of the bacterial suspension. For disease development, slices were incubated at 28°C for 72 h in a humid box. After incubation, 200 mg of rotten potato tissue was collected and resuspended in 2 ml of Ringer's buffer supplemented with an antioxidant, 0.02% diethylthiocarbamic acid (Acros Organics, Geel, Belgium). Fifty microliters of the suspension was transferred to the well in a freshly prepared potato slice. Bacteria were transferred from one slice to another 10 times in total. Serial dilutions of homogenized rotten potato tissue were plated on CVP at each transfer. The total numbers of GFP-positive and GFP-negative bacterial colonies producing cavities on CVP were counted and the percentage of GFP-positive cavity-forming colonies was calculated. Three replicate potato slices were used and the experiment was repeated twice. If present, GFP-negative cavity-forming bacteria were randomly chosen at each time point and checked by a *Dickeya* spp.-specific polymerase chain reaction (PCR,) based on ADE1/ADE2 primers

(ADE1: 5'-GATCAGAAAGCCCCGAGCCAGAT-3', ADE2: 5'-CTGTGGCCGATCAGGATGGTTTGTCTGTC-3'), to verify the identity of bacteria (24). For this, cells were collected from a suspected colony using a sterile toothpick and resuspended in 50 µl of 5 mM NaOH. Suspensions were boiled for 5 min at 95°C and put on ice for 1 to 2 min. One microliter of the cell lysate was used as a template in the *Dickeya* spp.-specific PCR. Amplified DNA was detected by electrophoresis in a 1.5% agarose gel in 0.5× TBE buffer stained with ethidium bromide (5 mg ml⁻¹). The expected fragment length amplified by the ADE1/ADE2 primers for *Dickeya* sp. was 420 bp.

Growth of the GFP-tagged strain. To assess bacterial growth under aerobic conditions, an overnight bacterial culture with a density of ca. 10⁹ cells ml⁻¹ in NBa was diluted 50 times in NBa. Bacteria were grown at 28°C with a shaking rate of 200 rpm. Growth rate was determined by measuring the OD₆₀₀ for a period of up to 25 h.

To evaluate growth under anaerobic conditions, 5 ml of liquid paraffin was added over 30 ml of the bacterial suspensions in PEB, prepared as described above for growth in aerobic conditions. Samples were incubated at 28°C without shaking. Growth was determined by measuring the OD₆₀₀ at the same time intervals as for growth in aerobic conditions.

Ability of the GFP-tagged strain to macerate tuber tissue. The ability of GFP-tagged *Dickeya* sp. IPO2254 to macerate potato tuber tissue was evaluated in a potato slice assay, as described for estimation of the GFP plasmid stability with some modifications. Instead of one, three wells per tuber slice were used. The diameter of rotting tissue was measured after 72 h incubation at 28°C. The result was compared with that of the wild-type strain and with a water control. The experiment was repeated twice.

Growth of potato plants and soil inoculation with GFP-tagged *Dickeya* sp. In a replicated experiment in 2008, minitubers of cultivar Kondor (Vitrocom), highly susceptible to black-leg pathogens (H. Velvis, *personal communication*), were planted in potting compost in 5-liter plastic pots in the greenhouse and grown at a 16 h/8 h (day/night) photoperiod, at 26 to 28°C and 70% relative humidity. Inoculation of soil was performed 3 weeks after planting, when plants were ca. 27 to 29 cm high and the stolons already formed. Plants were watered up to 1 h before soil inoculation. The lower part of the pots (ca. 40%) was immersed for 40 min in suspensions of *Dickeya* sp. IPO2254 containing 10⁸ CFU ml⁻¹ bacteria in water, or in bacteria-free sterile water. Half an hour before soil inoculation, while avoiding disturbing the plants, 30% of roots were cut off aseptically with a knife without removing the plants from pots, from half of the number of plants. Plants with damaged and undamaged root systems were inoculated in the same way. After inoculation, plants were left unwatered for 24 h. The greenhouse experiments were conducted in April/May and July/August 2008. In total, 36 plants were used; per replication we used 6 plants inoculated with water (control), 6 plants with intact roots inoculated with GFP-tagged *Dickeya* sp. IPO2254, and 6 plants with damaged roots inoculated with the same strain. As well as sampling, plants were observed weekly for development of disease symptoms.

Sampling of potato plants for CVP plating. Plants were sampled 1, 15, and 30 days postinoculation (dpi). At each time point, four plants per treatment were sampled. One gram of soil was randomly collected from each pot and separately suspended in 2 ml of 1/4-strength Ringer's buffer (Merck) supplemented with 0.02% diethylthiocarbamic acid (Arcos Organics). The suspended soil was shaken for 10 min at 100 rpm and 100 µl of the undiluted, 10 times and 100 diluted samples were plated on CVP containing cycloheximide (100 µg ml⁻¹) (Sigma) and if appropriate with ampicillin (150 µg ml⁻¹) (Sigma).

Per plant, the total root system was collected and processed separately. Roots were washed with water to remove soil particles, sterilized in 70% ethanol for 1 min, washed three times

with water for 1 min, incubated in 1% sodium hypochlorite (commercial bleach) for 4 min and finally washed three times with water for 4 min. Roots were weighed and Ringer's buffer (Merck) was added to twice the weight. Each sample was crushed in a Universal Extraction bag (BIOREBA) using a hammer. Extracts were plated as described for soil samples. To check the sterilization of root surface, the last washing water was collected, centrifuged (8,000 rpm, 10 min), and the pellet was resuspended in 2 ml of Ringer's buffer. Three times 100 µl was plated on CVP and plates were incubated for bacterial growth and cavity formation at 28°C for 16 h.

Six individual roots, three stolons, and six progeny tubers with the diameter in range between 1 to 3 cm from each plant were randomly chosen. These were sterilized, crushed and plated in the same way as described for total roots. Each root, stolon, and progeny tuber was processed separately.

From each plant, two or three 0.5-cm-thick fragments from different stems cut 5 cm above ground level were jointly collected. The stem cuttings from each individual plant were sterilized, crushed, and plated in the same way as described for the total root system.

Microscopic observations. Sample preparation. Three roots with a length of at least 30 cm, two stolons, and two stems were cut randomly from every plant inoculated with the GFP-tagged strain, and two leaves were also collected from a plant expressing blackleg symptoms. The cut roots, stolons and stems were washed and sterilized before microscopic observation as described for CVP plating.

ESM. Each root and stolon was cut into fragments of 1.5 to 2 cm long and each stem into fragments of 0.5 cm thick. Leaves were used without further cutting. Fragments were embedded in liquefied PT medium (32) cooled down to 45 to 50°C containing cycloheximide (200 µg ml⁻¹) and if required with ampicillin (150 µg ml⁻¹) in petri dishes. After the medium had solidified, the plates were sealed with parafilm to prevent drying and incubated for 2 days at 28°C. Samples were examined for the presence of *Dickeya* sp. IPO2254 under 495 nm blue light using an epifluorescence stereo microscope (ESM) (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 CLSM (Leica DM5500Q) were prepared in the same way as for the epifluorescence microscope. Most samples were counter-stained just before microscopic observations. For this, plant parts were washed from the agar and incubated for 0.5 to 1 min in propidium iodide (PI) (20 µg ml⁻¹) (Invitrogen, Carlsbad, CA) solution. Samples were washed briefly in demineralized water and inspected under the microscope.

For excitation of GFP and PI, a 488 nm blue laser was used. For GFP, a 505 nm emission filter and for PI a 620 nm emission filter was used. Photographs were taken with a Leica Digital System (Leica) combined with a CLSM microscope using 10x and 63x water immersion objectives.

Isolation of the GFP fluorescent bacteria from the infected plant material. GFP-tagged bacteria were isolated from plant parts harboring GFP-tagged bacteria 30 dpi. Four roots, two 2 cm long stem cuts taken 10 cm above the ground level, and one leaf were cut into small pieces and incubated in 0.5 ml of Ringer's buffer for 20 min with shaking, and 100 µl of each suspension was plated onto TSA containing ampicillin (150 µg ml⁻¹). GFP-positive colonies were collected from the plates.

Identification of GFP fluorescent bacteria by *Dickeya* spp.-specific PCR. For characterization of the reisolated bacteria, a colony-PCR procedure was used. Cells from a suspected colony were collected from TSA with a sterile toothpick and processed in the same way as colonies sampled for the pPROBE-AT-*gfp* plasmid stability assay.

Identification of reisolated *Dickeya* sp. by repetitive element PCR fingerprinting. For purification of genomic bacterial DNA,

the Qiagen Genomic DNA purification Kit (Qiagen) for gram-negative bacteria was used according to the manufacturer's instructions. Repetitive element PCR fingerprinting (rep-PCR) was done on 12 randomly chosen reisolates of GFP-tagged *Dickeya* sp. as described before (46) using REP1R/REP2I primers (REP1R: 5'-IIIICGICGICATCIGGC-3', REP2I: 5'-ICGICTTATCIGGCCTAC-3') with the following modifications. The DNA concentration was adjusted with demineralized, sterile water to a final concentration of approximately 100 ng µl⁻¹. Rep-PCR was performed in a total volume of 28 µl using 6 U of *Taq* polymerase (Roche) per reaction. Amplified DNA was analyzed by electrophoresis in a 1.5% agarose gel in 0.5× TBE buffer and stained with ethidium bromide (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 (Bio-Rad) was used as a size marker.

Statistical analysis. Bacterial count data were analyzed with ordinary linear regression using the statistical software package GenStat (26). To achieve approximate normality, the data were log transformed after adding a value of 1 to avoid taking logs of zero. Effects were considered to be significant at $P \leq 0.05$ and pair-wise differences were obtained using the *t* test. Plates overgrown, due to high densities of cavity-forming bacteria on CVP, were recorded as uncountable, taking the value 10⁶ CFU g⁻¹ as a likely cut-off level (censored observations). We used estimation of expected values for the censored observations based on normality assumptions as described in Schmees and Hahn (39).

Data were analyzed according to the experimental design in which two replicated greenhouse experiments were done with treatments having six replications (plants) each. The linear model considered was a complete block design with replicates as complete blocks, main effects for time and treatment and the two-way interaction between time and treatment.

RESULTS

Construction of GFP-tagged *Dickeya* sp. Transformation of *Dickeya* sp. IPO2222 with pPROBE-AT-*gfp* resulted in 27 transformants, from which a highly fluorescent colony was selected (*Dickeya* sp. IPO2254). Repeated transfer of GFP-tagged bacteria onto TSA plates supplemented with ampicillin (150 µg ml⁻¹) or in liquid NBa showed that the transformant expressed GFP in a stable way. The presence of pPROBE-AT-*gfp* was proven by plasmid DNA purification and agarose gel electrophoresis (data not shown).

Maintenance of pPROBE-AT-*gfp* in *Dickeya* sp. in non-selective conditions. The stability of GFP expression in *Dickeya* sp. IPO2254 was evaluated in a potato slice assay. With a 3-day interval, *Dickeya* sp. IPO2254 was transferred from a potato slice derived from a *Dickeya* sp.-free minituber to a new slice, for a period of 1 month (10 times in total). During the first 9 days (three transfers) no loss of plasmid was observed. After 21 days, 95% of cells were still GFP-positive and after 30 days, 83% of the colonies still expressed GFP (data not shown). GFP-negative cavity-forming bacteria collected at each time point, were all positive in a *Dickeya* sp.-specific PCR (Fig. 1).

Growth of GFP-tagged *Dickeya* sp. GFP-tagged *Dickeya* sp. IPO2254 displayed similar growth characteristics as the parental wild-type strain *Dickeya* sp. IPO2222 under aerobic (in NB broth) and anaerobic (in PEB medium) conditions, indicating that the growth rate was not affected either by the presence of the pPROBE-AT-*gfp* or by expression of the GFP protein (data not shown).

Tuber tissue maceration capacity of GFP-tagged *Dickeya* sp. The ability of the *Dickeya* sp. IPO2254 to macerate potato tubers tissue was investigated using a potato slice assay. After incubation of slices for 3 days at 28°C, the diameter of the rotting tissue was not significantly different from that of the parental wild-type strain *Dickeya* sp. IPO2222 (data not shown).

Colonization of potato plants followed by CVP plating.

Population dynamics of *Dickeya* sp. IPO2254 in soil and in plants were examined by CVP plating. One day after soil inoculation the marker strain was found in all soil samples. Following a rapid decrease, populations stabilized at a low level of 10^2 to 10^3 CFU g^{-1} soil during a 30 days experimental period (Fig. 2).

Densities of internal root populations of GFP-positive cavity-forming bacteria in both damaged (cut) and intact (uncut) roots were high 1 day after soil inoculation (10^4 to 10^6 CFU g^{-1}) (Fig. 3). No statistically significant increase in densities of root populations was found during the course of the experiment, not even in plants showing visible blackleg symptoms. A significant decrease of density was found 15 dpi in roots derived from root-damaged plants, but the initial densities of GFP-tagged *Dickeya* sp. were restored after 30 days. At 1 and 30 dpi but not after 15 dpi, bacterial densities in plants with uncut roots were significantly lower than those in plants with cut roots. No visible symptoms were observed in roots.

Relatively low population densities of *Dickeya* sp. IPO2254 (1 to 1,000 CFU g^{-1} of plant tissue) were detected in stems, stolons, and progeny tubers of potato plants with cut and uncut roots 15 and 30 dpi (Fig. 2). No statistical differences in population densities were found in tissues derived from plants with damaged and intact roots. (Fig. 2). At 1 dpi no stolons and progeny tubers were present, whereas stems were not analyzed at 1 dpi. For stems from plants with uncut roots and stolons from cut and uncut roots a significant increase in population densities was found from 15 to 30 dpi. No GFP-tagged *Dickeya* sp. was found in any sample of water-inoculated control plants.

Microscopic observations of plant colonization patterns.

ESM. Plant parts were analyzed with an ESM at a low magnification of 2.5 to 10 times. At 1 dpi, higher densities of *Dickeya* sp. IPO2254 were found on the surface of small roots (diameter: 0.25 to 0.5 mm) than on the surface of larger roots (diameter: 1 to 3 mm) (data not shown). At 1 dpi, bacteria were also observed inside approximately 20% of the embedded roots after disinfection.

Fig. 1. Maintenance of pPROBE-AT-*gfp* in *Dickeya* sp. IPO2254 during growth on potato slices. Potato slices of minitubers cv. Kondor were inoculated with 10^8 CFU ml^{-1} *Dickeya* sp. IPO2254; slices were incubated for 3 days. Bacteria were harvested from rotten potato tissue and transferred to a fresh potato slice. Bacteria were harvested 10 times at 3-day intervals. At every transfer, serial dilutions of rotten potato tissue were plated on crystal violet pectate, and the percentage of green fluorescent protein-positive cavity-forming *Dickeya* sp. was calculated.

Fig. 2. Population dynamics of *Dickeya* spp. IPO2254 in soil, roots, stems, stolons, and progeny tubers taken from plants grown in inoculated soil. Samples were taken from plants with an undamaged (intact) and a damaged root system. Samples were surface-sterilized before extracting the bacteria. Plant and soil extracts were plated on crystal violet pectate 1, 15, and 30 days post-soil inoculation. Stem cuttings were taken 5 cm above the ground level. The average of the predicted values is shown from four plants per time point. Statistical analysis was done per subsample (soil $n = 4$, roots $n = 4$, stems $n = 4$, stolons $n = 12$, and progeny tubers $n = 12$). Values followed by identical characters are not significantly different ($P = 0.05$).

A**IPO2254** *(Continued on next page)*

Fig. 3. Colonization of potato roots and stems with green fluorescent protein (GFP)-tagged *Dickeya* sp. IPO2254 using epifluorescence stereo microscopy. Plant parts, embedded in PT agar and incubated for 1 to 2 days at 28°C, were screened for a GFP-signal. **A**, Fragments of potato root. At 1 day post-soil inoculation (dpi), GFP-positive bacterial colonies on unsterilized roots were found on roots. After surface sterilization, a GFP signal was found in vascular and pith tissue of roots 1, 15, and 30 dpi. **B**, Cross sections of surface-sterilized potato stems embedded in PT agar. GFP signal was present in xylem and parenchyma tissue at 15 and 30 dpi, but not at 1 dpi (data not shown). Control, samples from water-inoculated plants.

At 15 and 30 dpi, the GFP signal was frequently found inside roots, stolons, and stem fragments taken 5 cm above the ground level (Fig. 3A and B). After 30 days, the GFP signal was detected in approximately 75% of the roots, 38% of the stems, and 50% of the stolons from plants with intact roots, and in 92% of roots, 50% of stems, and 50% of stolons from plants with a cut root system (Fig. 4).

CLSM. Plant parts were analyzed with a CLSM at a magnification of 640 to 1,000 times. Detailed studies on the localization of *Dickeya* sp. IPO2254 in plant tissues using CLSM showed that bacteria were mainly present in the vascular tissue of roots and stems. In roots, *Dickeya* sp. IPO2254 was found in the pith (in medulla and cortex), both inter- and intracellularly. In stems, bacterial cells were found inside and between the xylem vessels and protoxylem cells (Fig. 5).

Symptom development. No symptoms were observed in the first two weeks after soil inoculation. After 2 weeks, symptoms started to develop and after 30 days all plants grown on *Dickeya* sp.-infested soil showed wilting and chlorosis of the leaves, irrespective of root treatment. One plant from a cut and another from an uncut treatment showed typical blackening and soft rotting near the stem base. Control plants only expressed some chlorosis of lower leaves due to ageing (data not shown). Trans-sections of the stems from plants showing blackleg symptoms revealed a hollowing of the stems by degradation of pith tissue and a browning of the vascular tissue.

Characterization of GFP-expressing bacteria from infected plant tissue. Plant samples with a GFP fluorescence signal ob-

served under the epifluorescence microscope were collected at 30 dpi and were extracted and plated on TSA. Twelve green fluorescent isolates from various plant parts were selected; eight from roots, two from stems, and two from leaf material. 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 were identical to strain *Dickeya* sp. IPO2254 used for soil inoculation and to the parental wild-type strain *Dickeya* sp. IPO2222 used for GFP tagging (data not shown).

DISCUSSION

In this study we have shown for the first time that root infection with *Dickeya* spp. from soilborne inoculum can result in the occurrence of typical blackleg symptoms and in a systemic colonization of potato plants. Systemic colonization of *Dickeya* sp. in stab-inoculated root cuttings of *Chrysanthemum morifolium* has been reported before (27) as well as colonization of xylem vascular tissue with *Dickeya* spp. in naturally infected tomato plants (2). However, the colonization of intact potato roots from an inoculated soil has never been reported.

Using a GFP-tagged strain, roots were externally and internally colonized within 1 day of soil inoculation, and within 1 month, systemic spread of bacteria could be visualized in stolons and progeny tubers by dilution plating, ESM, and CLSM.

Fig. 3. (Continued from preceding page).

Fig. 4. Percentage of samples embedded in PT agar found infected with green fluorescent protein-tagged *Dickeya* sp. IPO2254, 1, 15, and 30 days post-soil inoculation. Plant parts were embedded in PT agar and incubated for 24 to 48 h at 28°C before screening with an epifluorescence stereomicroscope. Samples were taken from plants with undamaged (intact) and damaged roots. From four plants in total 12 roots, 8 stolons, and 8 progeny tubers, were screened per time point. Statistical analysis was done per subsample (roots, stolons, progeny tubers). Values followed by identical characters are not significantly different ($P = 0.05$).

The identity of the GFP-positive bacteria in roots, stems, and leaves was confirmed by isolation, followed by colony PCR using *Dickeya* sp.-specific primers and rep-PCR analysis, showing that the fluorescence was not due to conjugative transfer of the plasmid to other bacteria or caused by autofluorescent microorganisms, such as fluorescent *Pseudomonas* spp., present in the same niches.

GFP possesses excellent features as a reporter protein and is broadly used for studying bacterial populations in soil and the rhizosphere, colonization of plant tissue by pathogenic bacteria and for tracing particular proteins in the cytoplasm (10,37). In our study, the GFP-tagged strain showed similar characteristics as the parental wild-type strain with respect to growth under aerobic and anaerobic conditions and in the ability to macerate potato tuber tissue. The expression of GFP did not significantly affect the important biological features of *Dickeya* sp. IPO 2254. This confirms the observations of other research groups using GFP-tagged bacteria to monitor the destiny of bacterial cells in the environment (10).

GFP expression in *Dickeya* sp. during growth in plant tissue under nonselective conditions was stable. Only ca. 15% of the cells were negative in GFP expression after a total period of 30 days and after transferring the actively growing cells 10 times to fresh potato slices. Also in other studies, pPROBE-plasmids carrying *gfp* and various antibiotic resistance genes were stable up to 80 generations (80 doubling times) without any plasmid loss (23). The generation time of *Dickeya* spp. was determined at approximately 54 min in a rich medium and 80 min in a poor medium, respectively (15,36). Assuming an average generation time of 60 min on potato slices, the pPROBE-AT-*gfp* carrying *gfp* gene in our study was steadily expressed in a large part of the *Dickeya* spp. IPO2254 population for more than 700 bacterial generations.

Low populations of GFP-tagged *Dickeya* sp. in potato tissue, embedded in PT medium, could be visualized with microscopical techniques after incubation. Microscopic observation of the GFP-tagged bacteria in plant tissue directly after harvest was often difficult due to the relatively low cell densities. Therefore, the embedded tissue was incubated for 24 to 48 h, enabling the bacterial cells to multiply. In this way we were able to monitor specifically culturable cells even if initial population densities were low.

Our microscopic observations may suggest that *Dickeya* sp. shares the same pattern as other root invading bacteria (21), comprising three stages. In the first stage, bacteria colonize the surface of lateral roots and junctions between the lateral and main roots. In the second stage, bacteria penetrate the roots and establish infection of the cortex, and in stage three, they move into parenchyma cells of the pith and into xylem vessels of the stems, from where they can easily spread towards distantly located plant parts. Such colonization was observed both for *Ralstonia solanacearum*, a gram-negative vascular plant pathogen (45) and for a nitrogen-fixing gram-positive *Bacillus megaterium* (21).

Root colonization from soilborne inoculum was found irrespective if roots were damaged or not, indicating that *Dickeya* sp. enters via natural openings that occur during main and lateral root formation. In general, small but significant differences were found between incidences in infected tissues from plants with intact and damaged roots. It has been reported that both plant-pathogenic bacteria, such as *R. solanacearum*, and endophytic bacteria can enter plant roots via natural openings (14,17,35). In our study, higher numbers of GFP-tagged *Dickeya* sp. were found on the surface of lateral roots than on the surface of primary roots 1 day post-soil inoculation (data not shown). Similar results have been reported in root cuttings grown under in vitro conditions, where *Dickeya* spp. was predominantly found near natural openings created during lateral root formation (43).

Infection of soil with *Dickeya* spp. can also occur via other routes. *Dickeya* spp. bacteria can be released from rotten tubers or from an infected root system. The motile bacteria can migrate via free water in soil up to a distance of 10 m (12). Continuous infection of soils can occur during rainfall, if high numbers of bacteria are released into the soil from decaying tubers. Soil may also be infected from infected haulms after haulm destruction, as they can contain large numbers of cells which may contaminate the soil after rainfall (29). Finally, soilborne inoculum of *Dickeya* spp., a broad host range pathogen, may originate from other infected hosts, including crops grown in rotation with potato and/or weeds (8,22).

The risk of infection of a potato crop from soilborne inoculum will be dependent on the survival of the bacteria in the soil. Survival of *Dickeya* spp. in soil seems to be relatively short with a maximum period of 6 months (20,34). In our studies, a 1,000-fold

Fig. 5. Colonization of potato stems and roots by green fluorescent protein-tagged *Dickeya* sp. IPO2254 at 30 days post-soil inoculation analyzed with confocal laser scanning microscopy after incubating embedded plant parts in PT agar for 2 days at 28°C. (In most cases, plant cells were counterstained with the red-fluorescing dye propidium iodide.)

decrease in populations was found within 15 days. However, *Dickeya* sp. could survive at low densities of ca. 10^2 to 10^3 CFU per gram of soil for 30 days. These densities may be sufficient to establish an infection when the conditions promote plant colonization. Moreover, it cannot be excluded that *Dickeya* spp. may survive longer in plant debris of host plants as it was reported for closely related *P. carotovorum* (5).

Systemic colonization of potato plants will be dependent on various factors such as potato cultivar, *Dickeya* species, initial inoculum present, soil moisture, soil type, temperature, and pH. In our experiments, all plants showed symptoms, of which two plants had a typical blackening of the stem base, wilting, chlorosis and necrosis of plant tissue. In the glasshouse, conditions were highly favorable for disease development. The potato cultivar Kondor is highly susceptible to blackleg-causing pathogens (H. Velvis, unpublished data). The *Dickeya* sp. biovar 3 strain used is highly virulent, causes high blackleg incidences in the field and has been dominantly present in seed potatoes in several European countries in the past 5 years (19,40,42). The temperature in the glasshouse was high, favoring bacterial proliferation and symptom expression. Roots were submerged for 40 min in a bacterial suspension, probably long enough to create low oxygen condi-

tions that can impair the host defense (29). Field studies are required to further assess the risks of systemic colonization of plants via soilborne inoculum.

In general, these results suggest that systemic colonization of potato plants from contaminated soils can be highly significant in the epidemiology of potato blackleg caused by *Dickeya* spp. Progeny tubers can be infected systemically at the stolon end before harvest, limiting the possibility of controlling blackleg during harvest and postharvest. To further estimate the role of stolon end infection, the frequency of blackleg development from infected stolon ends relative to infected periderm needs to be investigated.

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