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Surveys to identify sources of blackleg-causing soft rot Pectobacteriaceae responsible for initial infection of potato crops

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Abstract Surveys were conducted at two locations in the Netherlands during the growing seasons of 2022 and 2023, to identify sources of blackleg-causing soft rot Pectobacteriaceae (BL-SRP) responsible for initial infections of potato crops. For this, a TaqMan assay for a *Pectobacterium brasiliense* variant was developed, to allow detection of all BL-SRP variants prevalent in the Netherlands. Leaves of second and third generation prebasic (PB2, PB3) and ware potato crops located near PB1 (minituber) crops (cv. Agria) were analyzed, as well as soil, weeds, flying insects and rainwater. BL-SRP variants detected in these field samples were compared with those detected in PB1 crops using (enrichment) TaqMan assays. Depending on location and year, the first BL-SRP infections in PB2 and PB3 crops were found early in the growing season and were of the same BL-SRP variants later detected in the PB1 crops. Soil samples were negative for BL-SRP. One hundred weeds were analyzed per location, of which some were positive for BL-SRP, but only those later in the growing season. BL-SRP contamination of insects was already detected early in the growing season. The percentage of positive insects varied between 1.6 and 7.8% depending on location and year. Contaminated insects belonged predominantly to the families Chrysopidae and Anthomyiidae. Rainwater sampled at different locations in the Netherlands were all negative for BL-SRP. We hypothesize that later generation potato crops located nearby are the main source of initial infection of PB1 crops with BL-SRP. Insects may play a role in the transmission of BL-SRP.

Keywords *Pectobacterium brasiliense* · *Pectobacterium parmentieri* · Enrichment TaqMan assays · Initial infection · Insects

Introduction

Soft rot Pectobacteriaceae (SRP) are comprised of the genera *Dickeya* and *Pectobacterium*, which are responsible for diseases in various important crops worldwide including potato, tomato, maize, cabbage, and ornamental plants (Van der Wolf et al., 2021a). In potato, SRP causes soft rot, blackleg and slow wilt diseases, resulting in yield loss, and the downgrading or rejection of seed lots (Toth et al., 2021).

During the last 50 years, blackleg in Europe was caused primarily by *D. dianthicola, D. solani, P.*

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atrosepticum, *P. brasiliense* and *P. parmentieri,* (Van der Wolf et al., 2021a). During the last decade in the Netherlands, specific genotypes (clades) of *P. brasiliense* (Jonkheer et al., 2021) caused more than 90% of potato blackleg in the Netherlands (Van Duivenbode, NAK, Emmeloord, the Netherlands, personal communication). *P. parmentieri* was only occasionally present, while *P. atrosepticum* and *Dickeya* species, the primary blackleg pathogens prior to 2010, were rarely detected. Prior to 2022, Clade 1 of *P. brasiliense*, a genetically homogeneous genotype, was mainly responsible for the disease, but in 2023 a second clade (Clade 2) of *P. brasiliense* emerged, which mainly comprised blackleg causing strains (Pardeshi et al., 2025; Van Duivenbode, NAK, Emmeloord, the Netherlands, personal communication).

Seed potatoes have been recognized as the most important inoculum source for SRP disease development (Czajkowski et al., 2011; Toth et al., 2021). However, seed potato cultivation starts with the use of minitubers or tubers from clonal selection (PB1 crop), free from blackleg causing SRP (BL-SRP). Nevertheless, in the first year of tuber multiplication in the field, infections with BL-SRP can occur. Depending on location and season, the incidence of infection was up to 8% and 39% for leaf and tuber samples, respectively (Van der Wolf et al., 2022). The relative contribution of different inoculum sources responsible for such early infection of field-grown plants is still largely unknown.

Infections may start from soilborne inoculum, but several studies indicated that SRP populations rapidly decline in soil (Toth et al., 2021). It is, however, difficult to conclusively exclude soil as a source of infection. SRP has been detected in fields in the absence of potato, which suggests that SRP may be endemic in soil (Perombelon & Hyman, 1989). In addition, detection of low SRP populations in soil in the presence of high background bacterial populations remains a challenge. Moreover, a role for nematodes, soilborne arthropods and molluscs, in which SRP may survive for longer periods than free in soil, cannot be excluded (Joynson et al., 2017; Nykyri et al., 2014).

A more likely pathway for initial infections is inoculum carried by rain, irrigation water, aerosols, insects, machines, fur, feathers or clothes, including boots and shoes, by animals or labourers (Van der Wolf et al., 2021c). The inoculum may be deposited on the haulms, after which wounds or natural openings, such as stomata and hydathodes, may serve as a port of entry to establish an infection (Kastelein et al., 2020). The inoculum may also directly contaminate soil or migrate via water from haulms into soil from which infections of tubers could occur (Kastelein et al., 2020). Transmission of SRP from infected haulms can also result in root infections and subsequently in infections of stolons and tubers (Czajkowski et al., 2010; Kubheka et al., 2013).

In this study, a comprehensive two-year survey was conducted to identify sources potentially responsible for initial infections of PB1 crops with BL-SRP. For this, later generation potato crops growing near to a PB1 crop, as well as weeds, soil, flying insects and rainwater were collected from two farms of seed potatoes growers, at two different locations in the northwestern part of the Netherlands, and analyzed with sensitive TaqMan assays. The two farm locations were selected based on a former two-year study (2019–2020), during which relatively high infection levels of BL-SRP were detected in PB1 cv. Agria crops, leading to questions regarding the source of the infections (Van der Wolf et al., 2022). Rainwater was also collected at 6 to 8 other locations distributed around the country. As far as we know, this is the first coherent study on different sources responsible for initial infection of seed potato crop with BL-SRP. It will support our understanding of the relative significance of the various sources.

The infection sources surveyed were selected on the basis of literature data. BL-SRP-infected later generation potato crops cultivated near a PB1 crop were analyzed as they are likely an important infection source. Weeds may be a reservoir, as in previous studies some of the SRP variants isolated from weeds were potato blackleg-causing species (Toth et al., 2021). Although BL-SRP are not considered true soil inhabitants, they may persist for a long time in soil in association with organic material, including roots of various plants (Burr & Schroth, 1977). In several studies flying insects were found contaminated with BL-SRP (Kloepper et al., 1979; Rossmann et al., 2018). Insects may carry the pathogen from an infection source to a PB1 crop, even if this infection source is distantly located. The presence of SRP in rainwater was also described before (Franc & Demott, 1998), but the risks of infection of a potato crop by contaminated rainwater have not been investigated yet.

Materials and methods

Location sites surveys

In 2022 and 2023, surveys for the contamination of insects, weeds and neighboring potato crops were done on location sites 7 and 9 (Fig. 1A) north of Amsterdam (NL) where commercial growers cultivated prebasic seed crops. PB1 crops raised from minitubers were grown adjacent to later generation seed potato crops or in the case of location 9, a ware potato crop. In both years, and at both locations, the occurrence of BL-SRP infections of a PB1 crop (cv Agria), which is relatively susceptible to blackleg (Gill et al., 2014), was studied during the growing season. In 2022, at location 7, minitubers were planted on April 28th, and at location 9 on May 3rd. In 2023, minitubers were planted at location 7 on May 25 and at location 9 on May 12. During the growing season, crops were fertilized and sprayed with crop protection agents following the conventional management of a seed potato crop.

Bacterial strains and growth conditions

The following BL-SRP strains were used as reference in the TaqMan assays: *D. solani* IPO2222, *P. atrosepticum* IPO1007, *P. parmentieri* IPO1955, *P. brasiliense* IPO3649 (Clade 1), and *P. brasiliense* NAK 751 (IPO4295) (Clade 2). *P. brasiliense* IPO4211, a rifampicin-resistant mutant of *P. brasiliense* IPO3649 (Van der Wolf et al., 2025), was used in studies on soil contamination. In addition, a set of *Dickeya* and *Pectobacterium* strains were used to determine the specificity of a newly designed TaqMan assay for *P. brasiliense* Clade 2 (Table S1). *Acidovorax cattleya* NBC 430 (IPO4006) was used as an extraction and amplification control for the multiplex TaqMan assays (Bonants et al., 2019). Isolates were stored at −80 °C on beads (Protect bacterial preservers; http://www.tsc-swabs.co.uk). Unless otherwise stated, bacteria were grown on tryptone soya agar (TSA; Oxoid) for 48–72 h at 27 °C. For isolation of bacteria from water samples and insects, samples were spread-plated in ten-fold serial dilutions prepared 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) on double-layer crystal violet pectate (DL-CVP) medium (Helias et al., 2012). When the rifampicin-resistant mutant was used, the medium was amended with 100 µg/ml of rifampicin. Plates were incubated for 4 days at 28 °C. Cavity-forming colonies typical for SRP were streaked to pure cultures on TSA.

Enrichment in liquid medium

For enrichment prior to TaqMan detection, the liquid medium 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) was used (Van der Wolf et al., 2017). Enrichment was done as described in Van der Wolf et al. (2022).

DNA extraction

After enrichment, each sample was supplemented with *Acidovorax cattleya* (Acat) using a fixed number of cells (Bonants et al., 2019). DNA-extraction was done as described by Van der Wolf et al. (2022), with modifications. Briefly, DNA was extracted using the DNA extraction sbeadex maxi plant kit (NAP41620, LGC Genomic GMGH, Berlin, Germany) in combination with the KingFisher™ Flex Purification System (ThermoFisher Scientific, Eindhoven, NL).

Development of a TaqMan assay for *P. brasiliense* Clade 2

A TaqMan assay for *P. brasiliense* Clade 2 was developed, based on sequences of an ATPase gene. The primer/probe combination was selected using PrimerQuest (IDT, Leuven Belgium) using default settings. Amplicons were checked for sequence similarity with non-target organisms using BLAST in the nucleotide database of the NCBI Genbank. The assay was evaluated for its sensitivity using a ten-fold serial dilution of a gBlock in 10 mM Tris–HCl, 1 mM EDTA (TE) buffer (pH 8.0) (Figure S1). The specificity was tested using 1 ng of DNA extracted from 20 target and 25 non-target strains (Table S1).

TaqMan assays

Primers and probes used in this study are listed in Table 1. Samples were analyzed with a multiplex

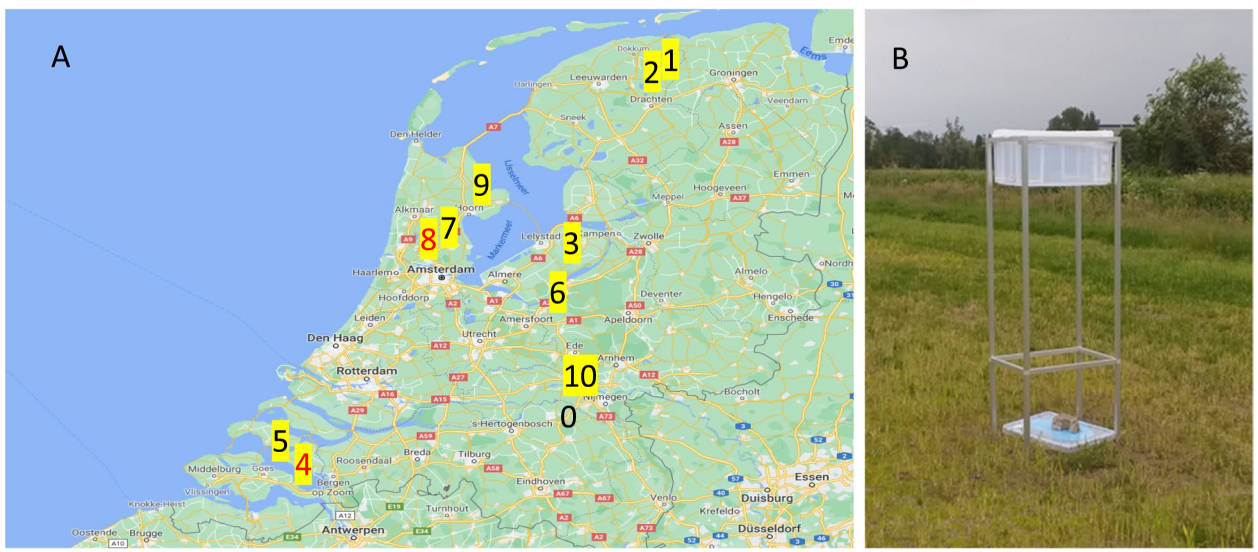

Fig. 1 **A**. Numbers 7 and 9 concern locations of seed potato farms surveyed in 2022 and 2023 in the Netherlands to identify sources responsible for infections of PB1 crops. Numbers 1 to 10 concern locations to collect rainwater in 2021 and 2022. In 2022, water was not collected at the red-marked locations (4 and 8). Location 10 concerns an open field of Wageningen University & Research. **B**. Construction used to sample rainwater

TaqMan assay to detect all BL-SRP prevailing in the Netherlands, i.e. *P. atrosepticum*, *P. brasiliense*, *P. parmentieri* and *Dickeya* species (Van der Wolf et al., 2022). All target-specific probes in the multiplex assay were labelled with FAM. Consequently, the assay did not distinguish between the individual BL-SRP species. In the multiplex TaqMan assay a test for Acat was also included by addition of a probe labeled with HEX. Samples positive in the multiplex assay were further analyzed by TaqMan assays to characterize the individual BL-SRP variants. For *P. brasiliense*, a triplex assay was used, in which one assay was able to detect all *P. brasiliense* strains, one assay was specific for Clade 1 and another assay was specific for Clade 2. The reaction mix consisted of 5 µL of the PerfeCTa Multiplex qPCR ToughMix, Low ROX enzyme mix (95149–250, QuantaBio, Beverly, MA, United States), to detect all *P. brasiliense* strains, 1.2 µM PcbrFw, 3 µM PcbrRv and 1 µM PcbrP (labelled with FAM), to detect Clade 1 strains, 0.3 µM TIR-F2, 0.3 µM TIR-R2, 0.1 µM TIR-P2 (labelled with YY/HEX), and to detect Clade 2 strains, 3 µM AAA116Fw, 3 µM AAA116Rv and 0.1 µM AAA116P (labelled with ATTO550). Water and 2 µl of sample were added up to a final reaction volume of 15 µl. The amplification was performed in a QuantStudio (AppliedBiosystems, Waltham, MA, USA) with the following program: after a hold of 2 min at 95 °C, the amplification took place during 40 cycles of 10 s at 95 °C and 30 s at 60 °C. Data analysis was done by automatic threshold calculation using QuantStudio 12KFlexSoftware v1.3. For identification of the other blackleg causing bacteria, simplex assays were used as described by Van der Wolf et al., (2022). In the multiplex assay, only reactions with Ct-values below 35 and a typical logarithmic curve were considered positive. In the simplex and triplex assays performed on the multiplex assay positive samples, Ct-values lower than 38 were considered positive.

Survey of potato crops

At different times during the growing season, three lower leaves per plant were sampled with pruning shears from 50 individual plants randomly selected, and collected in a labeled extraction bag (Bioreba, Reinach, Switzerland). Pruning shears were disinfected between each plant. Samples were stored overnight at 4 °C. The next day, after weighing the leaves, samples were crushed with a sample crusher (AAA LAB equipment B.V. Roelofarendsveen, NL). Ringer solution supplemented with 0.02% Sodium diethyldithiocarbamate (DIECA) was added to the Bioreba bag in double the amount of the weight of the leaves and samples were further crushed. One ml of leaf extract was transferred to a 1.2 ml collection tube of an 8-well strip placed in a 96-well microtube rack (19,560, Qiagen) to be analyzed for direct detection with the TaqMan assays. Racks were centrifuged for 15 min at 6000 rpm and the supernatant was discarded. After sealing the tubes with 8-cap strips, samples were stored at −20 °C until the DNA extraction was performed. For enrichment, another one ml of the same leaf extract was transferred to a 12 ml sterile tube (Greiner Bio-One, 164,162) already containing nine ml of PEB. After gentle mixing, tubes were closed, and samples were incubated for 3 days at 21 °C without agitation to allow enrichment under low oxygen conditions. Samples were homogenized and one ml of extract was directly pipetted into a 1.2 ml collection tube of an 8-well strip and processed as described for direct detection of BL-SRP. As a control for the enrichment, 100 µL of plant extract, 800 µl of PEB, and 100 µl of a suspension of *P. brasiliense* IPO3649 at 10^5 cfu, 10^4 cfu, 10^3 cfu or 0 cfu/ml in Ringers were pipetted in a 1.2 ml collection tube. Tubes were closed and incubated for 3 days at 21 °C without agitation. After enrichment, samples were processed as described before.

In 2022, at both locations the PB1 cv. Agria and a nearby located PB2 cv. Agria crop were sampled six times (7 June, 13 June, 20 June, 27 June, 5 July and 11 July) (Table 2). The PB2 crops were at a minimum distance of 26 m from the PB1 crop at location 7 and 56 m at location 9. In 2023, the PB1 cv. Agria was sampled twice, in the early and late phase of plant development (Table 2). At location 7, the PB1 cv. Agria crop, as well as two PB2 crops were sampled in the early phase of plant development, i.e. cv. Agria located at 4 m from the PB1 crop and cv. Alouette located at 67 m, and a PB3 crop cv. Fontane located at a 103 m from the sampled PB1 crop. Similarly, at location 9, a PB2 crop cv. Fontane located at 36 m, a PB3 crop cv. Agria at 6 m and a ware potato crop cv. Agria at 141 m were sampled in the early phase. In 2023, four composite samples of 50 minitubers of cv. Agria used for the PB1 crop were tested by the NAK (Emmeloord, the Netherlands) and found to be free of BL-SRP (data not shown).

Table 1 Primers and probes used in this study

Assay[1]	Strain	Name	Sequence	Target	Reporter dye	Addition	Reference
M, S	*Dickeya* sp.	Dickeya Fw284	tgtgcgttttcgggctactc	potassium transporter Kup			Zijlstra et al. (2019)
		Dickeya Rv284	ccctgtcttctgttat-caattcattaac				
		Dickeya P284	aaccagaataaggccc		FAM	MGB-NFQ	
M, S	*Pectobacterium atrosepticum*	ECA-CSL-1F	cggcatcat-aaaaacacgcc	unknown			Humphris et al. (2015)
		ECA-CSL-89R	cctgtgtaatatccgaaa-ggtgg				
		ECA-CSL-36 T-P	acattcaggctgatattcc-ccctgc		FAM	ZEN/IBFQ	
M, S	*P. parmentieri*	PwF1	tctgttcaatgt-caacgcaggta	*mdh*			Van der Wolf et al. (2017)
		PwR1	aggtaaccgcaattt-gctcaa				
		PwP1	tgtgcgcaacctg		FAM	MGB-NFQ	
M	*Acidovorax cattleya*	Acat 2-F	tgtagcgatccttcacaag	Unknown			Bonants et al. (2019)
		Acat 2-R	tgtcgatagatgctcacaat				
		Acat 2-Pr	cttgctctgcttctctat-cacg		HEX		
S	*D. dianthicola*	DdiFw	gccgtatccatcatgct-tacc	*dnaX*			Van der Wolf et al. (2014)
		DdiRv	aacgggcga-tagtcgtcttg				
		DdiP	tttccggcactcgg		FAM	MGB-NFQ	
S	*D. chrysanthemi*	Fw2	cgatttcccggcaagtgt	*dnaX*			Van der Wolf et al. (2014)
		Rv2	tggcaaaagggctgaattg				
		LNA probe 3	cgccgTCActccc		FAM	LNA[2]	
S	*D. zeae*	Fw4	tcccgcactaaagtt-gaaga	*dnaX*			Van der Wolf et al. (2014)
		R43	gcgagctggcgcgtatt				
		probe	cgcgagactTACtg-gataacgt		FAM	LNA	
S	*D. solani*	SOL-C-F	gcctacaccatcagggc-tat	Unknown			Pritchard et al. (2013)
		SOL-C-R	acactacagcgcgcat-aaac				
		SOL-C-P	ccaggccgtgctc-gaaatcc		FAM		
S	*D. dadantii*	Fw2	cccggtttcgcaattcag	*dnaX*			Van der Wolf et al. (2014)
		Rv3	gggcgtaggcaagac-gacta				
		Probe	tttcgccAACaaacggg		FAM	LNA	
S	*D. dieffenbachiae*	Fw2	gaattgcgaaaccgg-gatta	*dnaX*			Van der Wolf et al. (2014)

Table 1 (continued)

Assay[1]	Strain	Name	Sequence	Target	Reporter dye	Addition	Reference
		Rv1	gatttcccggcaggtatcg				
		Probe	cggctaCACcctgc		FAM	LNA	
S	*D. fangzhongdai*	DfF	cttcgccgcccaggtatttt	*fusA*			(Tian et al. 2016)
		DfR	atcagggcgtgaccttcgtt				
		DfP	tgctgcagactcgatcag-gttctga		FAM	ZEN/IBFQ	
M, T	*P. brasiliense*	PcbrFw	tgcgggttctgcgtttc	*araC*			Van der Wolf et al. (2017)
		PcbrRv	tggcgcgttcgcaatat				
		PcbrP	caaggcacgatacg		FAM	MGB-NFQ	
T	*P. brasiliense* (Clade 1)	TIR-F2	agataaacaagc-gagggttga	Toll-like receptor			Van der Lee et al., (2023)
		TIR-R2	atctatctcccatttcac-ccaag				
		TIR-P2	aaatacagcctccatta-gagtttccc		YY/HEX	ZEN/IBFQ	
S	*P. brasiliense* (Clade 2)	AAA116FW	agaccttgagccgtcattt	*ATPase*			This publication
		AAA116Rv	ttgtttcaacccattc-cacaatag				
		AAA116P	catctgggtttaagt-caatagttgcga		ATTO550		

[1] M in Multiplex assay

S in Simplex assay

T in Triplex assay

[2] LNA bases are shown in capital letters

Survey of weeds

In 2023, during the cropping season, at locations 7 and 9, weeds were collected twice, five weeks apart. Most weeds were collected in a PB1 crop (cv. Agria), but some were also taken from spray paths, a ditch side, or growing in another potato field directly neighboring the PB1 crop, to test for contamination with BL-SRP. Weeds were identified via visual observations supported by the use of the "PlantNet" app (https://identify.plantnet.org/nl). At each sampling date and location, 40 to 60 individual plants were collected. Weeds collected early in the growing season were relatively small and entire plants were analyzed. Just before haulm destruction, leaves and stems, and in some cases the upper root part of weed plants were collected in extraction bags (Bioreba, Reinach, Switzerland). Samples were stored overnight at 4 °C and processed in a similar way as described for the potato plants.

Analysis of BL-SRP in soil

On April 5th 2023, at locations 7, 20 soil samples of ca. 500 g at a depth of 15 cm were collected from a field just before the targeted minituber (cv. Agria) crop was planted. In total 20 samples, evenly distributed over a plot of 400 m^2 were collected using an auger. Between each sampling, the auger was cleaned with water and disinfected with 70% ethanol. For many years, grass had been grown in this plot. At location 9, 20 samples were collected from a field, at approximately 300 m distance from the plot in which the targeted minitubers were planted. Tulip bulbs had been cultivated in this plot in 2022, after which the plot was left fallow. Similarly, on 26 April 2022, soil

Table 2 Percentage plants positive in enrichment TaqMan assays for blackleg causing soft rot Pectobacteriaceae (BL-SRP) during a survey of potato crops (PB1 and neighbouring crops) grown on two locations in the Netherlands and sampled at different moments in the growing seasons of 2022 and 2023

Sampling date	Location	Crop, cultivar	% of samples positive in the multiplex assay for BL-SRP[2,3]	Range Ct-values positive samples in multiplex assay	% samples positive in simplex TaqMan assays[1]				
					Pbr, Clade 1	Pbr, Clade 2	Pbr, Mix Clade 1 + 2	Pbr, other clades	Ppar
7–6–2022	7	PB1, Agria	0		0	0	0	0	0
13–6–2022	7	PB1, Agria	0		0	0	0	0	0
20–6–2022	7	PB1, Agria	0		0	0	0	0	0
27–6–2022	7	PB1, Agria	0		0	0	0	0	0
5–7–2022	7	PB1, Agria	2	23.8	2	0	0	0	0
11–7–2022	7	PB1, Agria	2	32	2	0	0	0	0
7–6–2022	7	PB2, Agria[4]	0		0	0	0	0	0
13–6–2022	7	PB2, Agria	4	31.4–34.7	4	0	0	0	0
20–6–2022	7	PB2, Agria	2	33.9	2	0	0	0	0
27–6–2022	7	PB2, Agria	0		0	0	0	0	0
5–7–2022	7	PB2, Agria	4	34.3–34.4	2	0	0	0	0
11–7–2022	7	PB2, Agria	0		0	0	0	0	0
7–6–2022	9	PB1, Agria	0		0	0	0	0	0
13–6–2022	9	PB1, Agria	4	34.5–34.7	4	0	0	0	0
20–6–2022	9	PB1, Agria	2	34.7	2	0	0	0	0
27–6–2022	9	PB1, Agria	0		0	0	0	0	0
5–7–2022	9	PB1, Agria	2	31.9	2	0	0	0	0
11–7–2022	9	PB1, Agria	0		0	0	0	0	0
7–6–2022	9	PB2, Agria[5]	0		0	0	0	0	0
13–6–2022	9	PB2, Agria	0		0	0	0	0	0
20–6–2022	9	PB2, Agria	0		0	0	0	0	0
27–6–2022	9	PB2, Agria	0		0	0	0	0	0
5–7–2022	9	PB2, Agria	0		0	0	0	0	0
11–7–2022	9	PB2, Agria	6	32.9–34.7	4	0	0	2	0
3–7–2023	7	PB1 Agria	0		0	0	0	0	0
3–7–2023	7	PB2, Agria[6]	4	31.3–33.5	0	0	0	0	4
3–7–2023	7	PB2, Alouette	8	26.7–33.6	2	2	0	2	2
3–7–2023	7	PB3, Fontane	20	16.0–34.1	4	8	0	2	2
9–8–2023	7	PB1 Agria	100	18.2–32.9	12	52	18	18	NT[8]
19–6–2023	9	PB1, Agria			0	0	0	0	0
19–6–2023	9	PB3, Agria[7]			0	0	0	0	0
19–6–2023	9	PB2, Fontane	2	35	0	0	0	0	2
19–6–2023	9	Agria, ware			0	0	0	0	0
25–7–2023	9	PB1, Agria	10	21.3- 32	0	0	0	8	0

[1] TaqMan assays for *Pectobacterium atrosepticum* (Patr) and *Dickeya* sp. were negative for all samples

[2] Leaf samples of individual plants (3 leaves per plant) were analyzed (N = 50)

[3] Samples were tested with a multiplex TaqMan assay detecting all blackleg causing soft rot Pectobacteriaceae (BL-SRP) prevalent in the Netherlands, i.e. *Pectobacterium brasiliense* (Pbr) *P. parmentieri* (Ppar), Patrand *Dickeya* species, but without the possibility to distinguish the species. Multiplex positive samples were analyzed with simplex TaqMan assays for the individual species listed and additionally for Clade 1 and Clade 2 of Pbr, both Clades comprise mainly virulent strains. Only results of simplex TaqMan assays are listed for which at least one time a positive result was found. For the multiplex TaqMan assay a cut-off Ct-value of 35 was

Table 2 (continued)

used. For the simplex TaqMan assays the cut-off Ct-value was 38

[4] In 2022, at location 7, the PB2 crop was located at a minimum distance of 26 m from the PB1 crop

[5] In 2022, at location 9 the PB2 crop was located at a minimum distance of 56 m from the PB1 crop

[6] In 2023, at location 7, PB2, Agria was located at a minimum distance of 4 m from the PB1 crop, PB2, Alouette at a minimum distance of 67 m, and PB3, Fontane at a minimum distance of 103 m

[7] In 2023, at location 9, PB3, Agria was located at a minimum distance of 6 m from the PB1 crop, PB2, Fontane at a minimum distance of 36 m, and Agria, ware at a minimum distance 141

[8] NT = not tested

samples were collected from a field plot in Wageningen (sandy soil, location Nergena) from which the previous year symptomatic potato plants were harvested. These potato plants were grown from tubers vacuum-infiltrated with the rifampicin resistant strain of *P. brasiliense* (IPO4211). In total 50 soil samples, evenly distributed over an area of 880 m^2, were collected.

Detection in soil

Soil samples were mixed in a plastic bag and 5 g of the sample were transferred to a 50 ml tube. A spoon of washed stones and 45 ml of sterile Ringers were added after which the closed tubes were shaken in a paint shaker (Minimix Auto, Merris Engineering Ltd, Ireland) for 90 s. One ml of the sample was centrifuged for 15 min at 6000 rpm and the pellet frozen at −20 °C. This sample was stored for quantification of the target with a TaqMan assay for samples that were positive in the enrichment TaqMan assay. Another one ml of the sample was analyzed with the enrichment TaqMan assay, by mixing the sample with nine ml of PEB. For soil sampled in Wageningen, PEB was supplemented with 100 µg/ml of rifampicin. Closed tubes were incubated for three days at 21 °C without shaking. One ml of the homogenized enriched sample was transferred to a 1.2 ml collection tube of an 8-well strip placed in a 96-well microtube rack and centrifuged for 15 min at 6000 rpm. The supernatant was removed, tubes were sealed, and pellets were frozen at −20 °C prior to DNA extraction and analysis. DNA was extracted from soil with the MagAttract PowerSoil Pro DNA kit (Qiagen, Venlo, NL) using a KingFisher Flex Purification System according to the manufacturer's instructions.

To determine the detection level of the enrichment multiplex TaqMan assay for *P. brasiliense* in soil, a ten-fold serial dilution of strain IPO3649 was added to the soils of both locations, while for soil from Wageningen, strain IPO4211 was added. Experiments were performed within one month after collecting the soil samples. Enrichment was started directly upon addition of the bacteria. The detection level was determined with and/or without rifampicin added to the PEB as needed. As a control, a ten-fold serial dilution of a gBlock in pure water with the *P. brasiliense* target sequences was tested in the TaqMan assay (Van der Wolf et al., 2022).

Sampling and analysis of BL-SRP on insects

Sticky traps of 10×25 cm (nr.210306456, Royal Brinkman or Russell IPM Ltd, Deeside, United Kingdom) connected to a wooden pole were placed at different times during the growing season at a height of 50 cm in a PB1 crop at both locations for a period of 3 to 4 days. Sticky traps with the captured insects were transported to Wageningen for further processing. If insects were relatively loosely attached to the traps, the insects were collected with a toothpick and/or a disinfected tweezer and transferred to a 1.2 ml collection tube of an 8-well strip placed in a 96-well microtube rack. If insects were firmly attached to the traps, a disk was punched from the trap with a disposable, ethanol-sterilized hole punch 0.4 or 0.6 cm in diameter (Vanem Equipment Manufacturing, Hoek van Holland, NL), so that the disk contained only one insect (Fig. 2). One ml of PEB was added to the collection tube containing the insect or disk so that the insect was well covered by the medium. Tubes were closed with a cap and incubated for three days at

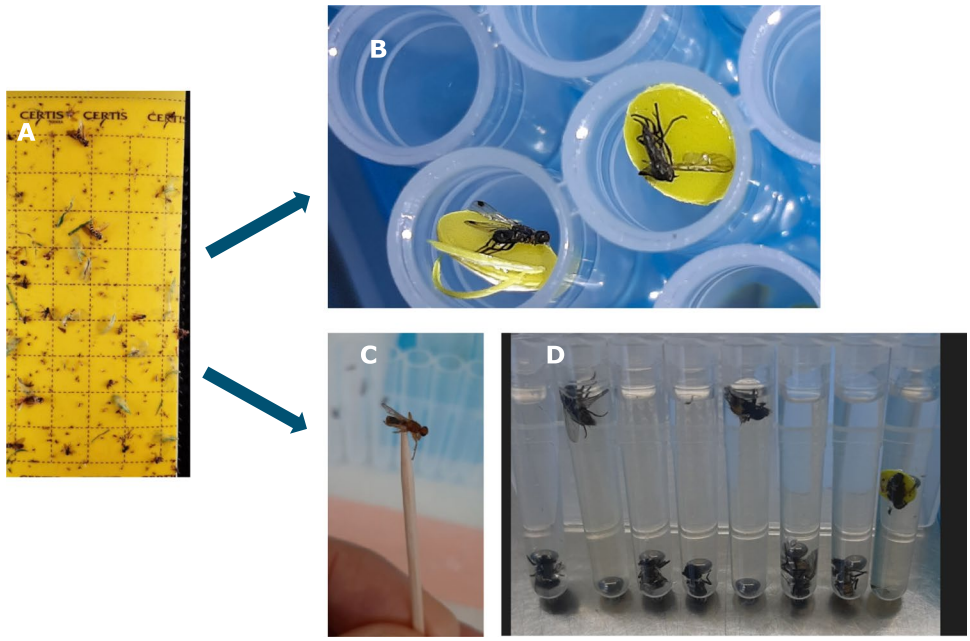

Fig. 2 Collection of insects from sticky traps in 2022. **A**. Sticky traps with trapped insects. **B**. In case insects could not be easily removed, punches with individual insects were taken and transferred to the well of a 24-wells plate. **C**. In case insects could be easily removed from the trap, they were collected with a toothpick and transferred to tubes (D) for further processing

25 °C without agitation. Tubes were then centrifuged for 15 min at 6000 rpm and stored at −20 °C without removing the supernatant until further processing. For BL-SRP testing, but also for identification of the insects, samples were processed as follows. Tubes were incubated overnight at −80 °C and placed for two days in an Epsilon 1–4 LSC freeze drier (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) for lyophilization using the basic program according to manufacturer's instructions. About 90 mg of 0.7 mm garnet purple beads, (OMN.19–644, Omni International, United States) were added to the tubes after which samples were processed by dry bead beating in a TissueLyser II (Qiagen, Venlo, The Netherlands) twice for one min at 30 Hz, while rotating the blocks between each bead beating step. DNA extraction was performed using the DNA extraction sbeadex maxi plant kit (NAP41620, LGC Genomic GMGH, Berlin, Germany) in combination with the KingFisher™ Flex Purification System (Automata Technologies, London, UK). The characterization of BL-SRP was performed following the multiplex TaqMan assay to detect all BL-SRP prevailing in the Netherlands according to the protocol previously described.

Identification of insects

The insects were identified using visual observations in combination with sequences of mitochondrial cytochrome c oxidase subunit (COI) using the LCO1490/HCO2198 primer set as described by Folmer et al. (1994). PCR amplification was conducted as follows: 98 °C denaturation (0.5 min), followed by 5 cycles of 98 °C (10 s), 45 °C (30 s), and 72 °C (30 s), followed by 35 cycles of 98 °C (10 s), 54 °C (30 s), and 72 °C (30 s), and a final elongation at 72 °C for 2 min. The total reaction volume was 50 µl, 5 µl of purified insect DNA, 2 µl of dNTPs (5 mM), 10 µl of 5×Q5 reaction buffer (New England Biolabs, Leiden, NL), 2.5 µl of the forward and reverse primer, both in a concentration of 10 µM (COI FW GGTCAACAAATCATAAAGATATTGG, COI RV TAAACTTCAGGGTGACCAAAAAATCA) and 27.5 µl of MilliQ water. The expected amplicon was 658 bp. The COI amplicon was Sanger sequenced in both directions (Macrogen, Basel, Switzerland). The

sequences were trimmed in the 3′ and 5′ regions and the forward and reverse sequences assembled into a consensus. The consensus sequences were used for species identification through the BLAST function of the NCBI database.

Survival on insects

To study the survival of BL-SRP on the insects, after trapping near a potato crop in Wageningen, they were taken to the laboratory and spray-inoculated with a suspension of *P. brasiliense* IPO 4211 or with water till the entire trap was covered with fluid. Traps were left in a flow cabinet until they were just dry, after which traps were placed back in the field. Insects on traps were analyzed immediately after spraying (T = 0), and at one day, two days, three days and six days after placing them in the field, using dilution plating on DL-CVP amended with rifampicin. Experiments were repeated twice. Three and 12 insects were analyzed in the first and second experiments, respectively, for the presence of the bacterium. During the experiments temperature and relative humidity were also recorded.

Survey of rainwater

Rainwater was collected in polystyrene containers (l*w*h, 0.33*0.55*0.32 m) with a lid (Fig. 1B). Just before sampling, the lid was removed, the containers were sterilized with alcohol and an insect repellent gauze (mesh size 1 mm) was secured over top of the container using an elastic band, to prevent contamination of rainwater with insects. Containers were placed on an aluminum stand (Tupola, WUR, Wageningen, NL) at a height of 1.5 m and secured in the open field at a minimum distance of 50 m from buildings to avoid contamination of splashing water from soil and/or roofs (Fig. 1B). In total ten stands with containers were made available and distributed at different locations in the Netherlands. Location 10 was an open field in Wageningen of Wageningen University & Research. In 2021, the other collection devices were distributed over nine potato farms located in different geographic regions in the Netherlands and in 2022 on seven farms (Fig. 1A). Four to eight hours before at least 4 mm rain was expected, stands with the containers were positioned in the open fields. Four to 24 h after rainfall, the water was collected. Rainwater was transported to Wageningen within 24–48 h and concentrated by filtration through a 0.45 µm MF-Millipore™ Membrane Filter (Mixed Cellulose Esters, 47 mm diameter, HAWP04700), Merck-Millipore (Darmstadt, Germany) using a Glass Vacuum Filtration Device, Sartorius (Goettingen, Germany). Bacteria collected on the filter were resuspended in 5 ml of Ringer solution. Samples were spread-plated undiluted and 10 times diluted on four DL-CVP-plates (100 µl per plate). Cavity forming colonies were analyzed by collecting bacterial slime with a sterile toothpick from the colonies, resuspension in sterile water and a TaqMan analysis of suspensions after boiling (5 min, 100 °C). One ml of the concentrate was mixed with nine ml of PEB in a ten ml tube (N = 2) for enrichment which was performed as described for insects. Supernatant was removed, tubes were sealed, and the pellets were frozen at −20 °C prior to DNA extraction and analysis. For a direct TaqMan assay, DNA was extracted using the DNeasy PowerWater Kit (Qiagen, Venlo, NL) according to the manufacturer's instructions.

Statistics

For analyzing the incidence of TaqMan positive samples in the studies on the survival of *P. brasiliense* on insects captured on sticky traps, analysis of variance was performed using Genstat (VSN International, 2015. Genstat for Windows 18th Edition. VSN International, Hemel Hempstead, UK. Web page: Genstat.co.uk.). Fisher's Least Significant Difference was used as a post hoc test (P = 0.05).

Results

Development of a TaqMan assay for *P. brasiliense* Clade 2

The threshold value of the Clade 2 assay was determined at a level of 100 copies of a gBlock (Figure S1). Using 1 ng of DNA of each strain per reaction, the assay gave a strong reaction with 20 *P. brasiliense* clade 2 strains with Ct-values between 19.0 and 23.5 (Table S1). No positive reactions were found with 14 non-target strains, including other

clades of *P. brasiliense*, seven strains of four *Pectobacterium* species and three strains of different *Dickeya* species.

Infections of PB1 crops

In 2022, the incidence of potato plant infections at both locations was low during the entire growing season and did not exceed 4% in the multiplex TaqMan assay for BL-SRP (Table 2). At location 7, during the first sampling date (7 June) no infections with BL-SRP in the PB1 cv. Agria crop were found after enrichment. By the end of the growing season (5 and 11 July), only 2% of the PB1 plants were positive in the TaqMan assay for *P. brasiliense* Clade 1. At location 9, there were also no infections in the PB1 crop at the first sampling date (7 June), but Clade 1 was detected at the second sampling date (13 June, 4%). In 2022, all samples positive in the multiplex assay were also positive in a simplex assay for Clade 1. No infections with *Dickeya* sp, *P. parmentieri* and *P. atrosepticum* were found. The possibility of mixed infections with Clade 1 cannot be excluded as we did not test for the presence of Clade 2 with a simplex TaqMan assay.

At the start of the survey in 2023, the PB1 cv. Agria crops were free from BL-SRP at both locations (Table 2). By the end of the survey (9 August) 82% of the plants at location 7 were infected with Clade 1, 52% with Clade 2, 18% with a mixture of Clade 1 and Clade 2, and 18% with *P. parmentieri* (Table 2). Interestingly, by the end of the growing season (25 July) at location 9 only 8% of the PB1 plants were infected and only with *P. parmentieri*.

Nearby potato crops

In 2022, haulms of a PB1 crop were sampled at both locations in addition to a nearby PB2 crop (cv. Agria). They were sampled six times (7 June, 13 June, 20 June, 27 June, 5 July and 11 July) to determine if infections of the PB1 crop could be explained by transmission of BL-SRP from the PB2 crop (Table 2). PB2 crops were at a minimum distance of 26 m and 56 m from the PB1 crops at locations 7 and 9, respectively. At location 7, the first infections with Clade 1 was found on 13 June in an early phase of plant development, but the infection incidence was low (4%) and did not increase during the growing season. At location 9, a low infection incidence with Clade 1 (4%) was found for the first time at a late stage of plant development (11 July).

In 2023, nearby crops were analyzed at an early and at a late stage of crop development. In three crops neighboring the PB1 cv. Agria crop at location 7, the first infections were found at an early phase of plant development (on 3 July). Infections were with *P. brasiliense* Clade 1 (up to 4%), Clade 2 (up to 8%), and *P. parmentieri* (up to 4%) (Table 2). No BL-SRP infections were detected in the PB1 cv. Agria crop with the multiplex TaqMan assay at that time. No infections with BL-SRP were found in the neighboring crops at location 9 at an early phase of plant development. Only 2% of the plants infected with *P. brasiliense* strains not belonging to Clade 1 or Clade 2 were detected.

Soil

Soil was sampled in April at both locations 7 and 9 just before planting the PB1 crops. None of the 40 samples (20 samples per location) tested positive in the enrichment multiplex TaqMan. The 50 soil samples from the Wageningen location, where a potato crop infected with a rifampicin resistant strain of *P. brasiliense* causing blackleg was harvested the previous season, were also negative. To estimate the sensitivity of the enrichment TaqMan assay for *P. brasiliense* (Clade 1) in soil, samples from the three locations were supplemented with a ten-fold serial dilution of the rifampicin resistant mutant of *P. brasiliense* IPO4211 and tested in a direct multiplex TaqMan assay (without enrichment), and in the TaqMan-assay after enrichment in PEB with and without supplemented rifampicin (Figure S2). The threshold level of the direct assay was approximately 10^5 cfu per gram of soil, for the enrichment assay in PEB without rifampicin the threshold was 10^2 cfu per gram, and in PEB with rifampicin, 10 cfu per gram. Results for the three soil locations were largely similar (data not shown) except that soil from location 7 and Wageningen supplemented with 10^2 cfu per gram were positive, but from location 9 was negative (Ct-value 39.6) in the enrichment without rifampicin.

Weeds

In 2023, weeds were collected in and near the PB1 crops at locations 7 and 9 when potato plants had just

emerged and again just before haulm killing. At location 7, early in the season (3 July), no BL-SRP were detected in weeds (Table 3). However, before haulm killing (9 August), 26% of the weeds were positive in the multiplex assay. In the simplex assays 23% of the weeds were contaminated with blackleg causing variants of *P. brasiliense* (Clade 1 and/or Clade 2). A relatively high percentage of Shepherd's purse (*Capsella bursa-pastoris)* was found to be positive for *P. brasiliense* Clade 1 (12.5%), Clade 2 (37.5%) or both clades (12.5%). We further found 1 out of 3 plantain plants and 1 out of 1 common knotgrass plants positive for Clade 2. Ct-values of the simplex TaqMan assays after enrichment for weeds contaminated with Clade 1 and/or Clade 2 were as low as 19.7 (Table S2). This indicates the presence of high populations of the BL-SRP in weeds, or that living bacteria present in the weeds had grown to high populations during enrichment. At location 9, early in the season (19 June), one *Chenopodium album* plant was found positive for *P. parmentieri* and one for *P. brasiliense* (not Clade 1 or 2) (Table 4). Just before haulm killing (25 June) no samples were positive for BL-SRP.

Insects as infection source

In the period between June 13 and July 8, 2022, insects were collected seven and six times at locations 7 and 9, respectively (Table 5). The number of insects sampled and analyzed per sampling time and location ranged from 16 to 92. In total, 361 samples were analyzed from location 7, and 304 samples from location 9. Three percent of samples (11 samples) were positive in the enrichment multiplex assay at location 7and 2.3% (7 samples) at location 9. Three of the positive samples at location 7 were positive in the simplex TaqMan assay for *P. brasiliense* Clade I, seven were positive for *P. parmentieri*, and for one sample the identity could not be determined. Four of the positive samples at location 9 were positive in the simplex TaqMan assay for *P. brasiliense* Clade 1, one was positive for *P. parmentieri*, and for two samples the identity could not be determined. In 2022, overall, 2.8% of the insects were contaminated with BL-SRP causing bacteria (*P. brasiliense* Clade 1 or *P. parmentieri*) at location 7, and 1.6% at location 9. The 2022 BL-SRP-contaminated insects were not identified.

In 2023, insects were collected four times at location 7 during June 26 and July 17, and five times during June 19 and July 17 at location 9 (Table 6). The number of insects sampled during each collection date ranged from 88 to 136 insects. In total 384 and 568 insects were sampled at locations 7 and 9, respectively. At location 7, 12.5% (48 samples) of all insects were positive in the multiplex assay for BL-SRP, but for 4.4% (17 samples) of these, all simplex assays were negative. Of the 48 positive samples, 18 were positive in the Taqman assay for *P. parmentieri* and 8 for *P. brasiliense* Clade 1, while Clade 2 was not found. *D. zeae* was found in 2 samples and no other *Dickeya* species were detected. Of the 41 samples positive in the multiplex assay from location 9, 18 were positive for *P. parmentieri* and 9 for *P. brasiliense* Clade 1, while Clade 2 was not detected. Overall, 7.8% of the insects from location 7 were contaminated with BL-SRP causing bacteria (*P. brasiliense* Clade 1 or *P. parmentieri*) and 4.7% from location 9.

In 2023, the reaction of most positive samples in the enrichment multiplex TaqMan assay were weak, with Ct-values ranging from 30 to 35 (Tables 7 and 8), except for two location-7 autumn houseflies contaminated with *D. zeae*, which yielded low Ct-values of 20.3 and 21.8, respectively. A lance fly from location 9 yielded a Ct value of 18.7 for *P. brasiliense* Clade 1.

No relationship was found between the percentage of insects positive in the multiplex TaqMan assay and time of sampling. In 2022, BL-SRP positive insects were found in samples taken June 13–27, but not in the June 27—July 8 sampling time. However, in 2023, insects positive for BL-SRP were found during the entire growing season at various rates.

Identification of insects contaminated with BL-SRP

Insects contaminated with BL-SRP in 2023 were identified on the basis of COI sequences (Table 7 and 8). The 27 contaminated insects from location 7 belonged to 12 different genera as did the 24 contaminated insects from location 9. Most of the contaminated insects were of the order Diptera (flies), but some were of the order Neuroptera (lacewings).

Survival on insects

Since the detection of BL-SRP on the surface of trapped insects with the enrichment TaqMan assay

Table 3 Weeds positive in enrichment TaqMan assays for blackleg causing soft rot Pectobacteriaceae (BL-SRP) collected from a PB1 potato field at location 7 sampled during a young potato plant stage (3 July 2023) and just before haulm killing (9 August 2023)

			Results 3 July[1]		Results 9 August						
				TaqMan positive		TaqMan positive[3]					
Name	Common name	(Bi)Annual/Perrenial	Nr tested	multiplex[2]	Nr tested	Multiplex	Pbr Clade 1	Pbr Clade 2	Pbr Clade 1 and 2	Pbr other clades	% BL-SRP[4]
Amaranthus deflexus	Low amaranth	A	1	0	0	NT	0	0	0	0	nd
Anthriscus sylvestris	Cow Parsley	A	1	0	0	NT	0	0	0	0	nd
Beta vulgaris maritima	Sea Beet	A/P	1	0	0	NT	0	0	0	0	nd
Capsella bursa-pastoris	Shepherd's purse	A	0	NT[5]	16	13	2	6	2	3	63
Carduus sp.	Thistle	A/B	12	0	6	0	0	0	0	0	0
Carex sp.	Cyperus	P	0	NT	1	NT	0	0	0	0	0
Chenopodium album	Lamb's quarters	A	4	0	6	3	0	0	0	3	0
Geranium sp.	Geranium	A	0	NT	1	0	0	0	0	0	0
Heracleum sp.	Hogweed	B	2	0	1	0	0	0	0	0	0
Lactuca virosa	Wild lettuce	A	4	0	1	0	0	0	0	0	0
Lamium sp.	Dead-nettles	A	0	NT	2	0	0	0	0	0	0
Lolium sp.	Grass	A/P	4	0	2	0	0	0	0	0	0
Plantago sp.	Plantain	P	4	0	3	1	0	1	0	0	33
Polygonum aviculare	Common knotgrass	A	3	0	1	1	0	1	0	0	100
Ranonkel sp.	Buttercup	A	3	0	0	NT	0	0	0	0	nd
Raphanus raphanistrum	Wild radish	A	1	0	0	NT	0	0	0	0	nd
Rorripa sylvestris	Creeping yellow cress	P	1	0	0	NT	0	0	0	0	nd
Senecio vulgaris	groundsel	A/B	0	NT	3	2	0	0	0	2	0
Sisybrium officinale	Hedge mustard	A	0	NT	1	0	0	0	0	0	0
Solanum nigrum	Black nightshade	A	2	0	2	2	0	0	0	2	0
Stellaria media	Chickweed	A	0	NT	4	2	0	0	0	2	0
Taraxacum officinale	Dandelion	P	1	0	0	NT	0	0	0	0	nd
Trifolium sp.	Clover	B	0	0	2	2	0	0	0	1	0
Unidentified	Weed	?	4	0	0	NT	0	0	0	0	nd
		Total	***45***	***0***	***52***	***26***	***2***	***8***	***2***	***13***	***23***

[1] On 3 July, entire weed plants were analyzed. On 9 august, the leaves and stems of weed plants were analyzed

[2] Plants were analyzed with an enrichment TaqMan assay. First, a multiplex assay was used detecting *P. brasiliense* (Pbr), *P. parmentieri* (Ppar), *P. atrosepticum* (Patr) and all *Dickeya* species, but without distinguishing the species. Multiplex TaqMan positive samples were analyzed with TaqMan assays for the individual species listed and additionally for Clade 1 and Clade 2 of Pbr, comprising mainly virulent strains. 'Pbr Clade 1 and 2', means that both clades were present in these samples. Occasionally, positive samples in the multiplex assay were not confirmed by the follow-up assays

[3] Samples were not tested in simplex assays for *P. parmentieri*, *P. atrosepticum* and *Dickeya* sp

[4]The percentage BL-SRP was calculated by dividing positive samples for Pbr Clade 1, Clade 2 and mixed infections of Clade 1 and Clade by the number of weeds tested

[5] NT = not tested; nd = not determined

Table 4 Weeds positive in enrichment TaqMan assays for blackleg causing soft rot Pectobacteriaceae (BL-SRP) collected from a PB1 potato field at location 9 during the young potato plant stage (19 June 2023) and just before haulm killing (25 July 2023)

Plant species	English name	(Bi)annual/ perennial	Data 19 June[1]		Data 25 July	
			Nr tested	Nr positive multiplex[2]	Nr tested	Nr positive multiplex
Senecio vulgaris	Groundsel	A	2	0	3	0
Chenopodium album	Lamb's quarters	A	9	1[3]	16	0
Capsella bursa-pastoris	Shepherd's purse	A	4	0	3	0
Rumex sp.	Sorrel	P	2	0	3	0
Polygonum aviculare	Common knotgrass	A	3	0	4	0
Persicaria maculosa	Peach herb	A	4	1[4]	4	0
Matricaria discoidea	Pineappleweed	A	6	0	5	0
Stellaria media	Chickweed	A	3	0	0	NT[5]
Fagopyrum esculentum	Buckwheat	A	2	0	0	NT
Phacelia sp.	Scorpianweed	A/P	2	0	2	0
Plantago sp.	Plantain	P	1	0	0	NT
Unidentified	Weed	?	1	0	2	0
Carduus sp.	Thistle	A/B	1	0	1	0
Urtica sp.	Stinging nettle	B/P	0	NT	3	0
Helianthus annuus	Sunflower	A/P	0	NT	1	0
Cosmea sp.	Garden cosmos	A	0	NT	1	0
Hypochaeris glabra	Smooth cat's ear	A	0	NT	1	0
Centaurea cyanus	Corn flower	A	0	NT	2	0
Malva sp.	Mallow	A/B/P	0	NT	2	0
Arabidopsis thaliana	Thale cress	A	0	NT	1	0
Arenaria serpyllifolia	Thyme-leaved Sandwort	A/B	0	NT	1	0
Taraxacum officinale	Dandelion	P	0	NT	1	0
Heracleum sp.	Hogweed	B/P	0	NT	3	0
Vicia sp.	Vetch	A	0	NT	1	0
		Total	***40***		***60***	

[1] On 19 June, entire weed plants were analyzed. On 9 August, the leaves and stems of weed plants were analyzed

[2] Plants were analyzed with an enrichment TaqMan assay. Firstly, a multiplex assay was used detecting *P. brasiliense* (Pbr), *P. parmentieri* (Ppar), *P. atrosepticum* (Patr) and all *Dickeya* species, but without distinguishing the species. Multiplex positive samples were analyzed with TaqMan assays for the individual species listed and additionally for Clade 1 and Clade 2 of Pbr, comprising mainly virulent strains

[3] Ct-value multiplex 35. Ct-value for *P. parmentieri* was 37.0

[4] C- value multiplex 33.6. Ct-value for the simplex assay for Pbr, was 37.4

[5] NT = not tested

would be affected if the bacteria died due to desiccation and UV radiation while on the traps, the survival of BL-SRP on trapped insect in the field was studied in two independent experiments. Sticky traps were placed in an open field in Wageningen (the Netherlands) for a period of 3–7 days to collect insects. Traps with insects were subsequently taken to the laboratory, sprayed with a suspension of *P. brasiliense* and returned to the field. *P. brasiliense* was detected on the insects by dilution plating up to two days after returning the traps to the field (Fig. 3). In the first experiment the numbers dropped from an average of 100 to a few cfu per insect in two days, and in the second experiment from 10 to a few cfu per insect in two days. Also, the incidence of contaminated insects decreased in two days; in the

Table 5 Number of Insects positive in enrichment TaqMan assays for blackleg causing Pectobacteriaceae (BL-SRP), collected from a PB1 potato field at two different locations (7 and 9) in the northwest of the Netherlands during the growing season of 2022

		TaqMan-positive[2]								
	Nr tested	Multiplex[1]	All Pbr	Pbr Clade 1	Pbr Clade 2	Ppar	All *Dickeya* sp.	*D. zeae*	Not determined[3]	% BL-SRP[4]
Location 7										
13 June	69	1	1	1	0	0	0	0	0	1.4
17 June	32	2	2	2	0	0	0	0	0	6.3
20 June	92	5	0	0	0	5	0	0	0	5.4
27 June	64	3	0	0	0	2	0	0	1	3.1
1 July	56	0	0	0	0	0	0	0	0	0
4 July	32	0	0	0	0	0	0	0	0	0
8 July	16	0	0	0	0	0	0	0	0	0
Total	*361*	*11*	*3*	*3*	*0*	*7*	*0*	*0*	*1*	*2.8*
% total samples		**3.0**	**0.83**	**0.83**	**0**	**1.9**	**0**	**0**	**0.27**	
Location 9										
13 June	64	4	2	2	0	1	0	0	1	4.7
20 June	76	1	1	1	0	0	0	0	0	1.3
24 June	48	2	1	1	0	0	0	0	1	2.1
27 June	72	0	0	0	0	0	0	0	0	0
4 July	28	0	0	0	0	0	0	0	0	0
8 July	16	0	0	0	0	0	0	0	0	0
Total	*304*	*7*	*4*	*4*	*0*	*1*	*0*	*0*	*2*	*1.6*
% total samples		**2.3**	**1.3**	**1.3**	**0**	**0.3**	**0**	**0**	**0.7**	

[1] Insects were analyzed with an enrichment TaqMan assay. First, a multiplex assay was used detecting all BL-SRP, i.e. *Pectobacterium brasiliense* (Pbr), *P. parmentieri* (Ppar), *P. atrosepticum* (Patr) and all *Dickeya* species, but without distinguishing the species. Multiplex positive samples were analyzed with TaqMan assays for the individual species listed and additionally for Clade 1 and Clade 2 of Pbr, clades comprising mainly virulent strains

[2] Reactions of TaqMan assays specific for *P. atroseptica*, *D. chrysanthemi*, *D. dieffenbachiae*, *D. dianthicola*, *D. solani* and *D. fangzhongdai* were all negative (data not shown)

[3] Occasionally, samples positive in the multiplex assay were negative in the follow-up assays and BL-SRP could not be identified

[4] Percentage BL-SRP calculated by the dividing the number of positives for Pbr Clade 1, Clade 2 and P. parmentieri by the total number tested

Table 6 Insects positive in enrichment TaqMan assays for blackleg causing Pectobacteriaceae (BL-SRP), collected from a PB1 potato field at two different locations (7 and 9) in the northwest of the Netherlands during the growing season of 2023

		TaqMan-positive								
	Nr tested	Multiplex[1]	All Pbr	Pbr Clade 1	Pbr Clade 2	Ppar	All *Dickeya* sp.	*D. zeae*	Not identified[3]	% BL-SRP[4]
Location 7										
26 June	96	12	1	0	0	11	0	0	1	11.5
3 July	96	15	2	1	0	6	2	2	6	7.3
10 July	104	8	1	1	0	3	0	0	5	3.8
17 July	88	13	4	4	0	4	0	0	5	9.1
Total	*384*	*48*	*8*	*6*	*0*	*24*	*2*	*2*	*17*	*7.8*
% total samples		**12.5**	**2.1**	**1.6**	**0.0**	**6.3**	**0.5**	**0.5**	**4.4**	
Location 9										
19 June	120	6	1	0	0	5	0	0	1	4.2
26 June	96	5	1	0	0	3	0	0	2	3.1
3 July	128	19	4	4	0	8	0	0	6	9.4
10 July	136	10	4	4	0	2	0	0	4	4.4
17 July	88	1	1	1	0	0	0	0	0	1.1
Total	*568*	*41*	*11*	*9*	*0*	*18*	*0*	*0*	*13*	*4.7*
% total samples		**7.2**	**1.9**	**1.6**	**0.0**	**3.2**	**0**	**0**	**2.3**	

[1] Insects were analyzed with an enrichment TaqMan assay. First, a multiplex assay was used detecting all BL-SRP, i.e. *Pectobacterium brasiliense* (Pbr), *P. parmentieri* (Ppar), *P. atrosepticum* (Patr) and all *Dickeya* species, but without distinguishing the species. Multiplex positive samples were analyzed with TaqMan assays for the individual species listed and additionally for Clade 1 and Clade 2 of Pbr, clades comprising mainly virulent strains

[2] Reactions of TaqMan assays specific for *P. atroseptica*, *D. chrysanthemi*, *D. dieffenbachiae*, *D. dianthicola*, *D. solani* and *D. fangzhongdai* were all negative (data not shown)

[3] Occasionally, sampes positive in the multiplex assay were negative in all follow-up assays and BL-SRP could not be identified

[4] Percentage BL-SRP calculated by the dividing the number of positives for Pbr Clade 1, Clade 2 and P. parmentieri by the total number tested

Table 7 Identification of insects positive in enrichment TaqMan assays for blackleg causing soft rot Pectobacteriaceae (BL-SRP) collected on yellow sticky traps in a PB1 potato field at location 7 in 2023

Insect species[1]	Common name	TaqMan assay[2]						Pathogen identification[3]
		All BL-SRP	Pbr (all Clades)	Pbr (Clade 1)	Ppar	*Dickeya* sp.	*D. zeae*	
Anthomyiinae sp.	Root maggots	30.8[4]			33.4			*P. parmentieri*
Chrysoperla carnea[5]	Common lacewing	33.3	34.8	35.5				*P. brasiliense* Clade 1
Contacyphon coarctatus[5]	Marsh beetles	31.4	31.5	32.2				*P. brasiliense* Clade 1
Delia florilega	Bean seed fly	33.7			32.7			*P. parmentieri*
Delia platura	Seedcorn maggot	34.8			32.9			*P. parmentieri*
Delia platura	Seedcorn maggot	30.8			32.4			*P. parmentieri*
Delia platura	Seedcorn maggot	34.5	34.3					*P. brasiliense* (clade unknown)
Delia platura	Seedcorn maggot	34			34.4			*P. parmentieri*
Delia platura	Seedcorn maggot	32.1			31.4			*P. parmentieri*
Gabrius breviventer	Rove beetle	32.6			31.9			*P. parmentieri*
Lasius niger	Black garden ant	34			33.4			*P. parmentieri*
Lotophila atra	Dung fly	34.1			34.8			*P. parmentieri*
Musca autumnalis	Autumn housefly	17.4				17.6	20.3	*D. zeae*
Musca autumnalis	Autumn housefly	19	nt	nt		19.1	21.8	*D. zeae*
Musca autumnalis	Autumn housefly	34.7			31.2			*P. parmentieri*
Musca autumnalis	Autumn housefly	35			34.5			*P. parmentieri*
Musca autumnalis	Autumn housefly	34.4			34.8			*P. parmentieri*
Musca autumnalis	Autumn housefly	33			35			*P. parmentieri*
Notiphila riparia	?	31.8			34.3			*P. parmentieri*
Platypalpus pallidiventris[5]	Dance fly	30.4	30.7	30.7				*P. brasiliense* Clade 1
Platypalpus sp.	Dance fly	34.3			34			*P. parmentieri*
Platypalpus pseudofulvipes	Dance fly	28.7	29.9	31.5				*P. brasiliense* Clade 1
Saltella sphondylii	?	32.9			32.5			
Saltella sphondylii	?	33.3			33.2			*P. parmentieri*
Sepsis thoracica	Back scavenger fly	29.6			34.9			*P. parmentieri*
Sepsis thoracica	Back scavenger fly	32.7	34.5	35.7	34.5			*P. brasiliense* Clade 1 + *P. parmentieri*
Sepsis thoracica	Back scavenger fly	32.7	33.3	33.4	31.5			*P. brasiliense* Clade 1 + *P. parmentieri*

[1] Identification using sequences of the mitochondrial cytochrome c oxidase subunit

[2] Insects were analyzed with an enrichment TaqMan assay. After enrichment, first a multiplex assay was used, detecting *Pectobacterium brasiliense* (Pbr), *P. parmentieri* (Ppar), *P. atrosepticum* (Patr) and all *Dickeya* species, but without the possibility to distinguish the species. Multiplex positive samples were analyzed with simplex TaqMan assays for the individual species listed and additionally for Clade 1 and Clade 2 of Pbr, both Clades comprise mainly virulent strains. Only results of simplex TaqMan assays are listed for which at least one time a positive result was found

[3] Identification based on the results of the TaqMan assays

[4] For the multiplex TaqMan assay a cut-off Ct value of 35 was used. For the simplex TaqMan assays the cut-off Ct value was 38

[5] Sequences of limited quality. No accession number available

Table 8 Identification of insects positive in enrichment TaqMan assays for blackleg causing soft rot Pectobacteriaceae (BL-SRP) collected on yellow sticky traps in a PB1 potato field at location 9 in 2023

Species[1]	Common name	TaqMan assay[2]				Pathogen identification[3]
		All BL-SRP	Ct Pcb PRI	Pbr (Clade 1)	Ppar	
Bellardia pandia	Emerald bottle fly	32.8[4]	34.4		35.9	*P. brasiliense* (clade unknown)
*Chrysoperla s*p.[5]	?	30.2	31	31.8		*P. brasiliense* Clade 1
Chrysoperla carnea[5]	Common lacewing	31			34.4	*P. parmentieri*
Chrysoperla carnea	Common lacewing	29.2			30.5	*P. parmentieri*
Chrysoperla carnea	Common lacewing	31.7	32.2	33.3		*P. brasiliense* Clade 1
Chrysoperla carnea	Common lacewing	35			34.1	*P. parmentieri*
Chrysoperla carnea	Common lacewing	35.2			34.7	*P. parmentieri*
Chrysoperla carnea	Common lacewing	30.4	31.5	31.2		*P. brasiliense* Clade 1
Chrysoperla lucasina	Common green lacewing	34.9			33.7	*P. parmentieri*
Delia platura	Seedcorn maggot	33.3			32.8	*P. parmentieri*
Delia platura	Seedcorn maggot	33.9			33.6	*P. parmentieri*
Delia platura	Seedcorn maggot	33.7			34.4	*P. parmentieri*
Delia platura[5]	Seedcorn maggot	32.1	33.5	34		*P. brasiliense* Clade 1
Delia platura	Seedcorn maggot	32.4	34.4	34.6		*P. brasiliense* Clade 1
Eupeodes corollae	Hover fly	28.9			34	*P. parmentieri*
Lasioglossum calceatum	Common furrow bee	31			34.2	*P. parmentieri*
Lygus piratenschip[5]	Tarnished plant bug	32.7			33.9	*P. parmentieri*
Lygus rugulipennis	?	34.1	35.5	35.8		*P. brasiliense* Clade 1
Melieria omissa	Bandless spotwing	34.6			32.5	*P. parmentieri*
Oscinella sp.	?	31.2	35.3	34.3		*P. brasiliense* Clade 1
Phaonia rufipalpis	?	32	33.3			*P. brasiliense* (clade unknown)
Platypalpus pallidiventris	Dance fly	28.8			33.6	*P. parmentieri*
Silba fumosa	Lance fly	18	18.7			*P. brasiliense* Clade 1
Syrphoctonus/Ichneumonidae[5]	?	22.7			33.8	*P. parmentieri*

[1] Identification using sequences of the mitochondrial cytochrome c oxidase subunit

[2] Insects were analyzed with an enrichment TaqMan assay. After enrichment, first a multiplex assay was used, detecting *Pectobacterium brasiliense* (Pbr), *P. parmentieri* (Ppar), *P. atrosepticum* (Patr) and all *Dickeya* species, but without the possibility to distinguish the species. Multiplex positive samples were analyzed with simplex TaqMan assays for the individual species listed and additionally for Clade 1 and Clade 2 of Pbr, both Clades comprise mainly virulent strains. Only results of simplex TaqMan assays are listed for which at least one time a positive result was found

[3] Identification based on the results of the TaqMan assays

[4] For the multiplex TaqMan assay a cut-off Ct value of 35 was used. For the simplex TaqMan assays the cut-off Ct value was 38

[5] Sequences of limited quality. No accession number available

first experiment from 100 to 33% and in the second experiment from 66 to 8.3%. A small difference in temperature and RH were measured between both experiments (20.5 °C versus 17.5 °C, and 71.4% versus 79.4% RH) which may explain the difference in decline of culturable bacteria.

Rainwater as infection source

Twenty rainwater samples were collected from ten locations in 2021 and 12 rainwater samples from eight locations in 2022 (Fig. 1B, Table S3). Samples were collected in 2021 from June 4 to 20, and in 2022

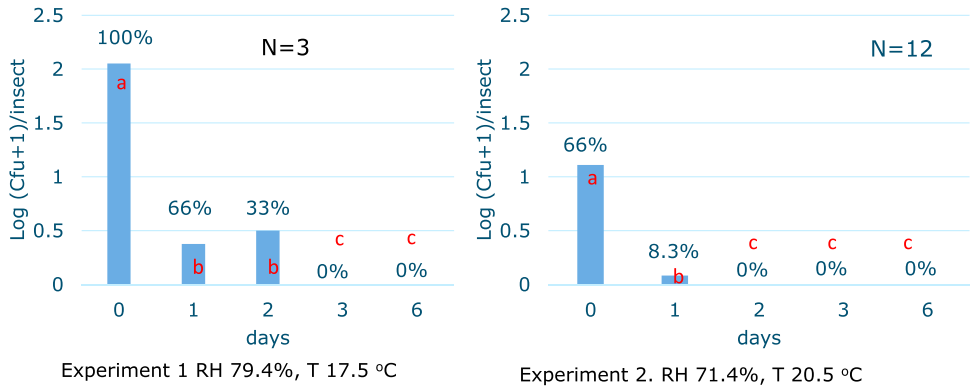

Fig. 3 Survival of *Pectobacterium brasiliense* on insects captured on yellow sticky traps in the field during two experiments varying in weather conditions. Average densities (log cfu + 1/insect) are shown, and the percentage of insects contaminated per time point. All water treated insects were negative. Values with a similar character (marked in red) were not significantly different (P = 0.05). Data on the average relative humidity (RH) and temperature (T) are included

from June 5 to 24. The volume of water varied per sampling date and per location from 0.3 to 2 L. The entire volume was concentrated by filtration, except for one sample in 2022 which was heavily contaminated with sand and from which only 100 ml could be filtrated due to plugging of the filter. In several cases, the concentrated bacteria from the rainwater formed cavities on DL-CVP. In 2022, 12 colonies were tested with the multiplex TaqMan assay for BL-SRP, but none were positive. The samples with concentrated bacteria were also tested in the enrichment TaqMan assay. Only one sample (location 3), collected in 2022 near Bant, heavily contaminated with sand, was positive in the multiplex TaqMan assay. Further characterization with the simplex TaqMan assays, indicated that the sample was contaminated with a variant of *P. brasiliense*, not belonging to Clade 1 or 2.

Discussion

By conducting surveys at two locations during two growing seasons, data was collected on infection sources and transmission pathways that can play a possible role in the infection of PB1 potato crops with BL-SRP. Results showed that young potato plants grown from minitubers were BL-SRP-free early in the season. However, just before haulm killing, the incidence of haulm infections can be high depending on the growing season and location. In 2022, a low infection incidence was found at both locations surveyed. In 2023, 82% of the PB1 plants at location 7 were infected with BL-SRP at the sampling date just before haulm killing, whereas haulms at location 9 remained largely pathogen-free.

Plant samples were not disinfected prior to enrichment so we could not distinguish superficial contamination from internal infections. However, in previous experiments, spray-inoculation of potato haulms with suspensions of *P. brasiliense* Clade 1 has shown that internal infections of leaves, stems and tubers can readily occur, and at high inoculation densities (above 10^5 cfu/ml) can result in symptomatic infections (Kastelein et al., 2020; Van der Wolf et al., 2025).

In 2022 and 2023, total precipitation during the growing seasons was similar. However, a relatively high level of precipitation was recorded early in the season in 2022, while in 2023 much of the precipitation occurred late in the growing season (Figure S3). Possibly the risk of infection for a PB1 crop will be greater when precipitation levels are high late in the growing season at a time when infection pressure is also high due to the presence of diseased potatoes growing nearby. Surveys done at the same locations (at that time indicated as locations D and E) were also done in 2019 and 2020 (Van der Wolf et al., 2022). In 2019, a year with a low level of precipitation during the growing season, infection incidences were low at both locations, but in 2020, a year with a high level of

precipitation, the incidences were high at both locations. This also indicates that the risk of infection depends on environmental factors during the growing season.

The level of precipitation during the growing season is considered a primary factor in the occurrence of blackleg infections (Aleck & Harrison, 1978; Pérombelon, 1992). Heavy rain results in splashing water and formation of aerosols which may contribute to the dissemination of the pathogen from an infection source (Fitt et al., 1983; Graham et al., 1977). Splash droplets can readily transport *P. carotovorum* ca. 3 m by a relatively low wind speed of 1 m s^{-1} (Fitt et al., 1983). In controlled laboratory studies, simulated rain hitting blackleg diseased potato plants, aerosolized populations of *P. atrosepticum* were generated, which lasted for up to 90 min in still air (Graham & Harrison, 1975). Pathogen populations will decline rapidly in aerosols, but may still be present at 100 m distance from an infection source (Perombelon et al., 1979). After a rain shower, free water in and puddling on soil can also cause translocation of bacteria (Ansermet et al., 2016). The distance of travel will be affected by soil particle size, but also by the presence of the microbiome, microflora and microfauna in soil, such as nematodes, earth worms and protozoa that may carry the pathogen (Yang & Van Elsas, 2018). Under moist conditions, tuber lenticels will open, creating a port of entry for the pathogen (Perombelon, 1976). Finally, water can cause anaerobic conditions favoring growth of BL-SRP, which are facultative anaerobes (Lund & Kelman, 1977).

A potentially high-risk factor responsible for contamination of a PB1 crop with BL-SRP is the presence of infected lower-grade potato crops growing nearby. We found PB2 and PB3 crops, grown near to PB1 crops, were already contaminated early in the growing season, before infections with PB1 crops were detected. The same haplotypes of *P. brasiliense* (Clade 1 and Clade 2) were found in the PB2 and PB3 crop as found in the PB1 crop in a later stage. The risk for carry-over of BL-SRP to PB1 crops will be dependent on the level of contamination of the lower grade crops as well as on the transmission rate of the pathogen. Dose–response studies in which the haulms of the blackleg susceptible cultivar Agria were spray-inoculated with *P. brasiliense* showed that low densities of 100 cfu per plant can be sufficient to establish an infection (Van der Wolf et al., 2025). We assume that the tubers of the PB2 and PB3 crops had become infected in a similar way as the PB1 crop, i.e. by transmission of BL-SRP from infected lower grade crops during field growth.

Soil cannot be excluded as an infection source, but we did not find any contamination of soils with BL-SRP sampled at locations 7 and 9, using a method allowing detection of as low as 10^2 cfu/g of soil. Moreover, the pathogen was not detected in soil sampled from an experimental field in Wageningen one year after harvesting a severely blackleg-diseased crop infected with a rifampicin-resistant strain of *P. brasiliense*. For these tests, a method was used that allowed detection as low as 10^1 cfu/g. Different experimental studies showed that SRP populations rapidly decline (De Boer et al., 1979) and that the frequency of detection is low (Pérombelon & Hyman, 1989). In a multiple-year rotation scheme, in which production of potatoes is followed by cultivation of other crops, the level of soil contamination during planting of seed potatoes is expected to be low. Based on this assumption, it is unlikely that soil-borne inoculum is responsible for the high infection levels occasionally found in the PB1 crops.

Weeds collected in or nearby PB1 crops became contaminated with BL-SRP late in the growing season. It is therefore likely that that the pathogen is transmitted from infected nearby located PB2 and PB3 crops to weeds, rather than being a primary infection source for a PB1 crop. Early in the growing season, BL-SRP was rarely detected on potato plants, but 25% of the plants were positive in the assays for the various BL-SRP variants, i.e. *P. brasiliense* Clade 1 and 2 and *P. parmentieri* just before haulm killing, at location 7. The same variants were found early in the growing season in the PB2 and PB3 potato crops.

Most of the weeds contaminated with BL-SRP were annuals that grew from seeds produced in previous years. It is expected that contamination of perennial weeds will potentially be important in the epidemiology of blackleg as they can carry the pathogen over multiple years. Notably, natural populations of *P. carotovorum* have been detected in true seed of annual crops (Doolotkeldieva & Bobusheva, 2022; Hadas et al., 2001). For example, seed transmission of *P. carotovorum* was recorded, resulting in diseased seedlings. It can therefore not be excluded that establishment of infections can occur via true seed produced by infected (annual) weeds.

At location 7 of our study, a large variety of weeds tested positive in the assays for BL-SRP just before haulm killing. In particular, in *Capsella bursa pastoris*, an annual brassica plant, both Clade 1 and Clade 2 of *P. brasiliense* were frequently detected. For the other weed species, the number of plants tested was too small to reliably estimate contamination incidences. Various weeds surveyed in potato fields have been found contaminated with SRP (Burr & Schroth, 1977; Gudmestad & Secor, 1985; Pérombelon & Hyman, 1989). In many cases, only *Pectobacterium carotovorum* was isolated. Only incidentally, the blackleg causing species were detected, such as *P. atrosepticum* in brassicas (Pérombelon & Hyman, 1989), *D. solani* in *Cyperus rotundus* (Tsror et al., 2010), *P. brasiliense* in *Malva nicaeensis* (Tsror et al., 2019), and *P. parmentieri* in unidentified weeds (Zoledowska et al., 2018).

Results of our surveys pointed to a role for insects in the dissemination of BL-SRP from infection sources to a PB1 crop. In 2022 and 2023, 2.2% and 6%, respectively, of trapped insects from the PB1 crop were contaminated with variants of BL-SRP that were also detected in infected potato plants. Flying insects may become contaminated by contact with infected plants close to a PB1 crop, but also by contact from infection sources at larger distances. Hover flies can migrate up to 70 km, and Drosophila at least 6 km (Coyne & Milstead, 1987; Lempke, 1972).

The percentage of contaminated insects determined in this study will be an underestimation of the actual contamination level. We collected insects from traps 3 or 7 days after they were placed in the field. On free moving insects, SRP can survive both internally and externally for at least 72 h, as shown in studies with *Drosophila* species (Brewer et al., 1980, 1981). However, bacteria on insects trapped on yellow sticky traps may die faster as they are exposed to adverse environmental conditions including desiccation and UV-radiation. We were unable to culture *P. brasiliense* from insects affixed on traps for three days. Dead bacteria will not multiply during enrichment, which implies that bacterial densities below the detection threshold of a TaqMan assay will not be detected using culturing methods. At least 10^4 cells per insect are required to obtain a positive TaqMan result (Rossmann et al., 2018). In our studies, Ct-values of the BL-SRP positive insects were often high (between 30–35), indicating no or only a limited multiplication of the bacteria during enrichment. It is therefore assumed that in many cases dead bacteria were detected before enrichment started.

Insects have been found to be carriers of SRP in other studies as well (Toth et al., 2021). In the USA, many genera of flies were found contaminated with SRP (Kloepper et al., 1979). In a study in Norway in which 2000 insects were analyzed with a quantitative PCR allowing detection of all SRP, 13% to 45% of the insects in and near potato crops were found positive. In particular, *P. atrosepticum*, the most prevalent BL-SRP in Norway, was detected, indicating a role for insects in the transmission of BL-SRP (Rossmann et al., 2018). In this study, enrichment techniques were not applied, which may have reduced the chance of detecting SRP. Therefore, the contamination levels in the Netherlands and Norway cannot be directly compared.

In this study a variety of different flies were contaminated with BL-SRP, with most of these belonging to the families Chrysopidae and Anthomyiidae. This suggests that the contamination was due to mechanical contact between BL-SRP and insects and not by a specific interaction with certain insect species. Nevertheless, it is striking that *P. brasiliense* Clade 2, which was detected in potato crops located nearby the targeted PB1 crop, has not been detected on insects.

As in Norway, *Delia* species were frequently contaminated with SRP. *Delia* species visit many plants known to be hosts of SRP and may transmit the pathogen from one host to another. In past studies, it was shown that *Delia platura* and *Drosophila melanogaster* are attracted to rotten tubers infected with *P. carotovorum* (Kloepper et al., 1981; Phillips & Kelman, 1982). In this way, flies may become contaminated with high densities of BL-SRP after which they may transmit the bacteria to a PB1 crop.

No indications were found for contamination of rainwater with BL-SRP in the Netherlands. In our two-year survey conducted during the growing seasons, 32 samples of 0.3–2 L of rainwater were analyzed. Only a single positive detection was found in 2023 using the multiplex TaqMan assay for BL-SRP, and further analysis showed that it was contaminated with a *P. brasiliense* strain not belonging to the virulent Clades 1 and 2. *Pectobacterium* species survive well in water with little loss of viability for over

150 days of storage (Van Doorn et al., 2011). If present in the rainwater samples, detection would have been likely.

The average amount of rain between April and July in the Netherlands is 240 mm which is equivalent to 2,400,000 L per ha (KNMI, years 1991-2020). If we assume the presence of 60,000 plants per ha, each plant will receive around 40 L of rainwater during the growing season. In 2021, a total of 30 L of rainwater were sampled. With a sample volume of 30 L tested being negative, the density of BL-SRP in rainwater will be low. We assume that each plant will become maximally exposed with a single cell via contaminated rainwater, insufficient to explain the relatively high disease incidences found in a PB1 crop at the end of the growing season. We therefore consider it unlikely that contaminated rainwater plays a role in the epidemiology of BL-SRP in the Netherlands.

Our findings differ from some previous reports. *P. carotovorum* was recorded in 80% of the samples collected from ocean water, rainwater, and aerosols in the United States, and, incidentally, also included *P. atrosepticum* (Franc, 1988; Harrison et al., 1987). The pathogen has also been found in 5% of the snow samples in the Rocky Mountains, usually originating from the Pacific Ocean (Franc, 1988). It was speculated that the presence of *P. carotovorum* in snow and rain is due to aerosolization of contaminated ocean water then being transported within cloud systems. In contrast, no Pectobacteriaceae were detected in fresh rainwater collected in China and South Korea (Cho & Jang, 2014; Lu et al., 2016).

In conclusion, it is suggested that infected lower grade potato crops located nearby PB1 crops (distance 4–141 m) play a role in the initial infections of PB1 crops with BL-SRP. Transmission of the pathogen may be conducted by insects, but dissemination of the pathogen by other means, such as via transport in or on soil during heavy showers, or via mechanical contact during cropping activities, cannot be excluded. It is assumed that the risk of infection will decrease if PB1 crops are grown in more isolated areas, at sufficient distance from other host plants.

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Author contributions Conceptualization, J.v.d.W.; Writing—Original Draft Preparation, J.v.d.W.; Writing—Review & Editing, M.K., O.M., L.P., and I.v.D.; Methodology, J.v.d.W., V.K., M.K., O.M. and I.v.D.; Supervision, J.v.d.W.; Project Administration, J.v.d.W.; Funding Acquisition, J.v.d.W. All authors have read and agreed to the published version of the manuscript.

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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

Accession numbers All nucleotide sequences of identified insects were deposited in the Genbank data base under the accession numbers PQ567964-PQ568006 (Table S4).

Conflicts of interest The authors declare no conflict of interest.

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