Systemic Colonization and Expression of Disease Symptoms on Bittersweet Nightshade (*Solanum dulcamara*) Infected with a GFP-Tagged *Dickeya solani* IPO2222 (IPO2254)

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Abstract

Colonization of *Solanum dulcamara* (bittersweet nightshade) plants by a GFP-tagged *Dickeya solani* type strain IPO2222 (IPO2254) was investigated by selective plating and epifluorescence stereomicroscopy (ESM), using in vitro plants and plants grown in compost soil. Replicated experiments were carried out in a growth chamber and the progress of infection and disease symptoms on tissue of the cultured plants, following leaf- and stem-base inoculations with bacteria, was evaluated. Microscopy observations were confirmed by spread-plating dilutions of plant extracts onto agar medium directly after the harvest. In experiments where the stem base of in vitro plants inoculated with a range of inocula of *D. solani* (10^4 to 10^8 colony forming units [cfu] ml⁻¹) was examined at 14 days post infection (dpi),

blackleg-like symptoms developed in more than 80% plants together with a reduction of the plant fitness (disease symptoms, weight, height, and appearance). In leaf-inoculated plants at 14 dpi, 15% of the plants exhibited severe blackleg-like symptoms. In detached *S. dulcamara* leaf assays, IPO2254 survived on the adaxial surface for 14 days at populations of 10^6 cfu per leaf. Thirty days after stem inoculation of plants grown in compost soil in pots, up to 10^4 cfu g⁻¹ of GFP-tagged *D. solani* were found inside the stems. *D. solani* were detected inside the vascular tissue (xylem vessels) of stems, in the pith tissue in roots, and on the internal surface of the stem hollow. The implications of *S. dulcamara* infection by *D. solani* for the long-distance dispersal of the bacterial inoculum are discussed.

Solanum dulcamara L. (bittersweet nightshade) is a perennial herbaceous weed, which in the last couple of centuries has been naturalized in many parts of the world. It is one of the 1,500 to 2,000 native *Solanum* species occurring in Europe and Asia (Weese and Bohs 2007). *S. dulcamara* has been introduced to North and South America, New Zealand, and Australia, and is now considered as an invasive weed (Forcella and Stephen 1988; Golas et al. 2010a). Bittersweet nightshade may grow in contrasting natural habitats from woodlands and scrublands to hedges and marshes and therefore it is now considered to have a worldwide distribution (Pegtel 1985). Moreover, *S. dulcamara* is often found in direct proximity to potato fields (Toth et al. 2011).

Together with other *Solanum* species present in Europe, e.g., *S. nigrum* L. (Flier et al. 2003), bittersweet nightshade is considered a weed of arable fields and an alternative (secondary) host for pathogens affecting potato (*S. tuberosum* L.) cultivations (Shah et al. 2010). For example, it is well established that *S. dulcamara* is a host for the quarantine pathogen *Ralstonia solanacearum*, the causal agent of bacterial wilt or brown rot of potato (Álvarez et al. 2010). It has been shown that *S. dulcamara* growing in ditches and near natural water sources used for irrigation of adjacent potato fields harbors relatively large *R. solanacearum* population and allows survival of the pathogen between cropping seasons while the primary host (potato) is absent (Elphinstone et al. 1998). Therefore, in several countries in Europe, *S. dulcamara* is a subject to extensive eradication programs from its natural habitats, aimed at preventing the spread of *R. solanacearum*. *S. dulcamara* is also a recognized alternative (secondary) host for *Phytophthora infestans*, an oomycete pathogen causing potato late blight, one of the most important nonbacterial potato pathogens worldwide (Golas et al. 2010b; van West and Vleeshouwers 2004).

Only limited data are available on the survival of soft rot *Enterobacteriaceae*: pectinolytic *Pectobacterium* spp. and *Dickeya* spp.

(Pérombelon 2002) on *S. dulcamara* and no extensive studies have been done so far to address the role of bittersweet nightshade in the spread and the survival of these pathogens in the environment. Olsson (1985) reported the isolation of *Dickeya* spp. from *S. dulcamara* growing in watercourses used for irrigation in Sweden (Olsson 1985). We have recently isolated *Pectobacterium carotovorum* subsp. *carotovorum* from bittersweet nightshade plants grown in a riparian environment in northern Poland. These isolates were virulent to potato tubers causing typical soft rot symptoms (Fikowicz-Krosko et al. 2017).

Soft rot *Enterobacteriaceae* (SRE), *Pectobacterium* spp. and *Dickeya* spp., are causal agents of blackleg, aerial stem rot, wet rot, and tuber soft rot of potato (*S. tuberosum*). Both genera are among the top 10 most important bacterial pathogens in agriculture (Mansfield et al. 2012). In Europe, *Dickeya* spp. cause vast economic losses in (seed) potato production, mainly due to the rejection and reclassification of seed lots. Since 2000, a new species, *Dickeya solani* (van der Wolf et al. 2014) has been found regularly in association with potato in many European countries, and also isolated from potato grown in Israel and Georgia. Therefore, the increasing losses in seed potato production attributed to *Dickeya* spp. infection might be related to the spread of *D. solani* (Toth et al. 2011; van der Wolf et al. 2014).

D. solani is mainly a seedborne pathogen (Czajkowski et al. 2009; Pérombelon, 2000); it is, however, well established that the infection of grown potato plants with pectinolytic bacteria may originate from other sources, e.g., contaminated equipment used for crop spraying, insects feeding on symptomatic plants, contaminated irrigation water, and/or aerosols (Elphinstone and Pérombelon 1986; Toth et al. 2011). Only limited data are available on the survival of pectinolytic bacteria on alternative (secondary) hosts including weeds (Toth et al. 2011; Tsrör et al. 2010), and even less is known regarding *D. solani*. It is, however, very likely that *Dickeya* spp., as broad host pathogens, can survive on different plant species as it happens in the case of *P. carotovorum* subsp. *carotovorum* (Ma et al. 2007; Pérombelon and Hyman 1989).

This study aimed to investigate the ability of a type strain GFP-tagged *D. solani* IPO2222 (IPO2254) to infect *S. dulcamara* plants grown in culture tubes or in pots with compost soil in a growth chamber to better understand the impact of *D. solani* and *S. dulcamara* interaction on the epidemiology of soft rot and blackleg of potato. The progression of infection and systemic colonization of plants were studied using a GFP-tagged *D. solani* strain and different inoculation points, i.e., roots and leaves, to evaluate the routes of systemic

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bacterial spread in growing plants. Epifluorescence stereomicroscopy (ESM) was used to localize and visualize the bacteria inside plant tissues in situ, and dilution-plating techniques were used to estimate the bacterial populations and assess *D. solani* population dynamics inside plants.

Materials and Methods

Bacterial strains, plants, and growth media. GFP-tagged type strain *D. solani* IPO2222 (IPO2254) (Czajkowski et al. 2010b; van der Wolf et al. 2014) was grown at 28°C for 24 to 48 h on tryptone soya agar (TSA) (Oxoid) supplemented with 100 µg ml⁻¹ ampicillin (Sigma). Liquid bacterial cultures were prepared in tryptone soya broth (TSB) (Oxoid) also supplemented with 100 µg ml⁻¹ ampicillin and grown at 28°C for 24 h with agitation (150 to 200 rpm).

S. dulcamara in vitro plantlets (accession A54750008, The Experimental Garden and Genebank, Radboud University Nijmegen, the Netherlands) were obtained from Prof. Titti Mariani from Department of Molecular Plant Physiology, Radboud University, Nijmegen, the Netherlands. They were maintained on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with Gamborg's vitamin mixture (Duchefa) (MS+G vit.), 30 g liter⁻¹ sucrose (Chempur), pH 5.8, and solidified with 7 g liter⁻¹ plant agar (Duchefa) at 22°C under 16/8 h photoperiod.

Growth and propagation of *S. dulcamara* in vitro plants. Forty milliliters of autoclaved MS+G vit. medium was poured into 77 × 77 × 97 mm Magenta culture vessels (Sigma) and closed with polypropylene Magenta caps (Sigma). Five nodal *S. dulcamara* explants per vessel were cultured 1 month prior use. All plant cultures were incubated under growth chamber conditions in an Innova 42R phytochamber (Eppendorf) equipped with a photosynthetic light bank (SYLVANIA, GRO-LUX, T8 15W, 8500K), in 16/8 h light/dark regime and temperature of 22 ± 0.5°C. Apical nodes of *S. dulcamara* plants with at least two pairs of leaves were harvested from 1-month-old stock cultures and placed individually on 10 ml of MS+G vit. medium, in De Wit culture tubes (Duchefa Biochemie bv.). Before inoculation with bacteria, plants were grown for an additional 2 weeks under similar conditions to allow growth of at least 1-cm-long roots and for the stem base cut wound to heal.

Inoculation of in vitro-grown *S. dulcamara* plants with *D. solani* IPO2254 to study systemic colonization initiated from stem base and roots. Inoculation of *S. dulcamara* plants with *D. solani* IPO2254. For inoculation of *S. dulcamara* plants, 10 µl of bacterial suspensions in 1/4 Ringer's buffer (Merck) containing 10⁴, 10⁶, or 10⁸ cfu ml⁻¹ of IPO2254 were pipetted into the interspace between the stem base and the MS medium of each individual plant. For negative control, instead of bacterial suspension, 10 µl of sterile 1/4 Ringer's buffer (Merck) was used. Four treatments were applied. Treatment A: plants inoculated with Ringer's buffer (control), Treatment B: plants inoculated with 10⁴ cfu ml⁻¹ of IPO2254, Treatment C: plants inoculated with 10⁶ cfu ml⁻¹ of IPO2254, and Treatment D: plants inoculated with 10⁸ cfu ml⁻¹ of IPO2254. Each treatment consisted of 20 plants grown in individual culture tubes (Culture Tubes "De Wit", Duchefa Biochemie bv.) and the experiment was repeated independently one time with the same setup.

Symptom development in vitro-grown plants after stem base and root inoculation. Plants were visually inspected 3, 7, 10, and 14 days post inoculation (dpi) for symptom development and were assessed for wilting, blackleg-like symptoms, stem desiccation, and plant death. The height of plants, from the surface of the agar medium to the plant's top, was assessed for each plant at each time point.

Sampling of *S. dulcamara* plants. Plants were sampled 3, 7, 10, and 14 dpi. At each time point, five plants per treatment were sampled. The plants were aseptically removed from the culture tubes and each (stem and leaves but no roots) was collected, weighed, and individually suspended in 1 ml of 1/4 Ringer's buffer (Merck) supplemented with 0.02% diethyldithiocarbamic acid (DIECA, antioxidant) (Arcos Organics) in 15 ml Falcon tubes. Each sample was crushed with a sterile, stainless steel manual homogenizer (Bionovo), and 100 µl of serial dilutions were plated in duplicate on TSA plates supplemented with 100 µg ml⁻¹ ampicillin and incubated for 24 to 48 h

at 28°C before GFP-positive colonies were counted. The experiment was repeated independently once and the results were averaged.

Inoculation of in vitro-grown *S. dulcamara* plants with *D. solani* IPO2254 to study systemic colonization initiated from inoculated leaves. Estimation of IPO2254 inoculum used for infection of leaves. Prior to other experiments, we assessed (1) the *D. solani* IPO2254 inoculum on the tip of forceps used for plant inoculation and (2) the bacterial inoculum on the leaf after the leaf was mechanically wounded and infected with IPO2254. For this, the tip of the forceps was dipped in 10⁷ cfu ml⁻¹ IPO2254 suspension in 1/4 Ringer's buffer (Merck) and immediately rinsed in 1 ml of 1/4 Ringer's buffer for 5 s, serially diluted in the same buffer. Then, 100 µl was plated on TSA supplemented with 100 µg ml⁻¹ of ampicillin and incubated at 28°C for 48 h. GFP-positive bacterial colonies were counted. The entire procedure was repeated six times for each of three independently prepared bacterial solutions (*n* = 18). Each time before sample collection, the forceps were sterilized with 70% ethanol, flamed, and cooled before being reused. Similarly, the 2-week-old in vitro-cultured *S. dulcamara* leaves (10 leaves in triplicate, *n* = 30) were inoculated with IPO2254 suspension using the forceps (as described above). Each wounded leaf was immediately transferred to a 1.5-ml Eppendorf tube and macerated in 1 ml of 1/4 Ringer's buffer with a sterile, manual homogenizer (Bionovo). The obtained suspensions were serially diluted and 100 µl duplicates were plated on TSA supplemented with 100 µg ml⁻¹ of ampicillin, and incubated in 28°C for 48 h before counting the GFP-positive bacterial colonies.

Inoculation of *S. dulcamara* leaves. Two-week-old *S. dulcamara* plants, grown individually in De Wit culture tubes as described above, were used. One leaf, taken from the second or third pair of leaves, was pinched with the tip of the forceps (0.4 cm²) dipped in the IPO2254 suspension of 10⁷ cfu ml⁻¹ in 1/4 Ringer's buffer as described above, without removing the plants from culture tubes and/or destroying the root system, giving a ~1 cm long wound. Each experiment consisted of 28 plants: six inoculated plants and one negative control plant, that were sampled 3, 5, 7, and 10 dpi for the isolation of bacteria and colony counting as described above. The experiment was repeated independently once with the same setup.

Symptom development in vitro-grown plants after leaf inoculation. Plants with inoculated leaves were visually inspected 3, 10, and 14 dpi for symptom development including wilting, typical blackleg-like symptoms, stem desiccation, and/or plant death. Plant height, from the surface of the agar medium to the plant's tip, was determined at each time point.

Microscopy observations of *S. dulcamara* in vitro-grown plants infected with GFP-tagged *D. solani* IPO2254. In vitro plants inoculated at stem base or leaf with IPO2254 were randomly collected for microscopy analyses 14 dpi. The haulm and the leaves were removed from the De Wit culture tubes and cleaned of the agar-medium residues using sterile water. The stems were cut into 0.5-mm slices and 0.5 to 1 cm pieces both crosswise and lengthwise. The leaves and roots were detached from the stems. At least 10 individual leaves, stems, and roots collected from at least 10 individual plants per experiment, together with uninoculated (control) samples, were used for microscopy studies. All tissues were embedded in PT agar medium (melted and cooled to 55°C) (Burr and Schroth 1977), supplemented with 100 µg ml⁻¹ ampicillin, in Petri dishes, and incubated for 48 h at 28°C (Czajkowski et al. 2010b). GFP-positive bacterial microcolonies were visualized at 495 nm blue light under Leica MZ10 F stereo-microscope UV imaging with an ET GFP filter and Leica DFC450C camera system as previously described (Czajkowski et al. 2010b).

Population dynamics of *D. solani* IPO2254 on *S. dulcamara* adaxial leaf surface in vitro. Two-week-old and ~10 cm high *S. dulcamara* seedlings were transplanted individually to 0.25-liter plastic pots filled with potting compost (COMPO SANA Universal Potting Compost, Compo) and kept in a growth chamber at 22°C and 16/8 h (day/night) photoperiod (white cool fluorescent light, Philips, TLD 58 W/840, 30 to 35 µmol m⁻² s⁻¹) and 80% relative humidity (RH) for rooting and shoot development. After 2 weeks, *S. dulcamara* leaves (third to sixth from the shoot tip) were detached from the plant. Two milliliters of IPO2254 suspension adjusted to 10⁸ cfu ml⁻¹ in sterile Ringer's buffer (Merck) was sprayed on the

adaxial surface of each leaf using a manual sprayer. Sprayed leaves were dried under the air flow and placed adaxial side up on 0.5% water agar (Oxoid) in square plastic Petri dishes (100 × 100 mm, Sarstedt). As control, sterile Ringer's buffer was used. The leaves were left to dry under the laminar flow, then transferred to the Petri dishes and kept in the growth chamber as described above. Samples were collected at 0, 1, 7, and 14 days and assayed for the presence of *D. solani* IPO2254. At each time point, five randomly chosen leaves were collected from five randomly chosen Petri dishes and separately shaken in 20 ml of Ringer's buffer in 50 ml Falcon tubes (Sarstedt) at 50 rpm at room temperature, each for 30 min, to remove bacterial cells from the leaf surface. The washings were serially diluted in Ringer's buffer, and 100 µl taken from the 10× and 100× diluted samples were plated in duplicates on TSA containing 100 µg ml⁻¹ ampicillin to select for GFP-positive colonies and 100 µg ml⁻¹ cycloheximide (Sigma) to prevent fungal growth before incubation. The plates were then incubated at 28°C and GFP-positive colonies were counted. The experiment was repeated once with the same setup and the results were averaged.

Growth of *S. dulcamara* plants in potting compost following stem-inoculation with *D. solani* IPO2254. Replicated experiments were performed in March 2017. *S. dulcamara* plants derived from apical meristems of culture tube-grown plants were collected and grown in individual 0.5-liter plastic pots in potting compost and incubated as described above for 14 days prior to inoculation with the bacteria. Plants were inoculated ($n = 10$ per experiment) as follows: 0.5 cm long transverse cut was made at 30 degrees on each stem 5 cm from the stem base; 10 µl (final concentration per inoculation point 10⁵ cfu) of the IPO2254 suspension in 1/4 Ringer's buffer was applied into the wound. As a control, plants were inoculated with 10 µl of 1/4 Ringer's buffer. After inoculation, the wound was covered in Parafilm (Parafilm M All-Purpose Laboratory Film, Bemis, WI) to protect it from desiccation. As with in vitro plants, *S. dulcamara* plants were visually inspected 14 and 30 dpi for infection development and were assessed for wilting, blackleg-like symptoms, stem desiccation, and plant death. Samples ($n = 5$) were collected at 14 and 30 dpi. For this, a 1.5 to 2 cm long stem fragment per plant was cut out 5 cm above the inoculation site and macerated in 1 ml of 1/4 Ringer's buffer supplemented with DIECA (Sigma), using a sterile manual homogenizer (Bionovo). Duplications of 100 µl of undiluted macerate were plated on TSA containing 100 mg liter⁻¹ ampicillin and 100 mg liter⁻¹ cycloheximide and incubated at 28°C for 48 h to allow the growth of GFP-tagged *D. solani* IPO2222. The experiment was repeated once with the same setup.

Statistical analysis. Bacterial count data were analyzed by ordinary linear regression using the statistical software package GenStat (Payne et al. 2007). To achieve the approximate normality, data were log transformed after adding value 1 to avoid taking logs of zero. Results were considered to be significant at $P = 0.05$ and pair-wise differences were obtained using the t test. Data were analyzed according to the experimental design in which two replicated experiments were done at each time point with treatments of replicated plants. The adopted linear model was a complete block design with replicates as complete blocks, main effects analyzed for time and treatment and the two-way interaction between time and treatment. Normal distribution was assumed for plant height and weight.

Results

Systemic colonization of in vitro *S. dulcamara* plants initiated at stem base and roots. Two independent experiments were conducted in June and July 2016. In each experiment, 20 plants per treatment were used and plant fitness and disease progression in plants inoculated with GFP-tagged *D. solani* IPO2222 (IPO2254) were investigated.

*Fitness of *S. dulcamara* plants and disease development.*

Treatment A. No visible symptoms were observed in *S. dulcamara* plants inoculated with sterile 1/4 Ringer's buffer (negative control) during the entire course of both experiments and at 14 dpi (end point). The average height and the average fresh weight were 7.73 cm and 151 mg, respectively (Fig. 1).

Treatments B, C, and D. General observations: in all treatments of *S. dulcamara* plants inoculated with *D. solani* IPO2254, five classes of symptoms were recorded (Fig. 1): (1) root colonization identified by a distinct turbidity of the MS medium around the roots, starting from the site of the inoculation and spreading down the roots; (2) embrowning of roots identified as a change of color from white/yellowish to brown/black; (3) blackleg-like symptoms – blackening and soft rotting of the stem above and below the inoculation point; (4) wilting and yellowing of leaves; and finally (5) plant death when the haulm rotted extensively, the stem collapsed, and the plant parts were difficult to distinguish from one another (Fig. 1). Additionally, inoculation of plants with IPO2254 resulted in the reduction of plant fitness manifested by a decrease in average height and weight of plants in comparison with the plants inoculated with Ringer's buffer only (negative control, *Treatment A*).

Treatment B. At 14 dpi in plants inoculated with 10⁴ cfu ml⁻¹ of IPO2254 symptoms included root colonization and embrowning, blackleg-like symptoms, and wilting and yellowing of leaves. These symptoms appeared after inoculation and progressed with time in both experiments. Root colonization could be observed as early as 2 dpi. Disease symptoms were observed in 87 and 100% plants in the first and the second experiment, respectively. In both experiments, the height and fresh weight of plants at 14 dpi were, on average, 5.82 cm and 68 mg, respectively.

Treatment C. In plants inoculated with 10⁶ cfu ml⁻¹ of *D. solani* at 14 dpi, all five types of symptoms were observed including plant death. Blackleg-like symptoms were observed in 93 and 100% plants in the first and second experiment, respectively. In both experiments at 14 dpi, the height and fresh weight of plants were, on average, 5.31 cm and 84 mg, respectively.

Treatment D. In plants inoculated with 10⁸ cfu ml⁻¹ of *D. solani* at 14 dpi, all five types of infection symptoms were observed as in treatment C. Interestingly, despite the higher inoculum than in treatments B and C, only 80 and 87% of plants showed disease symptoms in the first and second experiments, respectively. In both experiments at 14 dpi, the height and fresh weight of plants were, on average, 5.26 cm and average 45 mg, respectively.

Overall, at 14 dpi, plants inoculated with IPO2254 were on average shorter in all three treatments (treatments B, C, and D) by 25, 31, and 32%, respectively, when compared with control plants (treatment A).

IPO2254 populations in *S. dulcamara* haulms The number of bacteria present in plant haulms after stem base inoculation was evaluated 3, 7, 10, and 14 dpi for each of the three inocula used in treatments B, C, and D of *D. solani* IPO2254 (Fig. 2). The values differed widely per plant and per treatment, but overall were not statistically different between replicated experiments 1 and 2, therefore the data were analyzed together (Figs. 2 and 3). Regardless of the initial inoculum used, all three treatments yielded symptomatic systemic colonization. In all treatments at 3 dpi, bacterial populations were high with an average of 10⁶ to 10⁷ cfu g⁻¹ of haulms. No further increase in IPO2254 number was recorded at 7 and 10 dpi but at the end of experiment (14 dpi), the bacterial populations reached 10⁷ to 10⁹ cfu g⁻¹ of haulm tissue. No GFP-positive, ampicillin-resistant colonies were found in any of the samples taken from plants inoculated with sterile 1/4 Ringer's buffer (negative control – treatment A) in the entire course of both experiments.

Systemic colonization of *S. dulcamara* plants initiated at leaves.

Two replicated experiments were conducted in January and February 2017. In each experiment, 28 plants were analyzed, with six plants inoculated with GFP-tagged *D. solani* per treatment and time point, and one control plant. Plant fitness and disease progression in plants inoculated with IPO2254 were monitored.

Determination of IPO2254 inoculum load delivered by the forceps method. The number of bacteria present on the tip of the forceps dipped in *D. solani* IPO2254 suspension (10⁷ cfu ml⁻¹) in Ringer's buffer was 10⁶ cfu ($n = 18$) on average, which resulted in 10⁵ *D. solani* cfu per infected leaf ($n = 10$).

Fitness of *S. dulcamara* plants and disease development after leaf infection. No statistically important differences between experiments 1 and 2 were found, and therefore the data were analyzed together. As with the stem base-inoculated in vitro plants, five classes of

symptoms were also observed in plants that were leaf-inoculated with *D. solani* using forceps (Fig. 4): (1) colonization of roots, (2) roots embrowning, (4) wilting, and (5) full plant maceration leading to plant death. In comparison with the root-initiated colonization, no plant developed blackleg-like symptoms (3); however, an additional class of symptoms was determined, namely (4a) maceration of the inoculated leaf (Fig. 1). After 3 dpi, the only symptom assessed in 30% of the *S. dulcamara* plants was maceration of the first inoculated leaf. All five classes of symptoms were observed after 14 dpi (end point); the most abundant were the wilting of the haulms and maceration of the inoculated leaf, which were observed in 70% of plants in both experiments. Fifteen percent of symptomatic plants were fully macerated at the end point of the experiment. In total, after 14 dpi, 80% of plants developed at least one of the five classes of symptoms (Fig. 4). At 14 dpi, the height of the plants inoculated with IPO2254 at leaf was on average 13% lower than in case of the control plants. In the course of both experiments, the control plants, inoculated with sterile 1/4 Ringer's buffer, did not exhibit any disease symptoms. The wounded

leaves remained green with some minor necrosis due to bruising. The average height of the control plants after 14 dpi was 6.9 cm.

IPO2254 populations in *S. dulcamara* haulms. Plant haulms collected after 3, 5, 7, and 10 dpi revealed the presence of IPO2254 in all *S. dulcamara* plants following leaf inoculation. The *D. solani* populations were stable, with an average of 10^7 to 10^8 cfu g⁻¹ of haulms during the entire course of both experiments (Fig. 5).

Microscopy observations of *D. solani* IPO2254 in inoculated *S. dulcamara* plants. In the stem base- and leaf-inoculated plants, at 14 dpi, GFP-positive microcolonies were found on and inside the *S. dulcamara* stems and roots that were collected, embedded in PT medium, and enriched for bacterial presence (Fig. 6). The fluorescence signal was not detected in leaves of the inoculated plants, but was predominantly localized in the stem and root vascular tissues as well as on the surface of the stem central hollow (outside the stem vascular system). In roots, the GFP signal was found both inside in the vascular tissue and on the outer surface (Fig. 6). The primarily inoculated leaves of the leaf-inoculated plants were not investigated

Fig. 1. Classes of symptoms observed in in vitro *Solanum dulcamara* plants after (1) stem base and root and (2) leaf inoculations with *Dickeya solani* IPO2254. Disease severity was assigned to five classes (categories): class (1) – root colonization identified as increase of turbidity of the MS medium around roots; class (2) – root embrowning identified as a change in root surface color from white/yellow to brown/black; class (3) – blackleg-like symptoms, blackening and softening of the stem above and below stem base; class (4) – wilting and yellowing of leaves; class (4a) – maceration of the inoculated leaf, (on leaf-inoculated plants only); class (5) – plant death with the symptoms of heavy rot of haulms.

microscopically due to extensive decomposition of plant tissue. *D. solani* IPO2254 was found in all treatments except for the negative control plants (treatment A).

Survival of *D. solani* IPO2254 on the surface of detached *S. dulcamara* leaves. In replicated experiments, survival of IPO2254 on the adaxial surface of detached *S. dulcamara* leaves was monitored weekly over a period of 14 days (Fig. 7). The number of bacteria differed widely per plant and per treatment, but was not statistically

significant different between experiments 1 and 2; therefore, these data were analyzed together. In both experiments, at 0 dpi, average populations of 10^6 cfu per leaf were found and at 1 dpi these populations remained unaltered. No further statistically significant changes in *D. solani* populations at 7 and 14 dpi in comparison with 1 dpi were observed in the course of both experiments (Fig. 7). No GFP-tagged ampicillin resistant bacteria were found on leaves not inoculated with IPO2254 (negative control) in both experiments.

Fig. 2. Disease symptoms observed after inoculation of stem and roots of *Solanum dulcamara* plants with *Dickeya solani* IPO2254, shown as percentage of affected plants. Plants were grown in vitro under growth chamber conditions at 22°C and 16/8 h photoperiod. In replicated experiments, 120 plants were used (60 plants per experiment) subjected to three inoculation levels, on 20 plants each: 10^2 , 10^4 , and 10^6 IPO2254 cfu per plant. Symptoms were evaluated at four time points: 3, 7, 10, and 14 days post inoculation. Percentages do not sum up to 100 as often several symptoms per plant were observed.

Fig. 3. *Dickeya solani* populations in culture tube-grown *Solanum dulcamara* haulms after stem base and root inoculations with IPO2254. Plants were grown in culture tubes under growth chamber conditions at 22°C and 16/8 h photoperiod. In replicated experiments, 120 plants were used (60 plants per experiment) divided to three inoculation levels, on 20 plants each, with IPO2254: 10^2 , 10^4 , and 10^6 CFU/plant. At each time point (3, 7, 10, and 14 days post inoculation), five samples per treatment were analyzed. Haulms were collected and weighed, and haulm extracts in 1/4 Ringer's buffer (Merck) were plated on tryptone soya agar plates supplemented with 100 μ g ml⁻¹ ampicillin and incubated at 28°C for 24 to 48 h. GFP-positive, ampicillin-resistant bacterial colonies were counted.

Presence of *D. solani* IPO2254 on *S. dulcamara* after inoculation of plants grown in potting compost. No ampicillin-resistant, GFP-positive bacterial colonies were found in samples of IPO2254-inoculated plants taken at 14 dpi (data not shown). Among 20 plants tested in both replicates ($n = 10$ per experiment) after 30 dpi, the GFP-tagged bacteria were detected in eight stems (four in experiment 1 with bacterial densities of 7×10^3 , 5×10^1 , 3.5×10^4 , and 2.6×10^4 per gram of plant tissue and four in experiment 2 with bacterial densities of 3.3×10^4 , 2.7×10^4 , 3×10^4 , and 7.9×10^3 per gram of plant tissue). Number of bacteria differed widely between plants, ranging from 10^1 to 10^4 cfu g⁻¹ of stem tissue. None of the plants inoculated with IPO2254 revealed any visible disease symptoms during the course of both experiments. Control plants inoculated with Ringer's buffer remained symptomless, and no GFP-tagged bacteria were isolated from the stems at any time point.

Discussion

In this study, we have shown for the first time that infection of culture tube-grown *S. dulcamara* with *D. solani* can result in the development of disease symptoms, similar to those observed on in vitro

Fig. 4. Symptoms observed after inoculation of leaves of culture tube-grown *Solanum dulcamara* plants with *Dickeya solani* IPO2254, shown as percentage of affected plants. Plants were grown in a growth chamber at 22°C and 16/8 h photoperiod. In replicated experiments, 56 plants were used (28 plant per experiment). Symptoms were evaluated at three time points: 3, 10, and 14 days post inoculation. Percentages do not sum up to 100 as often several symptoms per plant were observed.

Fig. 5. *Dickeya solani* populations in culture tube-grown *Solanum dulcamara* haulms after leaf inoculation with IPO2254. Plants were grown in a growth chamber at 22°C and 16/8 h photoperiod. In replicated experiments, 56 plants were used (28 plant per experiment). At each time point (3, 5, 7, and 10 days post inoculation), six plants per treatment were sampled together with one control plant. Haulms were collected and weighed, and haulm extracts, prepared in 1/4 Ringer's buffer (Merck), were plated on tryptone soya agar plates supplemented with 100 µg ml⁻¹ ampicillin and incubated at 28°C for 24 to 48 h. GFP-positive, ampicillin-resistant bacterial colonies were counted.

cultivated potato (*S. tuberosum*), and in systemic colonization of the plants. Despite the fact that the pectinolytic bacteria have been repeatedly reported on *S. dulcamara* grown in natural habitats (Fikowicz-Krosko et al. 2017; Olsson 1985; Toth et al. 2011), no extensive studies have been done to assess the role of bittersweet nightshade in the spread and survival of pectinolytic bacteria in nature. Likewise, no detailed studies have been conducted to evaluate the

Fig. 6. Fluorescence microscopy of tissue culture *Solanum dulcamara* plants inoculated with IPO2254 at stem base and roots (A) and leaf (B). Samples of *S. dulcamara* roots and stems were collected 14 days post inoculation for microscopy observations and were embedded in the PT medium and incubated for 48 h at 28°C to enrich bacterial cultures and GFP signal inside plant tissues. At least 10 individual random stem and root samples collected from at least 10 individual plants per experiment were used for microscopy observation at magnification of 2.5× to 10×. GFP signal was visualized at 495 nm blue light under Leica MZ10 F stereomicroscope UV imaging with an ET GFP filter and Leica DFC450C camera system. Representative images are shown.

routes by which *D. solani* invades *S. dulcamara*. A better understanding of *D. solani* virulence and symptom expression on *S. dulcamara* can lead to identification of new critical target points leading to successful reduction of the spread of pectinolytic bacteria in the environment and development of appropriate procedures involving weed control. Furthermore, this study shows for the first time that the axenically grown (weed) plants can be used successfully to study the effect of alternative hosting of SRE. Once introduced to culture tubes, these plants are grown under strictly controlled conditions and therefore the results are highly reproducible. Similarly, as it was reported previously, the pectinolytic bacteria are unable to grow in in vitro culture media without the presence of the host plants (Leifert and Waites 1992). Therefore, the possibility of obtaining false-positive colonization and infection results in the assay are minimal. Consequently, this may serve as a method of choice for the first screening of other wild plants as *Pectobacterium* spp. and *Dickeya* spp. hosts. This proposed screening method can be easily scaled up if required. However, it must be pointed out here that symptoms observed in in vitro grown plants does not necessarily reflect the natural infection occurring under field conditions.

A systemic colonization of potato plants by fluorescently labeled *Pectobacterium* spp. and *Dickeya* spp. has been discussed in several studies (Czajkowski et al. 2010a; Golan et al. 2010; Kubheka et al. 2013), but the colonization of *S. dulcamara* by a GFP-tagged *D. solani* has not. Using a GFP-tagged strain and two routes of inoculation ([1] stem base together with roots and [2] leaves), a rapid colonization of in vitro *S. dulcamara* was visualized by dilution plating and fluorescent microscopy.

The infection of *S. dulcamara* plants grown in tissue culture with *D. solani* was rapid. The first symptoms of root colonization after pipetting the bacterial culture in the interspace between the agar medium and the stem base were observed 2 days after the inoculation. This was comparable with symptom development in the roots of in vitro potato plants in our earlier studies (Czajkowski et al. 2015). Similarly, after leaf inoculation, the first symptoms were recorded in a relatively short time (3 dpi) but leaves had to be wounded mechanically to allow bacteria to enter the leaf tissue. In the detached leaf assay, non-wounded *S. dulcamara* leaves did not exhibit any disease symptoms when sprayed with the bacterial inoculum on the adaxial side of leaves. This is also in agreement with our former studies on the colonization of potato leaves by *D. solani*, where the use of a carborundum powder to abrade the leaf surface was required for the bacteria to penetrate plant tissue (Czajkowski et al. 2010a). Overall, our observations indicate that *D. solani*, under in vitro conditions, is highly virulent not only in potato as was reported previously (Toth et al. 2011), but probably also in closely related plant species such as *S. dulcamara*. In our former studies, *S. tuberosum* plants grown under the same in vitro conditions and inoculated with *D. solani* IPO2254 via application of bacteria into the interspace between stem base and agar medium, expressed severe disease symptoms including typical blackleg and plant death in the majority of plants (Czajkowski et al. 2015).

D. solani could penetrate intact roots of the in vitro grown plants, which may indicate that it could behave alike under natural conditions, although this still needs to be experimentally confirmed. In potato fields, *D. solani* can be released from a rotting mother tuber or from an infected root system and migrate with water in the soil up to a distance of 10 m (Graham and Harper 1967), far enough to find a new host. A chronic infestation of soil can take place during rainfall if high numbers of bacteria are released into the soil from decaying tubers or blackleg-affected stems (Pérombelon 1982). Similarly, in our study, *D. solani* could survive on the leaf surface for at least 14 days, from where it could further spread to other plants via wind blow or with rain aerosols.

A systemic colonization of plants was recorded regardless of the inoculation routes chosen, indicating that it cannot be excluded that *S. dulcamara* may also be infected in nature with *D. solani* via an air-, water-, and/or soilborne inoculum. *D. solani* is not considered under normal circumstances to be a soilborne nor airborne pathogen, but in fields where infected crops are grown or drainage water from such fields is used in cultivation areas, the bacteria might be transferred with air or water. It is well established that under field conditions,

infected potato tubers release SRE into the soil (Burr and Schroth 1977; Czajkowski et al. 2010b; Pérombelon and Hyman 1989; Powelson and Apple 1984), where the bacteria may spread and colonize roots of other host and nonhost plants, including *S. dulcamara*. Similarly, an aerial infection may occur from neighboring fields as bacteria of the *Dickeya* spp. may be transferred by wind, rain water, or aerosols to plants growing close to infected potato fields (Graham and Harrison 1975; Pérombelon 2002; Pérombelon et al. 1979). Moreover, *S. dulcamara* may get infected via insect transmission of bacteria (Grenier et al. 2006; Kloepper et al. 1981).

A systemic infection of stem base/root-inoculated in vitro *S. dulcamara* plants, together with the emergence of disease symptoms, can take place under a range of bacterial inocula. This suggests that under favorable conditions, the infection of a secondary host under in vitro conditions can develop even with a very low level of inoculum, ~100 cfu per plant. Essentially, similar results were obtained for potato (Toth et al. 2011). Data concerning SRE populations on weeds under natural conditions is limited. Although it is probable that many varieties of weeds may harbor SRE (de Boer 2003), few, if any, would enable the bacteria to survive in a systemic infection that could lead to a disease development as shown in this study with *S. dulcamara*. These results highlight the potential of *S. dulcamara* and other Solanaceae weed plants as a reservoir for *D. solani* in the environment.

To date, only several reports have shown the presence of SRE on weeds growing in the vicinity of potato fields. Tsror et al. (2010) reported that *Cyperus rotundus* (nut grass) growing near potato fields in Israel may be latently infected by *D. solani* (Tsror et al. 2010). We have isolated *P. carotovorum* subsp. *carotovorum* from symptomless roots of *S. dulcamara* growing in a potato cultivation region in northern Poland (Fikowicz-Krosko et al. 2017). This indicates that weeds, especially if they grow near potato fields, may become a reservoir (alternative host) for *Pectobacterium* spp. and *Dickeya* spp. The sizes of SRE populations in and on weeds are usually small (~10 to 100 cells per plant as observed in our studies, unpublished data), but we can speculate that in the worst-case scenario, when a highly disease-susceptible potato cultivar was concerned and favorable weather conditions occur, this reservoir might be sufficient to support infection in potato crops.

The fact that *D. solani* does not cause disease development on *S. dulcamara* plants grown in potting compost is of rather minor importance. In contrast to the culture tube-grown plants, *S. dulcamara* grown in potting compost in a growth chamber setup failed to develop disease symptoms after being inoculated with 10^5 cfu per plant

Fig. 7. Population dynamics of *Dickeya solani* IPO2254 on adaxial surface of *Solanum dulcamara* leaves. In replicated experiments, 40 leaves (20 leaves per experiment) were sampled at four time points (0, 1, 7, and 14 days post inoculation [dpi]). At each sampling time, five leaves (third to sixth from the shoot terminal) were detached per plant, spray-inoculated with 2 ml of 10^8 cfu ml⁻¹ IPO2254 bacterial suspension in 1/4 Ringer's buffer, and incubated adaxial side up on 0.5% water agar in square plastic Petri dishes (100 × 100 mm). At 0, 1, 7, and 14 dpi, five randomly chosen leaves from five randomly chosen Petri dishes were collected and individually shaken in 20 ml Ringer's buffer in 50-ml Falcon tubes at 50 rpm at room temperature for 30 min to rinse bacterial cells off the leaf surface. The serially diluted washings were plated on tryptone soya agar plates supplemented with 100 µg ml⁻¹ ampicillin and incubated at 28°C for 24 to 48 h and GFP-positive, ampicillin-resistant bacterial colonies counted.

by stem puncturing. It is likely that the in vitro plants, due to the applied growth method, were more susceptible to bacterial infection (Pierik 1997), or simply the presence of saprophytic microflora prevented *D. solani* proliferation and reaching a threshold level for virulence expression (Charkowski 2006). Then again, no symptoms caused by pectinolytic bacteria on field-grown *S. dulcamara* and other weeds like *C. rotundus* were described and all reported isolations were done from symptomless plant tissues. Likewise, *D. dianthicola*, bacteria closely related to *D. solani*, was found to infect *S. dulcamara* under glasshouse conditions, but the observed symptoms were only minor (Elphinstone 2008). This may indicate that, although *Dickeya* spp. and *Pectobacterium* spp. may systemically infect common weeds including *S. dulcamara*, the expression of disease symptoms is altered for an unknown reason.

It can also be that the soil-grown *S. dulcamara* plants were cultivated under conditions not promoting *D. solani* infections. However, 40% of these symptomless plants were systemically infected with IPO2254 populations in stems of 10^1 to 10^4 cfu g⁻¹ plant tissue at 30 dpi. Similar populations of IPO2254 were found in stems of potato plants growing in soil infested with 10^8 cfu ml⁻¹ *D. solani* (Czajkowski et al. 2010b) or following an axial leaf inoculation with 10^8 cfu ml⁻¹ *P. carotovorum* subsp. *carotovorum* of plants grown under greenhouse conditions (Rahimian and Mitchell 1984).

Microscopy observations of in vitro *S. dulcamara* tissues inoculated with GFP-tagged *D. solani* showed the same colonization pattern as observed in IPO2254-inoculated potato plants (Czajkowski et al. 2010a, b). In both cases, IPO2254 colonized the root pith tissue and stem xylem vessels. Although the knowledge on *D. solani* colonization patterns of weeds (including *S. dulcamara*) is limited, we can assume that in general it would be similar as for most known plant-associated bacteria (Alfano and Collmer 1996; Romantschuk 1992). The systemic colonization of *S. dulcamara* under natural conditions remains largely unknown and requires further studies.

In conclusion, our results suggest that *S. dulcamara* has a potential to be an important reservoir for SRE bacteria in the environment, facilitating *Pectobacterium* spp. and *Dickeya* spp. survival, hence playing an important role in the blackleg epidemiology. Further investigations are needed to evaluate the risk of a *S. dulcamara*-potato cross infection with SRE bacteria.

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