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The relation between dose and infection incidence determined for *Pectobacterium brasiliense* spray-inoculated on potato haulms

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Abstract In field experiments in the Netherlands, risks were assessed for haulm and tuber infections of potato plants with a blackleg-causing strain of *Pectobacterium brasiliense* after spray-inoculation of haulms with the pathogen. For this, young potato plants (cv. Agria) grown from minitubers were inoculated with different densities (0, 10^1, 10^3, 10^5 or 10^8 cfu/ml) of a rifampicin resistant mutant of *P. brasiliense*. During inoculation, contamination of the soil with inoculum was avoided as much as possible. In an additional treatment, soil was inoculated directly around the plants with 10^8 cfu/ml. In 2021, a relatively rainy growing season, an inoculum density of 10^3 cfu/ml produced a significantly higher disease-incidence in haulms than found in the water-treated control. However, also in the haulms of the negative control, infections were found, probably due to dissemination of the pathogen with water in soil. All plants yielded infected tubers, also plants of the negative control. At an inoculum density of minimally 10^5 cfu/ml symptomatic plants were found. In 2022, a year with less rainfall during the growing season, only an inoculum density of 10^8 cfu/ml resulted in a significantly higher infection-incidence in the haulms than found in the negative control. In contrast, an inoculation density of 10 cfu/ml resulted in a significantly higher infection incidence of tubers than found in the water control. In 2022, symptomatic plants were only observed after inoculation of soil with 10^8 cfu/ml.

Keywords Rifampicin resistant mutant · Enrichment TaqMan assay · Blackleg · Risk assessment · Weather conditions

Introduction

Bacterial soft rot diseases, caused by *Dickeya* and *Pectobacterium* species, devastate a variety of important crops worldwide including potato, tomato, maize, cabbage, banana and ornamental plants (van der Wolf et al., 2021a). These genera were recently reclassified from the Enterobactericeae into the Pectobacteriaceae (Adeolu et al., 2016). In potato, soft rot Pectobacteriaceae (SRP) can cause soft rot during storage and blackleg during cropping. In particular blackleg causing SRP (BL-SRP) are responsible for economic damage due to downgrading, rejection of seed lots and loss of productivity (Czajkowski et al., 2011; Dupuis et al., 2021).

Infected seed tubers are the most important source responsible for blackleg (Czajkowski et al., 2011; Toth et al., 2021). To avoid blackleg diseases, potato

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cultivation starts therefore with the use of progeny tubers produced from *in vitro* propagated plantlets (minitubers) or tubers from clonal selection, which are free of BL-SRP (Velvis & Van der Wolf, 2008). Unfortunately, already in the first year of multiplication, infections can occur (Van der Wolf et al., 2022). If infections result in tuber maceration, a rapid spread of the pathogen within a seed lot can occur, in particular during mechanical harvesting, sorting and grading (Pérombelon & Kelman, 1980).

Several sources of infection have been identified responsible for initial infections of a pathogen-free seed lot. The presence of inoculum in soil cannot be excluded, but is unlikely as the pathogen cannot survive in soil for several years (Czajkowski et al., 2011). A more likely pathway for initial infections is via inoculum that is carried by rain, aerosols, insects, machines, furs, feather, or laborers (van der Wolf et al., 2021b). The inoculum may come in contact with haulms, after which wounds or natural openings, such as stomata or hydathodes, may serve as a port of entry to establish an infection (Kastelein et al., 2021). The inoculum may move down with water from haulms into soil after which infections of tubers can occur (Kastelein et al., 2021). In theory, migration of the pathogen from contaminated haulms can also result in root infections and consecutively in systemic infections of stolons and tubers (Czajkowski et al., 2010b; Kubheka et al., 2013). Inoculum carried through the air may also be deposited directly on the soil surface and translocated with water to infect roots and tubers. Finally, inoculum may be transported underground from infected plants to a pathogen-free crop, carried by water or by soil inhabitants, such as nematodes ((Pérombelon et al., 1987; Nykyri et al., 2014).

The aim of this study was to extend our knowledge on the risks of inoculum deposited on haulms, by establishing the relationship between inoculum dose and the incidence of infected or symptomatic plants. Dose–response relationships were already established for *P. brasiliense*, *P. atrosepticum* and *D. solani* after vacuum-infiltration of seed tubers with these pathogens (Van der Wolf et al., 2016). Even a low inoculum density of 1000 cfu per ml resulted in a high disease incidence (> 20%), irrespective of the species. In addition, dose–response studies upon spray inoculation of leaves were done under glasshouse conditions of *D. solani* and *P. parmentieri* (Kastelein et al., 2021). For *D. solani*, a low inoculum density of 100 cfu per ml resulted already in an effective colonization of intact leaves, but for *P. parmentieri* a 100-fold higher density was required to establish an infection.

In this study, field experiments were conducted, in which potato plants were spray-inoculated with different densities of a blackleg-causing strain of *P. brasiliense* (Clade I), a variant widely spread in the potato ecosystem in the Netherlands (Jonkheer et al., 2021). In 2014, *P. brasiliense* was described for the first time in the Netherlands, and is currently the dominant potato blackleg causing species (Nunes Leite et al., 2014; Van Duivenbode (NAK, Emmeloord, personal communication)). *Pectobacterium brasiliense* has a broad host range including important crops as potato, tomato, pepper, banana and cabbage (Oulghazi et al., 2021). Populations in potato show a high genetic diversity, but only a few haplotypes are able to cause blackleg (Jonkheer et al., 2021; Van Duivenbode (NAK, Emmeloord, personal communication)).

Materials and methods

Bacterial strains and growth conditions

P. brasiliense IPO3649 (Clade 1) was used in this study, while *Acidovorax cattleya* NBC430 (*Acat*) (Naktuinbouw, Roelofarendsveen, NL) (= IPO4006) was used as a extraction and amplification control in the multiplex TaqMan assays (van der Wolf et al., 2022). Briefly, a fixed amount of *Acat* cells was supplemented to each sample before DNA-extraction, such that use of an *Acat* specific TaqMan assay resulted in a Ct-value of approximately 30. For studies on population dynamics in field experiments a spontaneous rifampicin resistant mutant of *P. brasiliense* (IPO3649) was generated, i.e. *P. brasiliense* (IPO4211). Isolates were stored at − 80 °C on beads (Protect bacterial preservers; tscswabs.co.uk). Unless otherwise stated, bacteria were grown on tryptone soya agar (TSA; Oxoid) for 48–72 h at 25 °C. For the generation of rifampicin resistant mutants, Luria Bertani Agar medium (LBA, 5 g Yeast extract; 10 g Peptone; 10 g NaCl, pH 7.2) was used. Enrichment in leaves was done in Pectate Enrichment Broth (PEB, ($MgSO_4.7H_2O$, 0.3 g; $(NH_4)_2SO_4$, 1.0 g; $K_2HPO_4.3H_2O$, 1.31 g; polygalacturonic acid, sodium salt (Merck Life Science, Amsterdam, The

Netherlands), 1.5 g; in 1 L demineralized water; pH 7.2) supplemented with 50 µg/ml of rifampicin (PEBr) (Van der Wolf et al., 2022). For detection of *P. brasiliense* by dilution plating, samples, ten-fold serially diluted in Ringers solution (2.25 g/L NaCl, 0.105 g/L KCl, 0.12 g/L CaCl and 0.05 g/L Na_2CO_3), were spread-plated on double-layer crystal violet pectate (DL-CVP) medium (Helias et al., 2012) amended with 50 µg/ml of rifampicin. Plates were incubated for 4 days at 25 °C. Cavity-forming colonies, typical for the growth of SRP were counted.

Generation of a rifampicin resistant mutant of *P. brasiliense*

To create a spontaneous rifampicin resistant mutant of *P. brasiliense* strain IPO3649, 50 µl of an overnight culture of IPO3649 was plated on LBA supplemented with 50 µg/ml rifampicin. The mutation rate was about $2 * 10^{-6}$. Single colonies were transferred to LBA with 100 µg/ml of rifampicin. The selected mutant grew well on LBA with rifampicin, whereas the wild type strain did not.

Soft rot inducing ability of mutant strains

The mutant strain was compared with the wild-type strain for its soft rot inducing ability on potato tuber slices according to the procedure described by Czajkowski et al. (2010b) with modifications. Tubers (cv. Agria) were treated with chlorine (5% v/v), ethanol (70% v/v), washed with water, and dried in a flow cabinet. Tubers were evenly sliced (1 cm) and small wells were punched in the center of each slice after which 20 µl of a suspension of approximately 10^8 cells per ml ($OD_{600} = \sim 0.1$) of the mutant or of the parental strain was pipetted into the well. Inoculation with 0.01 M PBS ((8 g/l NaCl, 1 g/l KH_2PO_4, 14.5 g/l Na_2 HPO_4 $0.12H_2O$) was used as a control. Slices were placed on wetted paper and incubated for 4 days at room temperature in closed Petri dishes. The diameter of the rotten potato tissue was measured along two perpendicular lines and the mean diameter per tuber (in cm) was calculated ($N = 9$).

Blackleg causing ability of mutant strain

The ability of the mutant to cause blackleg was compared with the wild-type strain in a field assay as described by De Haan et al. (2008) with few modifications. Seed tubers of cultivar Agria were inoculated on 21 April 2021 with 10^6 cfu/ml of the pathogen suspended in water, or tubers were mock-inoculated with water. Tubers were submerged in the suspension in a milk bucket connected to a vacuum pump and left for 10 min under vacuum. After release of the vacuum, tubers were left in the suspension for 10 min more, followed by one day of drying in a ventilated open space. On 22 April 2021, tubers were planted in five randomized blocks of 20 tubers each block in an experimental field with sandy soil in Wageningen (NL). After emergence, plants were weekly observed for symptoms during a period of two months.

Field experiments

In in 2021 and in 2022, field experiments were conducted in Wageningen (NL) to study the relationship between dose and response (infection, symptom development) after spray-inoculation of haulms of young potato plants (cv Agria) with the rifampicin-resistant strain of *P. brasiliense*. Haulms of young potato plants (6 leaves stage) grown from minitubers were spray-inoculated with 50 ml of a suspension of different densities (0, 10^1, 10^3, 10^5, 10^8 cfu/ml) of a rifampicin resistant mutant of *P. brasiliense*. In an additional treatment, soil was inoculated directly around the plants with 10^8 cfu/ml. Experiments were performed in a randomized block design, with 5 blocks per treatment (Figures S1 and S2). The number of plants per treatment was dependent on the inoculum density: the higher the density, the lower the number of plants

Table 1 Treatments to determine the relation between dose of *Pectobacterium brasiliense* and infection incidence after spray-inoculation of haulms of young potato plants

Treatment	Inoculum density (cfu/ml)	Total nr of treated plants	Inoculation site
A	10^8	50	Haulms
B	10^5	100	Haulms
C	10^3	250	Haulms
D	10^1	250	Haulms
E	0	50	Haulms
I[a]	10^8	50	Soil

[a]As a control, inoculum was supplemented directly to the soil, near to the stem base of the plant (Treatment I)

(Table 1). We hypothesized that the infection incidence was positively related to the inoculum dose. Tubers were planted manually at a distance of 30 cm in the row and 75 cm between rows. The developing crop was sprayed with crop protection agents as in conventional seed potato crop cultivation. In 2021, tubers were planted on April 22nd and the inoculation was performed on June 8 th and in 2022 tubers were planted on April 26 th and inoculation was performed on June 2nd. In the first year, a back sprayer with pressure was used to spray in 3 s approximately 50 ml of bacterial suspension. To minimize contamination of soil during inoculation, two plastic trays (53 X 32 cm) (Modiform No. 2057, size 50 ×30 cm, Leusden, NL) were placed around the stem base to cover the soil; each tray was incised such that they could be wrapped around the stems leaving a minimum area of soil uncovered. Despite our efforts, inoculation liquid ran from stems resulting in some contamination of soil. To further limit soil contamination, in 2022, the inoculum volume was reduced to approximately 10 ml, but the densities increased for each treatment with a factor 5 to end up with a similar inoculation dose per plant. In addition, we used a manual sprayer allowing a more focused inoculation. Leaf and tuber samples were collected between 9 and 19 July in 2021 (78–88 dpi) and between 13 and 25 th of July in 2022 (78–91 dpi). Two samples per plant were collected separately, i.e., a composite sample of three aging leaves low in the canopy, and a composite sample of all progeny tubers per plant (manually harvested). Pruning shears were disinfected with 70% ethanol between the sampling of each plant to prevent cross-contamination. The leaves were transferred to labelled extraction bags (Bioreba, Reinach, Switzerland) suitable for extract preparation using a mechanical crusher. Per plant, up to 10 tubers were collected and collectively placed with the corresponding label in a plastic bag. Samples were stored in the cold room (4 °C) during the night to be processed the next days.

In 2021, soil samples (200 g per sample, close to and around the stems) were collected directly after inoculation on the 22nd of April, on the 9 th of June and on the 19 th of July. Ten samples (2 per block) from the soil inoculated treatment were analyzed using dilution plating on DL-CVP with rifampicin.

Sample processing

In 2021, soil samples were manually homogenized in a plastic bag. Five grams of soil were suspended in 45 ml of sterile Ringers supplemented with 0.02% diethanol-dithiocarbamate (DIECA) in a 50 ml tube containing sterile small stones. Tubes were placed in a paint shaker (Minimix Auto, Merris Engineering Ltd, Ireland) and soil was homogenized for 90 s, after which 50 µl of the undiluted, 10 times, 10^2 times, 10^3 times and 10^4 times diluted extract in Ringers was dilution plated (in duplicate) on DL-CVP with rifampicin. Colonies were counted after 2 day incubation at 25 °C.

Leaves were weighed and crushed with a sample crusher (AAA LAB equipment B.V. Roelofarendsveen, NL). Ringer solution supplemented with 0.02% DIECA was added to the Bioreba bag in double the amount of the weight of the leaves and the sample was further crushed. For detection with TaqMan assays without prior enrichment, one ml of the leaf extract was transferred to a 1.2 ml collection tube of a 8-well strip placed in a 96-well microtube rack (Qiagen, 19560). Ninety-six-well racks were centrifuged for 15 min at 6000 rpm and the supernatant was discarded. After sealing the tubes with 8-cap strips, plates were stored in the freezer until the DNA extraction was performed. For detection with prior enrichment, another one ml of leaf extract was transferred to a 12 ml sterile tube (Greiner Bio-One, 164162) already containing 9 ml of PEB-r. After gentle mixing, these samples were incubated for 3 days at 21 °C still standing. After homogenization of the samples, one ml of the enriched leaf extract was directly pipetted in a 1.2 ml collection tube of a 8-well strip placed in a 96-well microtube rack. The 96-well racks were centrifuged, sealed and stored as described for the detection method without prior enrichment.

Per plant, eight tubers were washed and placed in a plastic bag suitable for vacuum (LDPE bags, transparent, 0.10 mm, 300 ×500 mm, VPP Packaging B.V., Bussum, NL). Per tuber, 5 ml of water was added, supplemented with 100 µg/ml rifampicin. Bags were vacuumed and incubated for 5 days at 25 °C to allow multiplication of the bacteria. One ml of the fluid that leaked from the tubers was collected in tubes as for the leaves, centrifuged for 15 min at 6000 rpm and the pellet was frozen at − 20 °C. As a control, a leaf extract and a sample from 10 tubers from a

plant selected of mock-inoculated fields, and assumed free of *P. brasiliense,* were supplemented with the mutant IPO4211 or water. For the method without prior enrichment a ten-fold serial dilution of 10^1 to 10^5 cfu/ml was used and for the enrichment method densities of 10^2 and 10^3 cfu/ml were used.

Pathogen detection

For detection of *P. brasiliense* in plant material, a (quantitative) TaqMan assay was used and an enrichment TaqMan assay, to detect low concentrations of the pathogen. Prior to the DNA extraction, all samples were supplemented with *Acidovorax cattleya* (*Acat*) to support control of extraction and amplification as described by Van der Wolf et al. (2022). The internal control was prepared by diluting 10 µL of an *Acat* cell suspension with an $OD_{600}$of 0.8 in 10 mL 0.01 M PBS To each sample, a chrome steel ball of 3.2 mm diameter was added as well as the lysis buffer supplemented with 1 µg RNase A and 50 µL of the internal extraction control. The samples were homogenized using the Tissuelyser II Qiagen twice for 20 s at 20 Hz, the plate being inverted between the 2 bead-beating steps. DNA extraction was performed using the DNA extraction sbeadex maxi plant kit (NAP41620, LGC Genomic GMGH, Berlin, Germany) according to the manufacturer's instructions in combination with the KingFisher™ Flex Purification System (Automata Technologies, London, UK). The sample was eluted in 100 µL of elution buffer PN from the sbeadex maxi plant kit.

For the TaqMan assay, *P. brasiliense* primers and probe were used, targeting *araC*, an arabinose- responsive transcriptional regulator, as described by Van der Wolf et al. (2017a). The reaction mix of 15 µL consisted of 3 µl of low ROX enzyme mix (95149 -250, QuantaBio), 0.18 µl of 10 µM PcbrFw primer (5'-TGC GGGTTCTGCGTTTC- 3'), 0.45 µl of 10 µM PcbrRv primer (5'TGGCGCGTTCGCAATAT- 3'), 0.15 µl of PcbrP labelled with FAM (5'-CAAGGCACGATA CG- 3'), 0.45 µl of 10 µM *Acat* 2-F primer (3'-TGT AGCGATCCTTCACAAG- 5'), 0.45 µl of 10 µM *Acat* 2-R primer (3'-TGTCGATAGATGCTCACAAT- 5'), 0.15 µl of 10 µM *Acat* 2-P probe (5'-CTTGCTCTG CTTCTCTATCACG- 3') labelled with HEX, 8.17 µl of purified water and 2 µl of sample. The amplification was performed in the QuantStudio (Applied Biosystems, Waltham, MA, USA) with the following program: after a hold of 2 min at 95 °C, the amplification took place during 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Samples were considered positive at a threshold level (Ct-value) lower than 35. In case, TaqMan results were negative for both *P. brasiliense* and *Acat*, results were omitted from the data set.

Pathogen densities were estimated on the basis of a calibration curve prepared with a ten-fold diluted gBlock comprising the target sequence in water (Figure S4). The sequence of the gBlock used was 5'-TGC TGAGCCTGATAATGGCGCTCGCCCGCGCTG GCACCGAACGTCGTCATCGTGACATGCTGC TGGCGAAACCCGATCGATGAGCCGCCGCAA TCGTAATAAGCAGACTCCCCTGCCAGTGCA AATGTCATGATCAGCATCGGCTGCGAATGC GGGTTCTGCGTTTCTTCCACCAAGGCACGATAC GGGCGATATTGCGAACGCGCCAGCGACAGA TCCTCATCAATACGGCTGAAATCCGACCAA CACTCACCGATGTTGCCTGGCAGTATTCGG CGCGAATCAGAACGATTCACCTCGCCCATC CGGCACCGGTCACCTGACGGTAGTCCCTCT CGCACCCGTTGCTGTCTTTGCG- 3'.

Statistics

The incidence (*I*) of infected tubers in composite samples was estimated using the statistical equation: I= {1 – [(N – p)/N]1/n}* 100, in which N is the total number of subsamples tested, p is the number of subsamples tested positive in the TaqMan assay, and n is the number of individuals per subsample (De Boer, 2002). For analyzing the mean of the diameters of the maceration zones in the maceration assay and the incidence of infected and symptomatic plants, analysis of variance was performed using Genstat (VSN International, 2015. Genstat for Windows 18 th Edition. VSN International, Hemel Hempstead, UK. Web page: Genstat.co.uk.). Fisher's Least Significant Difference (LSD) was used as post hoc test ($P = 0.05$).The area under disease progress curve (AUDPC) was calculated using the method described by Praschivu et al. (2013)).

Results

Virulence rifampicin resistant strain

The maceration capacity of the rifampicin resistant strain of *P. brasiliense* IPO4211 did not significantly

differ from its parental wild-type strain IPO3649 in a potato disk maceration assay (Table S1). The mean e diameters of the zone of decay for IPO3659 and IPO4211 were 1.91 and 2.01 cm, respectively. In 2021, a field experiment was conducted to compare the virulence of the two strains using vacuum-infiltrated tubers. The disease incidence of the rifampicin resistant strain at the last day of observation (20 days after inoculation) was 62%, significantly less than the wild-type strain (88%) (LSD = 2.44 at a P-value of0.05) (Fig. 1). The area under the disease progression curve for the wild-type strain was 809 compared with 366 for the mutant. The mock-inoculated tubers did not yield symptomatic plants. Although the mutant was less virulent than the parental strain, the rifampicin resistant mutant was used in follow-up experiments. Symptom expression with the mutant was still considerable and due to the high risk for influx of (wild-type) *P. brasiliense* from the environment in open field experiments, which could interfere with our trials, the use of a marked strain was considered as essential. After all, the influx of *P. brasiliense* may result in an overestimation of the infection incidences due to spray-inoculation of haulms.

Dose response studies

In 2021 and 2022, field experiments were conducted in Wageningen (NL) to determine the relationship between the inoculum density of *P. brasiliense* and the risks for infection of leaves and tubers after spray-inoculation of haulms with *P. brasiliense*. The growing season of 2021 (1 May till 15 July) was relatively wet with a total precipitation of 2036 mm in the period between planting and sampling as measured at weather station Veenkampen located approximately three kilometers from the experimental field. The growing season of 2022 was relatively dry with a total precipitation of 1715 mm. In 2021, the average temperature till the first half of May was relatively low, but thereafter average temperatures in 2021 and 2022 followed the same trend. In Figure S3, details on the average precipitation and temperature in the two seasons are provided.

In 2021, a positive correlation was found between the inoculum density and the infection incidence of the haulms. Using the enrichment TaqMan assay, at high inoculum densities of 10^8 or 10^5 cfu/ml, leaf infections were found in all plants tested (Fig. 2A). At an inoculum density of 10^1 cfu/ml the incidence dropped significantly to 70%, but even in the water control around 50% of plants were infected. Also in the TaqMan assay without prior enrichment, a dose–response relation was found, with 87% samples positive using an inoculum density of 10^8 cfu/ml and 43% at 10^1 cfu/ml. In this analysis 40% of the mock-inoculated plants were positive (Fig. 2B). Almost all tuber samples were positive in the enrichment TaqMan assays, including 100% of the tuber samples from the mock-inoculated plants (Fig. 2C). Symptomatic plants, showing a reduced growth, wilting, upward folded top leaves with a dark grey

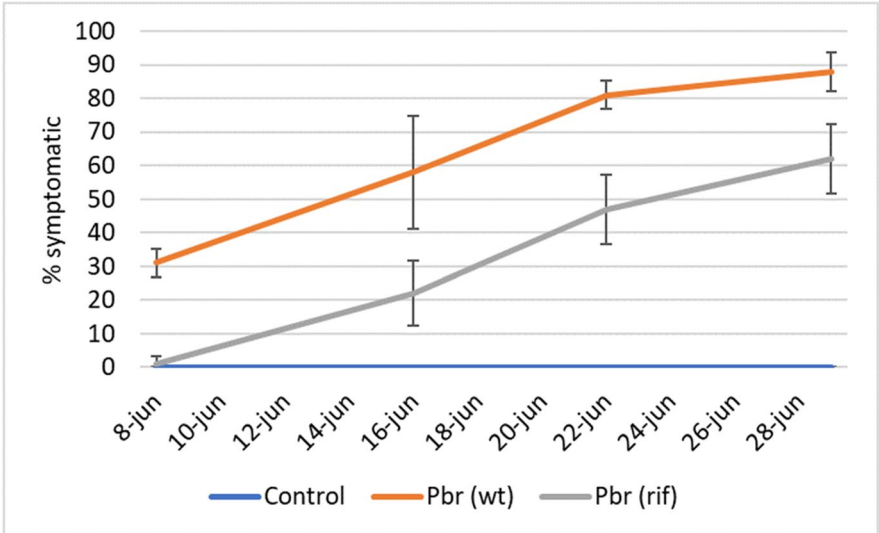

Fig. 1 Disease development during the growing season of 2021 for seed potatoes vacuum-infiltrated with a wild-type (wt) strain of *Pectobacterium brasiliense* (IPO3649) and its spontaneous rifampicin resistant mutant (IPO4211). Seed potatoes were planted in a sandy soil. Vertical lines indicate the standard deviations

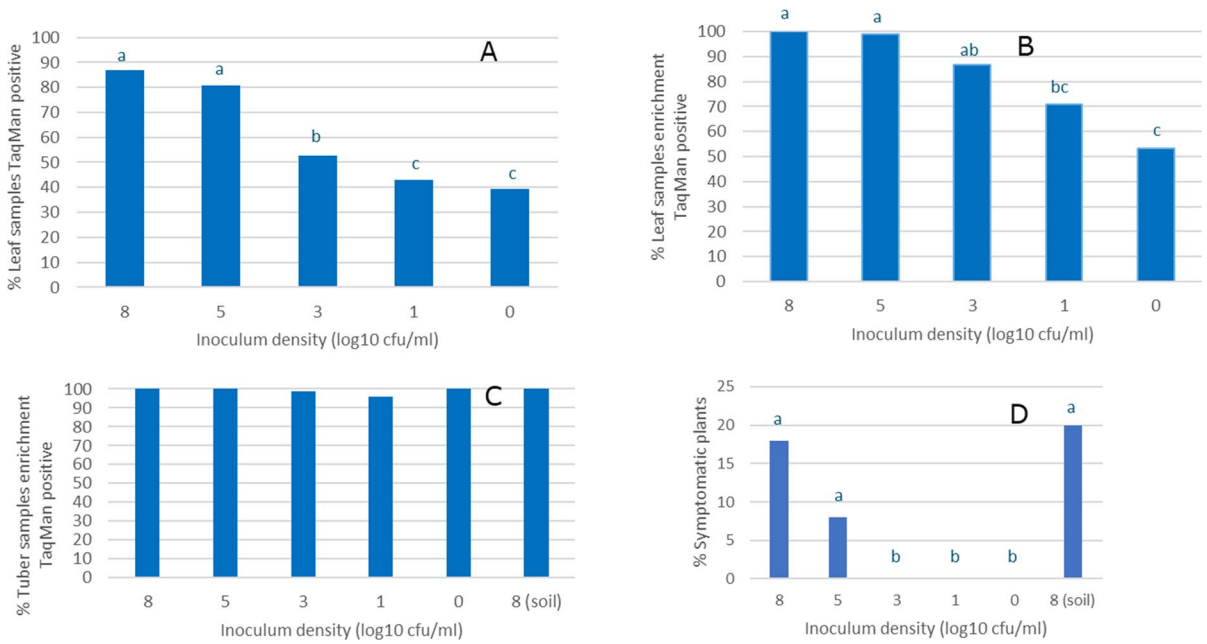

Fig. 2 Dose-response studies in 2021 to determine the relation between inoculum density and infection- and disease-incidence. Haulms of young plants growing in sandy soil in Wageningen (NL) were spray-inoculated with 50 ml of different densities of a rifampicin-resistant mutant of *Pectobacterium brasiliense,* or soil was inoculated around plants with a density of 10^8 cells/ml (8 (soil)). **A** Percentage of haulms positive in a TaqMan assay (LSD = 27.1), **B** Percentage of haulms positive in an enrichment TaqMan assay (LSD = 30.3), **C** Percentage of daughter tubers positive in an enrichment TaqMan assay (percentages were not significantly different), **D** Percentage of blackleg-diseased plants (LSD = 13.8). Percentages with an identical letter did not significantly differ ($P = 0.05$)

discoloration were only found at the two highest inoculum densities of 10^8 and 10^5 cfu/ml, with incidences of respectively 18 and 8% (Fig. 2D).

Concentrations in leaf extracts were estimated on the basis of a calibration line, generated by testing a ten-fold serial dilution of the gBlock in the TaqMan assay for *P. brasiliense* (Figure S4). The mean concentrations in leaves were estimated between 2520 cfu/ml for the highest inoculum dose and 400 cfu/g for the mock-inoculated plants (Table 2). For the highest inoculum dose of 10^8 cfu/ml, estimated concentrations in the leaf extracts ranged between $2 * 10^2$ and $2 * 10^6$ cfu per gram of leaf material, while in mock-inoculated plants the estimated concentrations ranged between 60 and 5000 cfu per gram of leaf material (Table 2).

In 2021, the estimated infection incidence of the individual tubers analyzed as composite samples of all tubers per plant ranged from 33.1% for an inoculum density of 10^1 cells/ml of ≥ 43.8% for a density of 10^5 cells/ml and ≥ 38.7% for a density of 10^8 cells/

Table 2 Concentrations of *Pectobacterium brasiliense* in potato leaf samples (in cfu/g) at the end of the growing season of 2021 after spray-inoculation of haulms of young plants with the pathogen in different densities

	TaqMan positive samples (Ct-values)[1]		Estimated density (log10 cfu/g)[2]	
Initial density (^{10}log cfu/ml)	Mean	Range	Mean	Range
8	29.5	19–33.2	3.4	2.3–6.4
5	29.4	21.8–34.9	3.4	1.8–5.6
3	30.3	22.3–34.9	3.1	1.8–5.5
1	30.1	22.7–34.9	3.2	1.8–5.5
0	32.0	28.3–34.8	2.6	1.8–3.7

[1] Ct-values lower than 35 were considered positive

[2] Densities were estimated on the basis of a calibration curve constructed using a ten-fold serial dilution of a gBlock (synthetic DNA)

ml. However, also the estimated incidence of the water-inoculated control was ≥38.7%, and therefore no correlation could be found between inoculum density and infection incidence (Table S2). In 2022, the estimated infection incidence of the individual tubers analyzed as composite tubers decreased from higher than 38.7% at 10^8 cfu/ml to 10.7% at the 10^1 cfu/ml, while the incidence of the water control was only 2.5% (Table S2). After soil inoculation, an incidence of higher than 38.7% was found.

After soil inoculation, 20% of the plants became symptomatic (Fig. 2D). The infection incidence and densities of *P. brasiliense* in soil were high one day after soil inoculation (90%, $7 * 10^3$ cfu/g) and decreased in the first week (60%, $8 * 10^1$ cfu/g), but were high again at 55 days after inoculation (90%, $7 * 10^3$ cfu/g) (Fig. 3). Soil sampled around the stem base of 10 plants from which haulms were spray-inoculated with 10^8 cfu/ml, was also infested at one day after inoculation. All soil samples were positive in the enrichment TaqMan assay with an mean concentration of $4 * 10^3$ cfu/g (range $4 * 10^1$ – $3 * 10^4$ cfu/g). This indicated that soil became infested during haulm inoculations, despite our efforts to avoid contamination.

In 2022 infection rates were lower. Unintentionally, seed potatoes, heavily infected with a wild-type strain of *P. brasiliense,* were planted in border rows to separate plots,, resulting in a high level of blackleg diseased plants. As a consequence, our target crop became (co)-infected due to cross-contaminations. On the 4th of June, blackleg diseased plants from the border crop were removed together with their mother tubers, but at that time dissemination of the pathogen already happened. Based on the results in the TaqMan assay without prior enrichment, only spray-inoculated plants inoculated with 10^8 cfu/ml showed a significantly higher infection incidence (85%) than all other treatments including the water control (Fig. 4A). The infection incidence of the other treatments were not significantly different from each other and varied between 11 and 43%. Similar results were found with the enrichment TaqMan assay, but for all treatments, a lower disease incidence was found than in the assay without prior enrichment, except for the treatment with 10^8 cfu/ml (Fig. 4B). Spray-inoculation of plants with 10^8 cfu/ml resulted in 100% infected tuber samples (Fig. 4C). The infection incidence decreased significantly with a decreasing inoculum density to 60% for inoculation with 10 cfu/ml. Only 18% of the mock-inoculated tuber samples were positive in the enrichment TaqMan assay. In 2022, none of the spray-inoculated plants became symptomatic, whereas 4% of the plants of the soil-inoculated treatment showed blackleg. After soil treatment with 10^8 cfu/ml, all tuber samples were positive in the enrichment TaqMan assay, while the infection incidence in leaf extracts did not exceed those of the mock-inoculated plants.

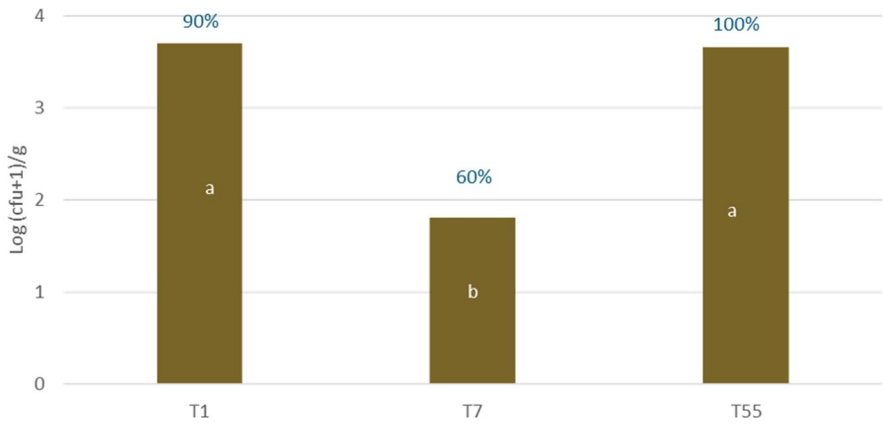

Fig. 3 Mean concentration of a rifampicin resistant *Pectobacterium brasiliense* strain in soil, after soil inoculation around the stem base of potato plants, at 1, 7 and 55 days post inoculation in a field experiment conducted in 2021. Above the bars the percentages of samples positive in a dilution plating assay are provided ($N = 10$). Values with an identical letter did not significantly differ ($P = 0.05$)

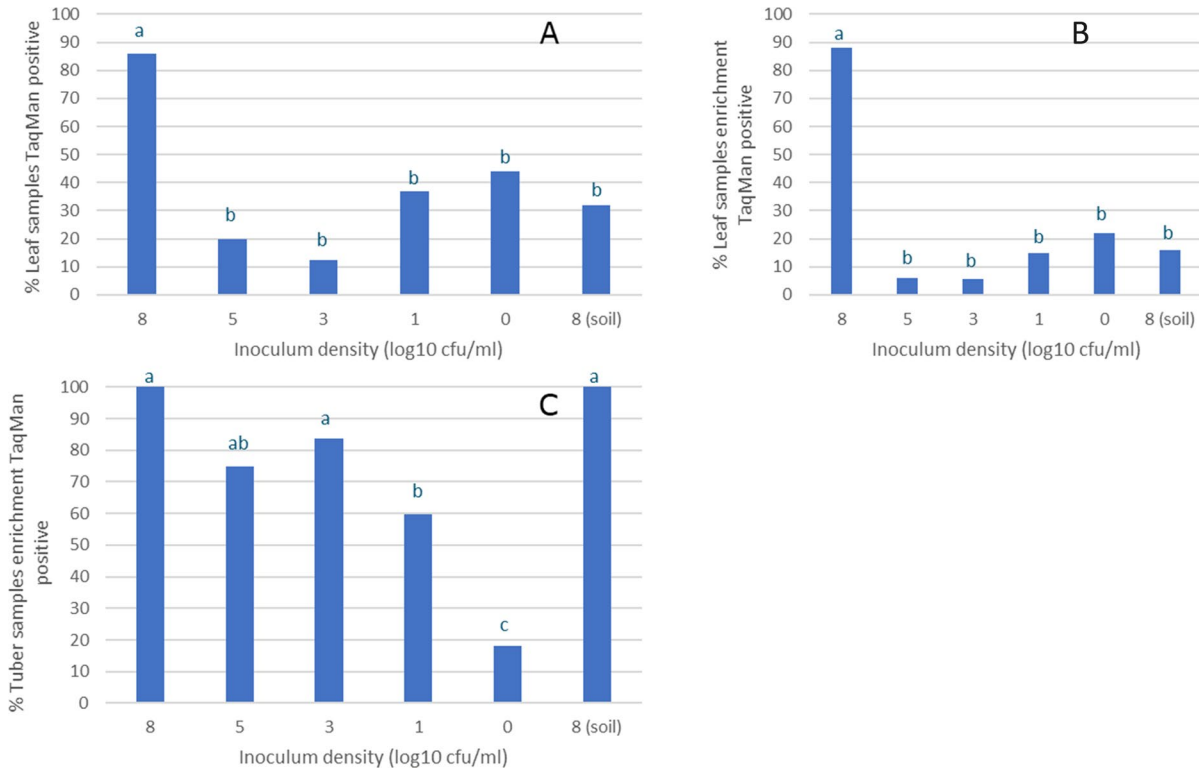

Fig. 4 Dose-response studies in 2022 to determine the relation between inoculum density and infection-incidence. Haulms of young plants growing in sandy soil in Wageningen (NL) were spray-inoculated with 50 ml of different densities of a rifampicin-resistant mutant of *Pectobacterium brasiliense,* or soil was inoculated around plants with a density of 10^8 cells/ml (8 (soil)). **A** Percentage of haulms positive in a direct TaqMan assay (LSD = 36.0), **B** Percentage of haulms positive in an enrichment TaqMan assay (LSD = 22.6), **C** Percentage of daughter tubers positive in an enrichment TaqMan assay (values were not significantly different). Plants did not develop symptoms except for 4% of plants after soil-inoculation (LSD = 26.8). Values with an identical letter did not significantly differ ($P = 0.05$)

Estimated mean concentrations in leaf extracts were largely similar for all treatments (Table 3). The maximum concentration in the leaf extract of an individual plant was estimated at a level of 10^7 cells per gram.

Discussion

In two-years field experiments, the risks for infection of potato plants was studied by spray-inoculation of haulms with different densities of *P. brasiliense*, a blackleg causing species, highly prevalent in the Netherlands (van der Wolf et al., 2021a). These studies are particularly of interest to assess the risks for initial infections of a high grade (pre-basic) seed potato crop. It is assumed that inoculum responsible for initial infections is mainly carried through the air, such as by insects, aerosols, rain water, machines, feathers, fur and clothes (Toth et al., 2021). It is expected that in general the inoculum dose deposited on haulms will be low.

The two experiments were conducted in a similar sandy soil in the Netherlands, using the same susceptible cultivar (Agria), the same rifampicin resistant bacterial strain in a similar series of densities, but results of the dose–response studies were different. Risks for infections of leaves after spray-inoculation of haulms seem to be particularly high during a rainy growing season. In 2021, a year with a relatively high level of precipitation, the incidence of leaf infections exceeded background levels already at a density of 10^3 cells per ml, whereas in 2022 an inoculum density of 10^8 cells per ml was required. In 2021, tubers

Table 3 Concentrations of *Pectobacterium brasiliense* in potato leaf samples (in cfu/g) at the end of the growing season of 2022 after spray-inoculation of haulms of young plants with the pathogen in different pathogen concentrations

	TaqMan positive samples (Ct-values)[1]		Estimated density (log10 cfu/g)[1]	
Initial density (^{10}log cfu/ml)	Avg	Range	Avg	Range
8	31.7	24.6–34.8	2.7	1.8–4.8
5	31.6	23.4–34.6	2.7	1.9–5.1
3	32.4	21.4- 34.8	2.5	1.8–5.7
1	31.0	16.8- 34.8	2.9	1.8–7.1
0	30.4	21.3–34.9	3.1	1.8–5.7

[1] Ct-values lower than 35 were considered positive

[2] Densities were estimated on the basis of a calibration curve constructed using a ten-fold serial dilution of a gBlock (synthetic DNA)

of all plants were infected, whereas in 2022, the incidence of tuber infections was density dependent. Nevertheless, in 2022 even at an inoculum density of only 10 cells per ml, tuber infection incidences were found exceeding the level of the mock-inoculated treatment. In general, after spray-inoculation of haulms, even at a low inoculum dose, the risks for tuber-infections is considered high, irrespective the growing season. This implicates that even at a low source strength, as will be the case during transmission of bacteria by contaminated insects, there is a high risk for tuber infections.

Insects are contaminated with a low number of SRP cells per insect, typically between 10 and 10.000 cells, from which part of the population may be non-viable, and only part of it will be deposited during contact with haulms (Rossmann et al., 2018). Also in other potential infection sources in the potato-ecosystem, such as in surface water, a low contamination level was detected of up to 10^2 cfu per ml (Jorge & Harrison, 1986). It is likely that haulms of a PB1 plant is often challenged with relatively low levels of living SRP cells.

Unfortunately, results of the 2022 experiments were affected by the use of seed tubers in the border rows, heavily contaminated with a wild-type strain of *P. brasiliense*. The higher incidences of infected haulms found with the TaqMan (Fig. 4A) compared with those of the enrichment TaqMan (Fig. 4B) can be explained by the fact that the wild-type strain of *P. brasiliense,* did not grow during incubation in PEB-r, whereas samples in the enrichment assay were diluted 10 times more than in the assay without prior enrichment.

In both years, plants of the water-treated controls became infected with the rifampicin resistant mutant of *Pectobacterium brasiliense.* In 2021, the plots of the controls were located at minimally 1.5 m from inoculated plots and in 2022 at 2.1 m, which seems insufficient to avoid cross-contaminations. The high incidence of tuber infections in the water control points to a dominant role for inoculum moving via soil water from the inoculated plants to the water-treated controls.

The concentration of *P. brasiliense* in leaf extracts of inoculated plants could be high, even at a low inoculation density. In 2021, an inoculum density of 10 cfu/ml resulted in an estimated maximum concentration of $6 * 10^5$ cfu/g, and in 2022 in $2.4 * 10^7$ cfu/g. It should be noted that the concentrations were estimated using a calibration curve of gBlocks diluted in water. A measurement of synthetic DNA in water will not be affected by a potentially incomplete DNA-extraction or by the presence of DNA-amplification inhibiting compounds. Therefore, the densities in the leaf extracts may have been underestimated.

The risks for plant infections with BL-SRP will not only depend on weather conditions (Perombelon et al., 1987; Czajkowski et al., 2011), but also on the aggressiveness of the strain (De Boer et al., 2012; de Haan et al., 2008), the susceptibility of the potato (Pasco et al., 2006; Yahiaoui-Zaidi et al., 2010), the microbiome (Kurm et al., 2024) and interactions with other pathogens (Rahimian & Mitchell, 1984b; Tsror & Hazanovsky, 2001) as have been shown for stem-inoculated potato plants (Rahimian & Mitchell, 1984a).

In former glasshouse studies, we demonstrated that after leaf inoculation, *P. parmentieri* and *D. solani* can enter hydathodes and stomata, and move into the vascular tissues to stem and even tubers (Kastelein et al., 2021; Czajkowski et al., 2010a). In a similar way, leaf and tuber infections may have occurred via systemic translocations of bacteria from leaves via stems to roots and tubers during our field experiments. Alternatively, bacteria may also leak from the surface of infected haulms with water to soil, thus causing lenticel infections of tubers (Fox et al., 1971;

Nielsen, 1978). Soil infestations may also result in root infections. In glasshouse experiments, using strains tagged with a fluorescent marker, root infections have been evidenced both for *D. solani* and *P. brasiliense* (Czajkowski et al., 2010b; Kubheka et al., 2013). In other studies, in which rifampicin resistant mutants of *P. carotovorum* and *P. atrosepticum* were used, it was found that under field conditions the populations of these bacteria declined rapidly and only low levels on the leaf surface persisted during the growing season (Burgess et al., 1994; Elphinstone and Pérombelon, 1986). It was suggested that the numbers that remain on the leaf surface were insufficient to contaminate progeny tubers. Only late in season, when populations increased in deteriorating leaves and leaf debris, a risk for infection of progeny tubers was found (Elphinstone & Pérombelon, 1985). We therefore assume that the risk for tuber contamination is relatively high at the end of the growing season.

During spray-inoculation of haulms, contamination of soil with the bacteria could not be entirely excluded. Already during the spray-inoculation bacteria may have dropped down and leaked into soil, but this may also have occurred during rain. In 2021, soil directly inoculated with high densities of *P. brasiliense* on the soil around young plants resulted not only in a high incidence of tuber infections, but also in a high incidence of leaf infections and a high percentage of symptomatic plants. In inoculated soil, population densities decreased in the first week, but later increased again, probably due to a leakage of inoculum from the underground parts of infected plants in soil, in particular from symptomatic plants. The dissemination of inoculum from symptomatic plants to neighboring plants via water in soil likely explains the high incidence of tuber infections in 2021.

Symptom expression is influenced both by weather conditions and inoculum densities. In 2021, spray-inoculation of haulms resulted in symptomatic plants at high densities of minimally 10^5 cfu/ml, whereas in 2022 no symptoms were observed after haulm inoculation. In field studies in 2013 and 2014, using seed tubers vacuum-infiltrated with *P. brasiliense* or *D. solani*, a positive correlation between inoculum dose and the incidence of symptomatic plants was found (Van der Wolf et al., 2017). In these studies, even a low dose (10^3 cfu/ml) resulted in a high infection incidence, indicating the aggressiveness of both pathogens.

To monitor inoculated bacteria, studies were conducted with a spontaneous rifampicin-resistant strain of *P. brasiliense*. In former studies with wild type strains, frequently a high background of the pathogen endemically present in the environment impacted the results in inoculated field plots (unpublished results). Use of the rifampicin-resistant strain will largely exclude interference by wild-type strains of *P. brasiliense* present in the potato ecosystem. The presence of rifampicin-resistant mutants of *P. brasiliense* in the potato-ecosystem is unlikely as it was shown that these mutants have a reduced fitness. In addition it is known that the *in vitro* mutation frequency resulting in rifampicin resistance is low and ranges from 10^{-10} to 10^{-6}, depending on the organism and the methodology used (Goldstein, 2014). Moreover, in our study, most samples were from non-symptomatic plants yielding low densities ($< 10^7$ cells/g) of the pathogen.

According to the evaluation in the field experiment, the mutant was virulent, although less than the parental wild-type strain. Rifampicin resistance is nearly always due to mutations in the β subunit of bacterial RNA polymerase (Goldstein, 2014). As for *P. brasiliense*, rifampicin-resistance has been found to affect the fitness for a number of other bacteria (Enne et al., 2004) (Hughes & Brandis, 2013; Wi et al., 2018). For future experiments, the use of another antibiotic resistant marker may be considered with a lower impact on virulence, but rifampicin is a relative strong inhibitor of background bacteria in or on growing media.

We conclude that during a dry season, the risks for haulm infections is low, but tuber infections can even occur after a low inoculum dose is deposited on haulms. We assume that tuber infections are predominantly caused by inoculum washed off from haulms during rain, after which bacterial cells migrate via the soil to the progeny tubers. During a growing season with a high level of precipitation the risks for both haulm and tuber infections are high, also after contact of haulms with a low inoculum dose.

The widespread presence of BL-SRP in the potato ecosystem, in combination with a high risk for infections, even at a low inoculum dose, makes it difficult if not impossible to exclude infections. A management strategy should therefore be focused on reducing blackleg development by using less susceptible

cultivars (Zeng et al., 2019), or seed lots with a higher level of suppressiveness (Kurm et al., 2024). It further implies that dissemination of the pathogen within or between seed lots should be reduced via hygiene and appropriate cultivation measures (Van der Wolf et al., 2021b).

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflicts of interest The authors declare no conflict of interest.

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