Strategische kennis voor de preventie van bacterieziekten in de pootaardappelteelt: eindverslag

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WAGENINGEN
UNIVERSITY & RESEARCH

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Research was conducted to infections of a (PB1-) potato crop grown from minitubers with blackleg causing soft rot Pectobacteriaceae (BL-SRP). For this, an enrichment multiplex TaqMan assay (EM-TaqMan assay) was developed, allowing to detect the various BL-SRP variants simultaneously with a sensitivity of 10-100 cells per gram plant material.
During four growing seasons (2019- 2022) PB1 crops were surveyed. Plant tissues (leaves, stems and tubers) were collected and analyzed using the EM-TaqMan assay. During wet growing seasons relatively high infection incidences were found both in leaves (8%) and tubers (≤ 42%), in particular with a virulent haplotype of *P. brasiliense*. During dry seasons, only low incidences were found.
It was found that *P. brasiliense* is able to enter a VBNC-state in the presence of cupper ions ($CuSO_4$). However, we were unable to find conditions to let the cells multiply again. Therefore we have no indications that VBNC play a role in the epidemiology of *P. brasiliense.*
The role of potential infection sources was studied. BL-SRP were not found in rain water, but 2.3% of the sampled insects at a PB crop were contaminated with BL-SRP, indicating that insects may be important in the transmission of BL-SRP from an infection source to a PB1 crop.
Inoculum deposited on haulms poses a high risk for tuber infections. This was studied by spray inoculation of haulms of young potato plants with different densities (10^2 – 10^9 cfu per plant) of a virulent, rifampicin resistant mutant of *P. brasiliense*. In a wet growing season, low densities could already result in haulm infections while all plants yielded infected tubers. In a dry year only at the highest inoculum densities haulm infections were found, but tuber infections even at the lowest densities.
Analysis conducted at the NAK to the role of environmental factors on the infection level of seed potatoes were done using data of seed lots tested in high classes in the period 2015-2018. Characteristics pointing to vertical infections (mother to daughter tubers) such as class/generation of a seed lot, shows a strong relationship with the presence of SRP in the daughter tubers. There is a difference in the infection risk between cultivars. Conditions positively related to the risk on horizontal transmission (infections from the environment) are soil characteristics, relative humidity, precipitation at the end of the growing season, and soil saturation, while temperature during the growing season shows a negative correlation with the presence of BL-SRP in daughter tubers.

Keywords: goose, deterrence, quantity regulation

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Photo cover: Survey on infections of a PB1 crop grown from potato minitubers with blackleg causing bacteria

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Samenvatting

Ontwikkeling van detectiemethoden
In het onderzoek naar initiële besmettingen van aardappelplanten met 'blackleg causing soft rot Pectobacteriaceae' (BL-SRP), die de ziekten zwartbenigheid en stengelnatrot ('bacterieziek') kunnen veroorzaken, is het niet alleen belangrijk te weten of deze aanwezig zijn, maar ook welke soort verantwoordelijk is voor de ziekte. *Dickeya solani* en *D. dianthicola*, *Pectobacterium atrosepticum*, en sommige varianten van *P. brasiliense*, zijn vaak agressief, *P. parmentieri* geeft een lagere ziekte-incidentie, terwijl er varianten van *P. brasiliense* zijn die geen bacterieziek veroorzaken. Resultaten uit vorig onderzoek (Deltaplan Erwinia 2.0) lieten zien dat de infectie-incidenties van BL-SRP in een PB1 gewas gegroeid uit miniknollen, laag zijn (≤ 3.4%)[1]. Er werden in dit project in de periode 2019- 2022 surveys uitgevoerd, waarvoor een gevoelige verrijkings-multiplex TaqMan assay werd ontwikkeld die alle BL-SRP varianten gevoelig aantoont, maar waarmee geen onderscheid gemaakt kon worden tussen de varianten. De gedachte was dat gedurende de surveys, (het geringe aantal) monsters dat positief zou zijn in deze verrijkings multiplex TaqMan assay daarna verder geanalyseerd zouden worden met reeds beschikbare simplex TaqMan assays specifiek voor de verschillende varianten. In alle gevallen werd een extractie- en amplificatiecontrole toegevoegd aan de monsters op basis waarvan een vals-negatieve reactie als gevolg van remming van de TaqMan reactie kon worden uitgesloten. De gevoeligheid van de multiplex en simplex TaqMan assays (zonder verrijking) lag tussen 1000 en 100.000 cellen per gram plantmateriaal. De gevoeligheid van de verrijkings-TaqMan assay lag tussen de 10 en 100 cellen per gram plantmateriaal. De methodes zijn met succes toegepast in het onderzoek naar initiële infectie.

Voor experimenteel onderzoek, waarbij planten bewust werden besmet, werd een spontane rifampicine resistente mutant van *P. brasiliense* geselecteerd. Deze merkerstam (mutant) is goed te onderscheiden van wild-type stammen die overal in de natuur voorkomen. Door rifampicine aan het groeimedium toe te voegen, worden de wild-type stammen uitgeschakeld. De groeisnelheid van de mutant en het vermogen om aardappelknollen te laten rotten waren vergelijkbaar met de wild-type stam. Echter, in een veldexperiment met geïnoculeerde knollen gaf de mutant wel significant minder zieke planten dan de wild-type stam (62 t.o.v. 88%). De mutant is toegepast in experimenteel onderzoek naar de relatie tussen de doses waarmee planten worden geïnoculeerd via bladbespuitingen, en de respons (aantal symptomatische en geïnfecteerde bladeren en knollen 8 weken na inoculatie).

Er werden gevoelige en betrouwbare methoden ontwikkeld voor het traceren en kwantificeren van BL-SRP tijdens surveys op basis van een (verrijkings) multiplex TaqMan assay. Het selecteren van een spontane rifampicine-resistente stam van een virulente stam van P. brasiliense maken veldstudies mogelijk naar de epidemiologie van de ziekteverwekker.

Viable but non culturable cells
De detectiemethode van de NAK is gebaseerd op uitgroei van SRP in een vloeibaar groeimedium met polypectaat onder anaerobe condities, waarna het pathogeen met een moleculaire toets (TaqMan assay) wordt aangetoond. Ook in onderzoek naar de populatiedynamica en bestrijding van SRP wordt veelal gebruik gemaakt van een combinatie van methoden gebaseerd op vermeerdering van de ziekteverwekker op of in media en een moleculaire toets. Verrijking verbetert de gevoeligheid van de detectiemethode alleen als de bacterie zich kan vermeerderen. De vraag is of BL-SRP ook in een Viable But Not Culturable (VBNC) vorm kunnen voorkomen, waarin ze wel levend, maar niet kweekbaar zijn. Dit zou met name voor detectie van lage dichtheden van de bacterie in grond een probleem kunnen vormen. Het bestaan van VBNC's is al voor een scala aan bacteriën, inclusief plant pathogene bacteriën aangetoond[2-5]. VBNC's kunnen onder specifieke omstandigheden wel weer 'gewekt' worden en dan ziekten veroorzaken. Voor *P. atrosepticum* is al aangetoond dat deze in een zgn. VBNC-toestand aanwezig kan voorkomen[6]. In dit project is het voor *P. brasiliense* onderzocht. Er werden condities aangelegd waarvan bekend zijn dat deze kunnen leiden tot cellen in een VBNC-toestand. Zo werden de cellen in een koolstof-vrij medium geïncubeerd, onder lage zuurstofspanning gehouden, met een koperoplossing (CuSO4) behandeld, met een hoge concentratie zout,

met bleek of ingevroren en daarna weer ontdooid. Alleen een behandeling met koper resulteerde in cellen die niet uitgroeien op een voedingsbodem maar volgens directe dood/levend kleuringen nog wel actief zijn. Voor deze dood/levend kleuring werden stoffen gebruikt die aantonen dat de celmembraan intact is (Syto9, propidium jodide), en stoffen die enzymactiviteit aantonen (carboxy fluoresceine diacetaat, propidium jodide). Na de kleuring worden de cellen onder de microscoop beoordeeld. Echter de cellen konden niet meer 'gewekt' worden, ook niet door het inoculeren van aardappelknollen of *in vitro* aardappelplanten.

De resultaten wijzen erop dat P. brasiliense mogelijk wel in een VBNC-toestand kan voorkomen, maar niet meer gewekt. We hebben geen aanwijzingen dat VBNC's een rol spelen in de epidemiologie van P. brasiliense.

Dosis respons studies
Er werd in 2021 en 2022 op een zandgrond in Wageningen veldexperimenten uitgevoerd, waarin de relatie werd vastgesteld tussen de dosis (hoeveelheid cellen van *P. brasiliense*) en de respons (infectiepercentage) bij inoculatie van het loof van aardappelplanten (cv. Agria). De planten gegroeid vanuit miniknollen werden in een jong (4-6 blad) stadium bespoten met dichtheden van 10^8, 10^5, 10^3, 10^1 of 0 cellen/ml van de rif-stam van *P. brasiliense*. Tijdens de inoculatie werd geprobeerd om contaminatie van de grond met het inoculum zoveel mogelijk te voorkomen. Ter vergelijking, werd in één van de behandelingen een hoge dosis van de bacteriesuspensie (^{108} cellen/ml) direct rond de planten op de grond aangebracht.

In 2021, waarin tijdens het groeiseizoen veel regen viel, werd al bij een inoculumdichtheid van 10^3 cellen per ml een significant hogere infectie-incidentie in het loof gevonden dan in de negatieve (water-behandelde) controle. Echter, ook in het loof van de negatieve controle werden infecties gevonden, waarschijnlijk doordat de bacterie zich ondergronds via regenwater had verplaatst naar naburige veldjes, en vandaar uit de planten systemisch hadden gekoloniseerd. De dochterknollen van vrijwel alle onderzochte planten waren besmet, ook die van de negatieve controle. Bij bladbespuitingen met een inoculumdichtheid van tenminste 10^5 cellen/ml werden symptomatische planten (achterblijvende groei, verwelking, donker grijsgroene samengeknepen topbladeren) gevonden. Ook na inoculatie van de grond met 10^8 cellen/ml was een hoog percentage van de planten symptomatisch.

In 2022, een jaar met betrekkelijk weinig neerslag, werd er alleen bij een inoculumdichtheid van 10^8 cellen/ml een significant hogere infectie-incidentie in het loof gevonden dan bij de negatieve controle. Tijdens dit seizoen, werden de resultaten beïnvloed door zware besmettingen met wild-type stammen van *P. brasiliense* vanuit de randrijen van aardappels die als buffergewas waren gebruikt. Na loofinoculatie waren de infectie-incidenties van dochterknollen al bij een inoculumdichtheid van 10^1 cellen hoger dan van de negatieve controle. Symptomatische planten werden alleen gevonden na inoculatie van de grond met 10^8 cellen/ml.

Geconcludeerd werd dat ook bij een groeiseizoen met veel regen, alleen na inoculatie van het loof met relatief hoge dichtheden (≥ 10^5 cellen/ml) er een risico is op symptoomontwikkeling. Tijdens een nat groeiseizoen, kunnen er symptoomloze infecties van loof al optreden bij 10^3 cellen/ml, maar bij een relatief droog groeiseizoen zijn hoge inoculum dichtheden nodig (10^8 cellen/ml). Al bij een lage inoculumdichtheid (10 cellen/ml), kunnen er na loofinoculaties, knolinfecties optreden. De hypothese is dat deze lage dichtheden met regenwater vanaf de buitenkant van het loof naar de grond lekken en zo knollen kunnen infecteren. Dit betekent dat ook bij een lage bronsterkte (denk aan insecten) er risico's zijn op knolinfecties.

Initiële infecties
In 2019 en 2020 werden er surveys gehouden op 5 verschillende Nederlandse pootgoedbedrijven om informatie te verzamelen over de infectie incidentie van BL-SRP in een aardappelgewas gegroeid uit miniknollen (PB1-gewas), alsmede over de verdeling van BL-SRP in individuele planten[7]. De resultaten van de surveys in 2019 en 2020 zijn beschreven in de publicatie https://www.mdpi.com/2076-2607/10/12/2504. Deze resultaten zijn niet in dit rapport opgenomen. In de laatste twee weken voor loofvernietiging, werden van 100-200 planten het jonge blad, de stengelbasis en de knollen verzameld en geanalyseerd met de verrijkings multiplex TaqMan assay op de aanwezigheid van *P. parmentieri*, *P. brasiliense*, *P. atrosepticum*, and *Dickeya* spp. Er werd steeds met cv. Agria gewerkt omdat dit ras relatief gevoelig is voor BL-SRP. In 2019, werden er op 4 bedrijven lage infectie-incidenties met *P. parmentieri* (1–6%) gevonden in

bladmonsters. De zwakke reacties in de TaqMan assay gaven aan dat er of zeer lage dichtheden in het blad aanwezig waren of dat (de meeste) cellen dood waren. Op één bedrijf werden reacties in bladeren gevonden met TaqMan assays die *D. zeae* and *D. chrysanthemi* specifiek aantoonde. In 2020, waren onderzochte PB1 gewassen op twee bedrijven (A en B) grotendeels vrij van infecties met BL-SRP. Op een derde bedrijf (C) werd een hoog percentage van besmette knollen gevonden (21%) met een assay voor *D. fangzhongdai*. Het pathogeen werd geïsoleerd en bleek in staat om, na vacuüm infiltratie van knollen, op het veld zwartbenigheid te veroorzaken. Op de laatste twee bedrijven (D en E) werden hoge percentages besmette knollen gevonden met assays voor *P. brasiliense* (35–39%) en *P. parmentieri* (12–19%), maar ook de percentages van bladeren besmet met *P. brasiliense* waren hoog (8%).

In 2021 en 2022 werd de speurtocht voortgezet op de twee bedrijven (D en E) die in 2020 te maken hadden met zware besmettingen met *P. brasiliense* in hun PB1 gewassen. Dit keer werden alleen de oudere bladeren (onder in het gewas) van 100 planten (cv. Agria) bemonsterd en geanalyseerd. In 2021, een relatief nat en koel jaar, werden opnieuw hoge besmettingspercentage bij beide telers gevonden. Bij teler D was 42% van de planten besmet met de virulente *P. brasiliense* bacteriën en 7% met *P. parmentieri*. Bij teler E waren deze percentages respectievelijk 16 en 0%. Op basis van een kwantitatieve TaqMan assay werd geschat dat de stengels tot 10^6 cellen per gram bladmateriaal konden bevatten. Er werden in dit jaar rottende stengels in het bemonsterde PB1 gewas bij beide telers gevonden die zeer hoge dichtheden *P. brasiliense* bevatten. In 2022, een relatief droog en warm jaar, waren de besmettingspercentages met BL-SRP laag (≤ 1%) bij beide telers. Op basis van de assay-waarden kon geconcludeerd worden dat ook de dichtheden laag waren.

Geconcludeerd werd dat hoge infectie incidenties met BL-SRP kunnen voorkomen in een PB1 gewas aan het einde van het groeiseizoen. De incidenties zijn afhankelijk van plaats en groeiseizoen. Bij relatief droog en warm weer werden er lage incidenties gevonden, maar bij nat weer kan de incidentie wel oplopen tot boven de 40%. De virulente groep van P. brasiliense is de meest voorkomende BL-SRP variant die gevonden werd in het PB1 gewas. Dit is op dit moment ook de dominante variant die door de NAK in symptomatische planten wordt gevonden (> 90%). Opvallend was dat infecties òf in de knollen òf in het blad van individuele planten werden gevonden, maar zelden in beide delen van de plant.

Infectiebronnen

Dichtbij gelegen PB2 gewassen
In 2022 werden tijdens het groeiseizoen bij telers D en E niet alleen een PB1, maar ook verschillende keren een dichtbij gelegen PB2 gewas (cv. Agria) bemonsterd, om te onderzoeken of de besmettingen in het PB1 gewas uit het PB2 gewas (cv. Agria) afkomstig konden zijn. In geen enkel jaar (2019-2022) werden in de bemonsterde PB1 gewassen typische bacteriezieke planten gevonden, maar wel in sommige aanpalende PB2 gewassen bij telers D en E (die niet zijn bemonsterd). Bij teler D lag het PB2 gewas dat in 2022 werd bemonsterd op 26 meter, en bij teler E op 59 meter van het PB1 gewas. De hypothese was dat het PB2 gewas eerder besmet zou zijn dan het PB1 gewas en zo een bron van infectie zou vormen. Echter 2022 was een droog en warm jaar en ook in het PB2 gewas waren de infectiepercentages in het loof laag.

Insecten
In 2022 is onderzoek gedaan naar de mogelijke rol van insecten bij de transmissie van BL-SRP. Bij telers D en E werden tijdens het groeiseizoen tweemaal per week (sticky) vangplaten opgehangen. De insecten op de vangplaten werden individueel bemonsterd en geanalyseerd met de verrijkings multiplex TaqMan assay. In totaal zijn er 665 insecten bemonsterd. Bij teler D was 3% en bij teler E 2.3% van alle insecten besmet, veelal met *P. parmentieri* of een virulente groep van *P. brasiliense*.

Regenwater.
De aanwezigheid van SRP in regenwater werd tijdens het groeiseizoen in 2021 onderzocht op 10 plaatsen en in 2022 op 8 plaatsen in Nederland. In totaal werden er 32 monsters geanalyseerd. Per monster werd 0.5-2 liter regenwater op 1.5 meter hoogte verzameld en op ruime afstand van gebouwen. Er werden in 2021 geen SRP gedetecteerd. In 2022 werd in één monster dat zwaar gecontamineerd was met gronddeeltjes, een niet-virulente vorm van *P. brasiliense* gedetecteerd.

Geconcludeerd werd dat insecten mogelijk een rol spelen bij ontstaan van initiële infecties omdat een relatief hoog percentage van de insecten BL-SRP bij zich dragen, de varianten die in Nederland aanwezig zijn in bacteriezieke planten. De insecten kunnen gecontamineerd raken door contact met besmettingsbronnen in een wijde omgeving van een PB1 gewas, omdat insecten zich over grote afstanden kunnen verplaatsen. We hebben niet aan kunnen tonen dat regenwater BL-SRP bevatten, en achten de kans dat besmet regenwater een rol speelt in het ontstaan van initiële infecties klein. Over de rol van infecties in een PB2 gewas of gewassen van een lagere klasse, aanpalend gelegen aan een PB1 gewas, bestaat nog onduidelijkheid.

Rol van omgevingsfactoren op besmettingsniveau pootgoed (bijdrage NAK)

Voor onderzoek naar de rol van omgevingsfactoren op het besmettingsniveau van pootgoed is door de NAK gebruik gemaakt van keuringsgegevens uit de periode 2015 en 2018. Reden hiervoor is dat er die periode een trial plaatsvond waarbij alle verhandelbare partijen PB1-S zijn getoetst op aanwezigheid van SRP in het nacontrolemonster. Een pootgoedpartij werd als besmet aangemerkt op het moment dat één of meer reacties in de nacontrole positief waren voor in ieder geval één van de SRP-soorten (NAK). Algemene kenmerken van de partij zijn afkomstig uit de aangifte. Omgevingskenmerken zoals weerskenmerken (KNMI, 2022) (bijvoorbeeld omgevingstemperatuur, relatieve luchtvochtigheid en zonne-instraling), grondkenmerken (bijvoorbeeld grondsoort en doorlatendheid), raskenmerken (EUROPotato, 2022) en omgevingsgewassen (RVO, 2022) zijn aan een partij gekoppeld op basis van de locatie van het perceel. Omdat ras, klasse en generatie van de partij al veel verklaren werden deze kenmerken altijd meegenomen (basiskenmerken) in het model en werd steeds alleen het te testen kenmerk toegevoegd aan de basiskenmerken.

Er werd gevonden dat kenmerken die duiden op verticale besmetting (van moeder naar dochterknol) zoals bijv. klasse/generatie of een besmette moederpartij, een sterke relatie vertonen met de aanwezigheid van SRP in de dochterpartij. Er is groot verschil in besmettingsrisico tussen rassen. Sommige rassen worden nauwelijks positief getoetst op SRP in het nacontrolemonster, andere rassen regelmatig. Omgevingskenmerken die mogelijk het risico op, of vatbaarheid voor, een horizontale besmetting (vanuit de omgeving) verhogen, vertonen ook een aanzienlijke relatie met de aanwezigheid van SRP in de nacontrole, maar niet zo sterk als kenmerken als klasse/generatie, kwaliteit uitgangsmateriaal of ras. Grondsoort, relatieve luchtvochtigheid en neerslag aan het einde van het teeltseizoen en de grondverzadiging die daarmee samenhangt vertonen allemaal een positieve correlatie met de aanwezigheid van SRP in de nacontrole. Temperatuur in het seizoen vertoont een negatieve correlatie met de aanwezigheid van SRP in de nacontrole.

Summary

Development of detection methods
Potato blackleg can be caused by various soft rot Pectobacteriaceae (SRP). In research to the initial infections of potato plants by blackleg causing SRP (BL-SRP), it is relevant which of these variants are present. *Dickeya solani* en *D. dianthicola*, *Pectobacterium atrosepticum*, and some variants of *P. brasiliense*, are often highly aggressive, *P. parmentieri* gives lower disease incidences, whereas other variants of *P. brasiliense* do not cause disease. Results from previous studies (Deltaplan Erwinia 2.0) indicated that a PB1 crop can be infected by BL-SRP, but the infection incidences are low (≤ 3.4%). In this project a highly sensitive multiplex TaqMan assay was developed able to detect all BL-SRP present in Europe, but the different BL-SRP variants could not be distinguished. We assumed that during surveys of a PB1 crop, an identification of the BL-SRP variant with specific simplex TaqMan assays would only be required for a relatively low number of samples positive in the enrichment multiplex Taqman assay. The multiplex assay included also an extraction- and amplification control supplemented to each reaction to exclude false-negative reactions as a result of inhibition of the reaction. The sensitivity of the multiplex and simplex TaqMan assays (without enrichment) was determined at a density between 1000 and 100.000 cells per gram of plant material. The sensitivity of the enrichment TaqMan assay was determined at a density of 10 to 100 cells per gram of plant material. The methods were successfully applied in epidemiological research on initial infections.

For experimental research, in which plants were infected on purpose, a spontaneous rifampicin resistant mutant of *P. brasiliense* was selected. This strain can be discriminated from wild-type strain ubiquitous present in the environment, by using a growth medium amended with rifampicin. The growth rate of the mutant and the tuber-maceration capacity was similar. However, in a field experiment with inoculated tubers, use of the mutant resulted in significantly less diseased plants than the wild-type strain (62 versus 88%). The mutant is used in dose-response studies, in which plants were inoculated via spraying of haulms. Eight weeks after the inoculation, the number of infected and symptomatic plants (leaves and tubers) was determined.

Sensitive and reliable methods were developed on the basis of (enrichment) multiplex TaqMan assays, to detect and quantify BL-SRP during surveys. The selection of a spontaneous rifampicin-resistant mutant of a virulent strain of P. brasiliense allows field studies on the epidemiology of the pathogen.

Viable but non culturable cells
The detection method for SRP, currently in use at the NAK, is based on an enrichment TaqMan assay, in which samples are first incubated in a (liquid) polypectate medium under anaerobic conditions, to allow selective multiplication of SRP. Enrichment procedures are also often used in studies on the population dynamics and management of SRP. Enrichment will increase the (diagnostic) sensitivity of the assay, but will not detect cells in a viable but non culturable (VBNC) state. The presence of the bacteria in a VBNC state could pose a problem, in particular to detect low densities of the bacteria in soil. The existence of VBNC's have been shown already for a variety of different bacteria, including plant pathogenic bacteria such as for *P. atrosepticum*. Cells in a VBNC state can be resuscitated under specific conditions. In this research project studies have been conducted to VBNC's for *P. brasiliense*. Bacterial suspensions were incubated under conditions known to induce cells in a VBNC state, such as in a carbon free growth medium, under anoxic conditions, in the presence of cupper (CuSO4), a high salt concentration, chlorine or by repeated freeze and thawing cycles of suspensions. Cells seem to enter the VBNC state only after a treatment with cupper, as they did not form colonies anymore on growth medium, but were vital according to live-dead staining methods, in which cells were microscopically examined after staining. For this live-dead staining methods, compounds were used indicating an intact cell membrane (Syto9, propidium iodide), and compounds indicating an enzymatic activity (carboxy fluorescein diacetate, propidium iodide). Nevertheless, we were unable to reactivate the cells, even not after incubating potato tuber tissue or by stem-inoculation of *in vitro* potato plants.

Results indicate that P. brasiliense may enter the VBNC state, but we have been unable to find conditions to let the cells multiply again. We have no indication that VBNC play a role in the epidemiology of BL-SRP.

Dose response studies
In 2021 and 2022, field experiments were conducted on a sandy soil in Wageningen, in which the relation was determined between the dose (number of cells of *P. brasiliense*) and the response (percentage infection and symptomatic plants) after haulm inoculation of potato plants (cv. Agria). Plants were grown from minitubers and spray-inoculated in a 4-6 leaf stage with 10^8, 10^5, 10^3, 10^1 and 0 cells/ml of the rifampicin resistant (rif) mutant of *P. brasiliense*. During inoculation, contamination of the soil with inoculum was avoided as much as possible. As a comparison, in one treatment soil was inoculated directly around the plants with 10^8 cells/ml.

In 2021, a relatively rainy growing season, already at an inoculum density of 10^3 cells/ml, a significantly higher disease-incidence in haulms were found than in the negative (water-treated control). However, also in the haulms of the negative control, infections were found, probably because the pathogen was translocated with rainwater to neighboring fields, from where plants were colonized systemically. All plants yielded infected tubers, also plants of the negative control. At an inoculum density of minimally 10^5 cells/ml symptomatic plants were found. Also after inoculation of soil with 10^8 cells/ml a high percentage of the plants became symptomatic.

In 2022, a year with a limited amount of precipitation during the growing season, only at an inoculum density of 10^8 cells/ml a significantly higher infection-incidence in the haulms was found than in the negative control. The experiment was slightly compromised by a high natural infection with a (wild-type) strain of *P. brasiliense* present in the border rows. After haulm inoculations, the infection incidences of the tubers were already higher than of the negative control at a density of 10 cells/ml. Symptomatic plants were only observed after inoculation of soil with 10^8 cells/ml.

In conclusion, during a rainy growing season, spray-inoculation of haulms with relatively high bacterial densities of ≥ 10^5 cells/ml gives a high risk on symptom development. Haulm infections can already occur at 10^3 cells/ml, but at a relatively dry growing season high inoculum densities are required (10^8 cells/ml). Tuber infections can already occur at a low inoculum level of 10 cells/ml during haulm inoculation, this is independent on the weather conditions. It is hypothesized that low densities deposited on the leaf surface, can wash off to the soil, resulting in tuber infections. This implicates that even a low source strength (for example transmission of bacteria from contaminated insects), there is a risk for tuber infections.

Initial infections studied via surveys
Information on the infection incidence of blackleg causing soft rot Pectobacteriaceae (BL-SRP) in potato crops grown from minitubers (PB1-crop) and the distribution of BL-SRP in individual plants was collected during a four-year's survey (2019 – 2022). In the first two years the survey was conducted at five growers located in the Netherlands and in the last two years at two growers. In the weeks before haulm destruction, leaves, stems and tubers of 100 or 200 plants of the susceptible variety Agria were analyzed separately for the presence of *Pectobacterium parmentieri*, *P. brasiliense*, *P. atrosepticum* and *Dickeya* spp. Extracted plant parts enriched for BL-SRP were analyzed with TaqMan assays specific for detection of blackleg causing (BL-SRP). In 2019, low incidences with *P. parmentieri* (1-6%) in leaves were found at four growers. At one grower, reactions were detected in leaves with TaqMan assays for *D. zeae* and *D. chrysanthemi*. In 2020, crops of two growers were largely free from BL-SRP. At one grower, a high infection incidence (21%) was found for *D. fangzhongdai* in tubers. The isolated pathogen was able to cause potato blackleg. At two other growers, high infection incidences in tubers were found with *P. brasiliense* (35-39%) and with *P. parmentieri* (12-19%), whereas also the incidence of leaves with *P. brasiliense* was high (8%). Results of the surveys in 2019 and 2020 are published in https://www.mdpi.com/2076-2607/10/12/2504. These results are not described in this report.

In 2021, only growers D and E were sampled. At each farm, from 100 plants, three ageing leaves per plant were sampled. Leaf samples were analyzed with an enrichment multiplex assay for BL-SRP, followed by simplex assays for the individual BL-SRP species. At grower D, 45% of the samples were BL-SRP positive: 42% with *P. brasiliense* and 7% with *P. parmentieri*. At grower E, 16% of the samples were BL-SRP positive:

only *P. brasiliense* was found. The estimated densities in the ageing leaves, as determined with a quantitative TaqMan assay ranged between 10^2 and 10^6 cfu per gram of leaf material. In 2021, the growing season was wet and in the PB1 crop already rotten stems were observed which all contained high densities of *P. brasiliense* as determined with a quantitative TaqMan assay, while some rotten stems also were infected with *P. parmentieri*. In 2022, relatively dry year with high temperatures, the infection percentages with BL-SRP were low (≤ 1%) at both growers. On the basis of the TaqMan values, it was also concluded that densities were low.

In conclusion, high infection incidences with BL-SRP in potato can been found in a PB1 crop at the end of the growing season. The risk for infections is dependent on location and growing season. At relatively dry and warm weather, low incidences were found, but at the end of a rainy season the incidences can be up to 40%. The virulent group of P. brasiliense is the most dominant BL-SRP variant found. This is also the dominant variant detected by the NAK in symptomatic plants. Interestingly, infected plants were infected in tubers or in leaves, but rarely in both organs.

Infection sources

Nearby located PB2 crops
In 2022, at growers D and E, not only a PB1, but also a nearby located PB2 crop (cv. Agria) was analyzed several times to determine if infections of the PB1 crop can be explained by transmission of BL-SRP from the PB2 to the PB1 crop. During the four-year's survey, symptomatic plants were never observed in PB1 crops, but incidentally blackleg-diseased plants were observed in PB2 crop. At grower D, the PB2 crop was located at 26 meters, and at grower E at 59 meter from the PB1 crop. Unfortunately (for the survey, not for the farmer), 2022 was a dry year and also the infection percentage of the PB2 crop was very low, despite the fact that in 2021 at the same farms high infection rates were found.

Insects
In 2022, research was conducted to the possible role of insects in the transmission of BL-SRP. At growers D and E, sticky yellow insect traps were placed twice a week. The insects on the traps were sampled individually and analyzed with the enrichment multiplex TaqMan assay. In total 665 insects were analyzed. At grower D, 3% and at grower E, 2.3% of all insects were contaminated, frequently with *P. parmentieri* or the virulent variant of *P. brasiliense.*

Rainwater
The presence of SRP in rainwater was investigated in the growing season of 2021 at 10 locations and in 2022 on 8 locations distributed over the Netherlands. In total 32 samples were analyzed. Per sample 0.5-2.0 liter of rainwater were collected in the open field at a height of 1.5 meter. Bacteria in rainwater was concentrated and analyzed by dilution plating on DL-CVP or by the enrichment multiplex TaqMan assay. In 2021, no BL-SRP were detected. In 2022, one sample, heavily contaminated with soil particles, was contaminated with a non-virulent variant of *P. brasiliense*.

Insects may play a role in the generation of initial infection, because a relatively high percentage of the insects carry BL-SRP, more specifically the variants present in blackleg diseased plants. Insects can become contaminated by contact with infection sources in a wide area around the PB1 crop as they can move over long distances. We have not found BL-SRP in rainwater and consider it as unlikely that they play an important role in the epidemiology. The possible role of infections in lower grade seed potatoes, growing nearby a PB1 crop, is still unclear.

Role of environmental factors on the infection level of seed potatoes (contribution NAK)
To study the role of environmental factors in the infection levels of seed potatoes, testing results were used from the period 2015 and 2018 collected by the NAK. In this period, in a trial, all tradable seed lots in the classes PB1 to S were tested on the presence of BL-SRP. From each seed lot 200 tubers were tested in 4 samples of 50 tubers. A seed lot was considered as infected if one or more samples were positive. The factors as weather conditions (KNMI) such as relative humidity and (sun) radiation, soil characteristics (e.g. permeability, percentage lutum), cultivar (EUROPotato) and surrounding crops were linked to location. Cultivar, class and generation were used as basic characteristics, because they are already known as

important variables explaining the risks on infections with BL-SRP. In the model the test characteristics was added to the basic characteristics.
Characteristics pointing to vertical infections (from mother to daughter tubers) such as class/generation of a seed lot, shows a strong relationship with the presence of SRP in the daughter tubers. There is a considerable difference in the infection risk between cultivars. Some cultivars are rarely tested positive on BL-SRP. Environmental conditions positively related to the risk on horizontal transmission (infections from the environment) are soil characteristics, relative humidity, precipitation at the end of the growing season, and soil saturation. Temperature during the growing season shows a negative correlation with the presence of BL-SRP in the daughter tubers.

1 Introduction

Soft rot Pectobacteriaceae (SRP) comprise the genera *Dickeya* and *Pectobacterium*, which are responsible for diseases in various important crops worldwide including potato, tomato, maize, cabbage, and ornamental plants [8]. In potato, SRP can cause soft rot, blackleg and slow wilt, responsible for high economic losses due to yield loss, and downgrading and rejection of seed lots [9].

Seed potatoes have been recognized as by far the most important source of disease development [9, 10]. Nevertheless, even after many years of research, knowledge on the source of initial infection is still limited. Seed potato cultivation starts with the use of minitubers or tubers from clonal selection, virtually free of SRP, but already in the first year of tuber multiplication, infections can occur [1, 7].

Infections may start from soilborne inoculum, but several studies indicated that the pathogen only survives for a limited period in soil, even in association with plant debris [9]. It is, however, difficult to conclusively exclude soil as a source of infection as detection of low inoculum densities in bulk soils remains a challenge. In addition, the role of nematodes, soilborne arthropods or mollusks in which SRP may live for longer periods than free in soil, cannot be fully excluded [11 12].

A more likely pathway for initial infections is via airborne inoculum that is carried by rain, aerosols, insects, machines, furs, feathers or clothes, including boots and shoes, by animals or laborers [13]. The inoculum may be deposited on the haulms, after which wounds or natural openings, such as stomata or hydathodes, may serve as a port of entry to establish an infection [14]. The inoculum may also migrate via water from haulms into soil after which infections of tubers can occur [14]. In theory, transmission from infected haulms can also result in root infections and consecutively in infections of stolons and tubers, but this pathway has not been studied yet.

The aim of our studies was to extend our knowledge on the sources responsible for and pathways of SRP resulting in initial infection of a potato crop. To make this possible, diagnostic methods were developed and/or optimized which were used in studies to address the following topics.

1. *The forming of cells of P. brasiliense in a VBNC state*. According to the literature, SRP can only survive for a limited period in soil, but can it be that cells at a certain time are not culturable but still alive? For *P. carotovorum* it has been found that they can exist in a 'viable but not culturable' (VBNC) state, if they are grown on an agar medium with a low carbon concentration [6]. As such, populations will be not detected using techniques based on cultivation. In this project the question is answered if cells of *P. brasiliense* can form cells in a VBNC state, and if yes, what their potential role is in the epidemiology. The effect of various environmental conditions which are known as inducive for the forming of cells in a VBNC state has been applied. Attempts were made to resuscitate cells present in a VBNC state.
2. *The distribution of BL-SRP in infected plants of a PB1 crop.* Where do the first infections of potato plants grown from SRP-free minitubers (PB1 crop) occur: in the tubers, the stem base, or the leaves? We assumed that tuber infections in the absence of leaf infections points to soil as inoculum source. Leaf infections without tuber infections indicates an important role of airborne inoculum. If both leaves and tubers of individual plants are infected, it is likely that the inoculum migrated from leaf to soil. To answer this question, surveys of PB1 crops on 5 farms in the Netherlands were conducted in 2019 and 2020. Results have been reported in Van der Wolf et al. (2022) [7].
   This final report does not include results of the surveys of 2019 and 2020. In this report, surveys at two farms conducted in 2021 and 2022 are described, in which only leaves were analyzed.
3. *Sources and carriers responsible for infection of a PB1 crop.* In literature, rain water has been indicated as a potential infection source [15, 16]. Therefore, in a two years survey, rain water was collected in the open space and in different locations in the Netherlands to estimate the presence and densities of BL-SRP. In the literature, also insects have been found contaminated with BL-SRP [17, 18]. In theory, insects can be an important carrier of the pathogen. A survey for the presence of BL-SRP on insects collected from sticky traps was done in PB1 crops at two farms. Finally, also a PB2 crop growing near the

PB1 crop was surveyed at different times during the growing season to determine if the PB1 infections could be explained by cross-contaminations from a nearby located PB2 crop.

4. Risks of contamination of haulms. In two-years field experiments, the risks of tuber infections after contamination (spray -inoculation) of haulms of young potato plants was investigated. The dose-response relation was estimated using a spontaneous rifampicin resistant strain of *P. brasiliense*.
5. The NAK investigated the role of environmental conditions on the infection levels of seed potatoes [19]. For this, data were used from testing of seed lots between 2015 and 2018. In this period, a trial was done in which all traded seed lots in the classes PB1 to S were tested for the presence of BL-SRP. The following factors were included in their studies: cultivar, class, generation, temperature, relative humidity, radiation, soil, and other crops grown in proximity of the seed potato crop. Data were used to develop a model in which these factors were used as predictors for the infection level of the seed lot. Results have been reported separately.

2 Materials and methods

2.1 Bacterial strains and growth conditions

D. solani IPO2222, *P. atrosepticum* IPO1007, *P. parmentieri* IPO1955, *P. brasiliense* IPO3649 were used in this study, while *Acidovorax cattleya* IPO4006 was used as a control for the multiplex TaqMan assays [7]. For field experiments, *P. brasiliense* IPO3649 and a spontaneous rifampicin resistant mutant of *P. Brasiliense* (IPO4211) was used. 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 plant extracts, the plant material was spread plated, in ten-fold serial dilutions 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 [20]. Plates were incubated for 4 days at 28 °C. Cavity-forming colonies typical for SRP were streaked to pure cultures on TSA.

2.2 Sampling of plant material

Generation of rifampicin resistant mutants of *P. brasiliense*
To create spontaneous rifampicin resistant mutants of *P. brasiliense* stam IPO3649, a suspension with an optical density at 620 nm of 0.1 (ca. 10^8 cells/ml) was first plated on a LB plate with 30 µg/ml of rifampicin. Growing colonies were transferred to Luria Broth (LB) or Trypticase Soy Agar (TSA) plates amended with 100 µg/ml rifampicin. The selected mutants grew well on this medium, whereas the wild type strain did not.

Two mutants were compared with the wild-type strain for their soft rot inducing ability on potato tuber slices (cv Alouette) according to the procedure described by Czajkowski et al. (2010) [21]. Briefly, tubers (cv. Alouette) were sliced (1 cm) and slices were subsequently treated with chlorine and water and dried in a flow cabinet. Small wells were punched in the center of each slice after which 20 µl of a suspension (OD600=0.1) of each mutant and of the parental strain was pipetted into the well. Slices were placed on wetted paper and incubated for 4 days at room temperature in closed Petri dishes. The average diameter of the rotten potato tissue was calculated (N=9). No difference in the maceration capacity was found. One of these strains (IPO4211) was used in field experiments.

The ability of bacterial isolates to cause blackleg in a field bioassay was tested as described by De Haan et al. (2008) [22] 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 1 day of drying in a ventilated open space. The wild type strain of *P. brasiliense* (IPO3649) was used as a positive control. On 22 April 2021, tubers were planted in 5 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 2 months.

VBNC experiments

In order to assess if SRP of *P. brasiliense* are able to form VBNCs, strain IPO3649 was incubated under conditions known to induce the VBNC state in other species. Those were incubation in a carbon-free medium (AB medium: 1 g/l NH_4Cl, 0.62 g/l $MgSO_4$ x 7 H_2O, 0.15 g/l KCl, 0.013 g/l $CaCl_2$ x 2 H_2O, 0.005 g/l $FeSO_4$ x 7 H_2O, pH 7.5), AB-medium with the addition of $CuSO_4$ at concentrations of 0.1 mM or 1 mM, AB-medium with the addition of 5% NaCl, AB-medium with the addition of 0.7 ppm NaClO and repeated freeze and thaw treatment. In addition, cells were also kept under near-anaerobic conditions by filling Eppendorf tubes to the rim with AB-medium. Bacterial suspensions of approximately 10^8 CFU/ml were inoculated into the different treatments. Treatments with AB-medium, $CuSO_4$, NaCl and NaClO were sampled daily and plated on LB medium to assess cultivability. The anaerobic and freeze and thaw treatment were sampled once a week.

Viability was assessed by microscopy after live-dead staining using the Live/Dead BacLight kit, which contains Syto9 and PI. In addition, samples from the $CuSO_4$ treatment were stained using CFDA (10 µM) and PI.

Resuscitation was attempted by washing cells 3 times in AB-medium and plating in LB-agar and into liquid PEB (Polypectate enrichment broth). In addition, washed cells were inoculated on potato slices and incubated at 25°C and injected into sterile *in vitro* potato plants. Plant material was extracted after 24 and 48 h and plated on LB-medium.

Field experiment: dose response

In 2021 and 2022, field experiments were conducted in Wageningen (NL) to determine which densities of *P. brasiliense* are required to establish an infection in case potato haulms (cv Agria) were spray-inoculated with a rifampicin-resistant marked strain of *P. brasiliense*. Treatments were organized in a randomized block design approach (Figure 1 and Figure 2).

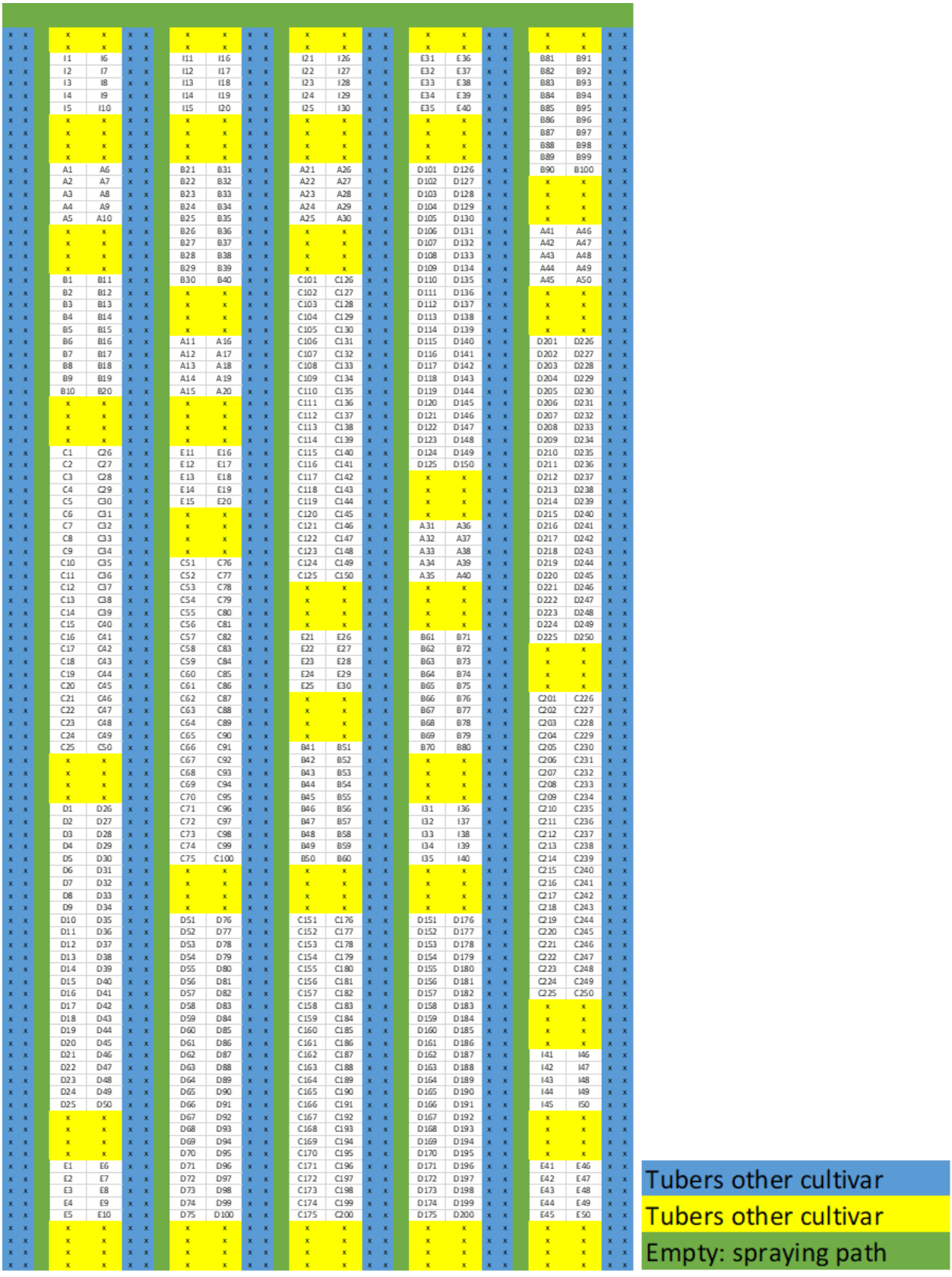

Figure 1 *Lay-out field experiment on dose-response in 2021. Haulms of young plants (cv Agria) were spray-inoculated with a rifampicin-resistant mutant of Pectobacterium brasiliense. A. 10^8 cells/ml, B. 10^5 cells/ml, C. 10^3 cells/ml, D. 10^1 cells/ml, E. 0 cells/ml (control) and I. soil was inoculated around the plants with a density of 10^8 cells/ml.*

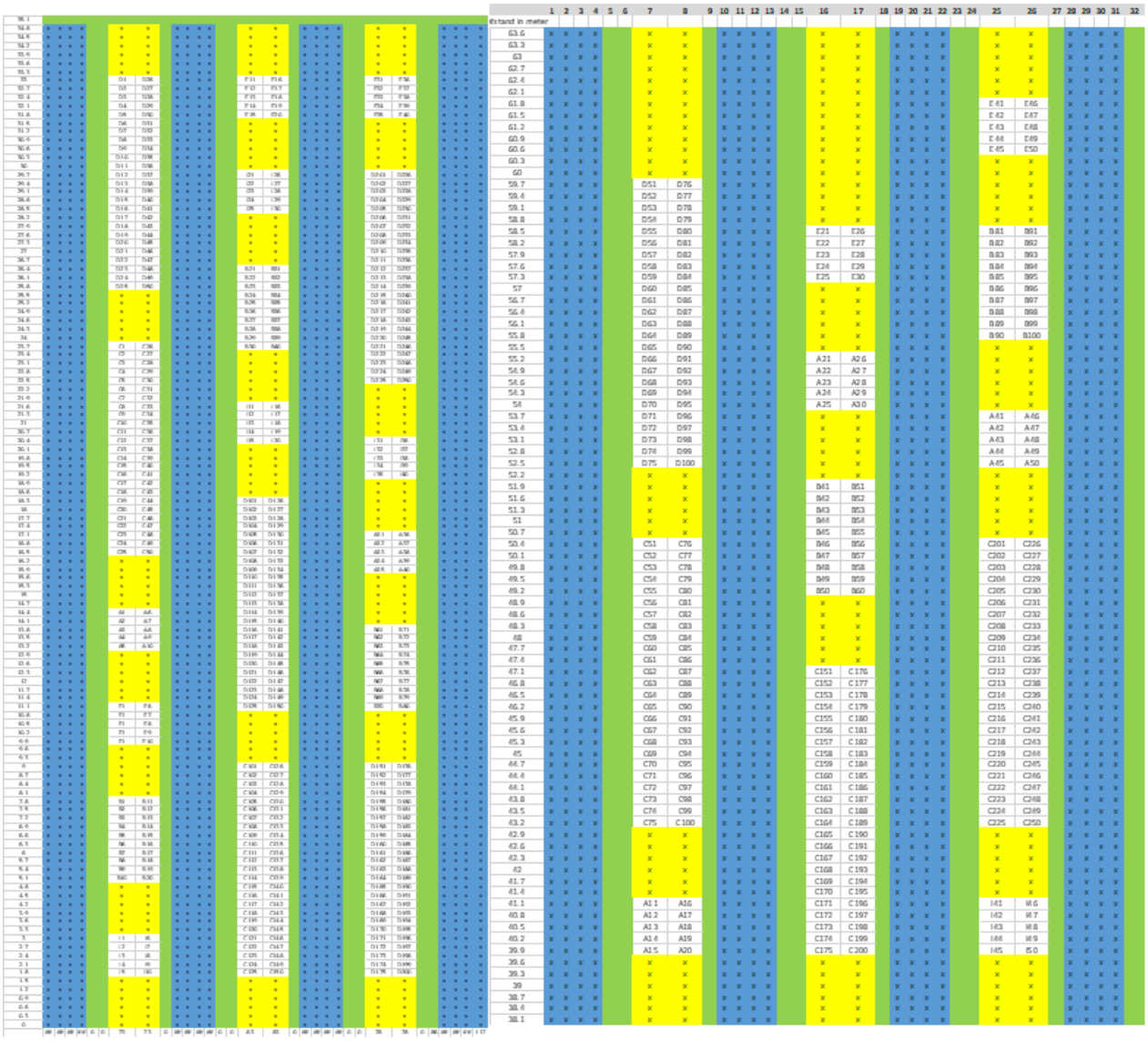

Figure 2 *Lay-out field experiment on dose-response in 2022. Haulms of young plants (cv. Agria) were spray-inoculated with a rifampicin-resistant mutant of Pectobacterium brasiliense. A. 10^8 cells/ml, B. 10^5 cells/ml, C. 10^3 cells/ml, D. 10^1 cells/ml, E. 0 cells/ml (control) and I. soil was inoculated around the plants with a density of 10^8 cells/ml.*

In 2021, tubers were planted on 22 April. In 2021, during spray-inoculation, a back sprayer with pressure was used. During spraying, approximately 50 ml of liquid was released in 3 seconds. To minimize contamination of soil during inoculation two plastic trays (53 X 32cm) (Modiform No. 2057, size 50x30 cm) 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, still inoculation liquid was running from stems downwards resulting in some contamination of soil. Therefore, in 2022, we reduced the inoculum volume to approximately 10 ml and increased the densities to end up with a similar inoculation dose per plant. We used a manual sprayer allowing a more focused spraying resulting in less contamination of the soil. Both experiments were performed in a randomized block design, with 5 blocks per treatment. The number of plants per treatment was dependent on the inoculum density, the higher the density, the lower the number of plants (Table 1). We hypothesized that the infection incidence was dependent on the inoculum dose.

Table 1 *Treatment coding to determine the dose – response for P. brasiliense after spray-inoculation of potato haulms. As a control, also inoculum was supplemented directly on the soil, directly around the stem base of the plant.*

Treatment	Density (cells/ml)	Total nr of plants	Inoculation site
A	10^8	50	Haulms
B	10^6	100	Haulms
C	10^3	250	Haulms
D	10^1	250	Haulms
E	0	50	Haulms
I	10^8	50	Soil

In 2021, samples were collected between 9 and 14 July. 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 well cleaned between each cutting and disinfected with 70% ethanol to prevent cross contaminations. The leaves were transferred to labelled Bioreba bags suitable for extract preparation using a mechanical crusher (AAA lab equipment, Roelofarendsveen, NL). All the tubers per plant were collected in separately labelled plastic bags. Samples were stored in the cold room (4°C) during the night to be processed the next day. In 2022, leaf samples were collected between 13 and 20 July in a similar way as in 2021.

Apart from this, soil samples (200 gram per sample, close to and around the stems) were collected directly after inoculation, (22 April), on the 9th of June and on the 19th of July. Ten samples (2 per block) from the soil inoculated treatment were analyzed using dilution plating on CVP with rifampicin. In 2022, on the 26th of April, 50 soil samples (between 25 and 15 cm deep) were randomly collected through the same 2021 field, at which time of sampling a winter wheat was growing. Soil extracts were analyzed in duplicate directly and after enrichment in pectate enrichment broth [23] supplemented with 100 µg/ml of rifampicin. After DNA extraction of the samples, a TaqMan PCR was performed.

Pathogen detection

For detection of BL-SRP in surveys, a direct (quantitative) TaqMan assay was used or an enrichment TaqMan assay as described previously [7]. Briefly, blackleg causing bacteria present in the Netherlands i.e. *P. atrosepticum* (Pat), *P. parmentieri* (Ppa), *P. brasiliense* (Pbr), *D. solani* and *D. dianthicola* were detected by a (multiplex) TaqMan. In case of a positive reaction in the (multiplex) TaqMan assay, the blackleg causing species was identified by a second series of simplex TaqMan assay, specific for the individual species or variant. For *P. brasiliense*, a triplex TaqMan assay was used comprising of a generic assay detecting all variants and two assays specific for a genetically homogeneous clade in which most strains can cause blackleg, while most strains outside this clade cannot (Van der Wolf et al., 2022). For the enrichment TaqMan, the protocol included incubation of samples under vacuum (anoxic) conditions, favoring selective growth of SRP preceding the DNA extraction and the TaqMan assays.

For the field experiments the following procedures were used. Leaves were weighed and crushed with a Sample crusher (AAA LAB equipment B.V. Roelofarendsveen, NL). The same weight of Ringers supplemented

with 0.2% DIECA was added to the Bioreba bag and the sample was further crushed. One ml of the extract was mixed with 9 ml of pectate enrichment broth supplemented with 100 µg/ml of rifampicin in 10 ml tubes that were incubated for 3 days at 21 °C. One ml was collected, centrifuged for 15 min at 6000 rpm and the pellet frozen at -20 °C prior to DNA extraction and analysis. Per plant, all tubers were washed and placed in a plastic bag. Per tuber, 5 ml water was added, supplemented with 100 µl/ml rifampicin. Tuber peel extracts spiked with IPO4211 (10, 100, 1000 and 10.0000 cells/ml) was used as a control for the enrichment. Bags were vacuumed and incubated for 5 days at 25 °C to allow multiplication of the bacteria. 1 ml of the fluid leaked from the tubers was collected, centrifuged for 15 min at 6000 rpm and the pellet frozen at -20 °C.

Surveys of PB1 and PB2 crops

In 2019 and 2020, farms A-E located in different regions in the Netherlands were surveyed for the presence of BL-SRP (Van der Wolf et al., 2022). In 2021 and 2022, PB1 crops at Farms D and E located in the Northwest of the Netherlands were sampled. These farms were selected, as in 2020 these farms suffered from high infections with *P. brasiliense* in their PB1 crop before harvest. In 2019 sampling was done between 25 June and 24 July, in 2020 between 18 June and 22 July, in 2021 between 26 June and 30 July and in 2022 between 7 June and 15 July. In 2019 and 2020, the top leaves of 100 and 200 individual plants, respectively, were sampled from each plant per grower. In 2021, deteriorating leaves of 100 individual plants per grower at the plant base were sampled, as it was assumed that higher incidences and densities of BL-SRP could be found in ageing leaves than in top leaves. Pruning shears were cleaned between each cutting and disinfected with 70% ethanol to prevent cross-contamination. Every 2–3 plants in a row were sampled. The sampling was only performed from the edge rows and along spraying paths. The leaves were transferred to a labeled plastic bag suitable for vacuum (LDPE bags, transparent, 0.10 mm, 300 × 500 mm, VPP Packaging B.V., Bussum, NL). Samples were transported at room temperature and stored in a cold room (4 °C) overnight to be processed the next day. The growing season of 2021 was very wet and rotten stems were found in the PB1 crop. In addition to the leaves, also 8 rotten stems were collected and analyzed for BL-SRP.

In 2022, the ageing leaves of a PB1 crop were analyzed again, but this time also the ageing leaves of a PB2 crop (cv. Agria), grown nearby the PB1. A scheme of the situation at Farm D and E is provided in Figures 3 and 4. It was hypothesized that infected PB2 plants could be an important source of infection for the PB1 crop.

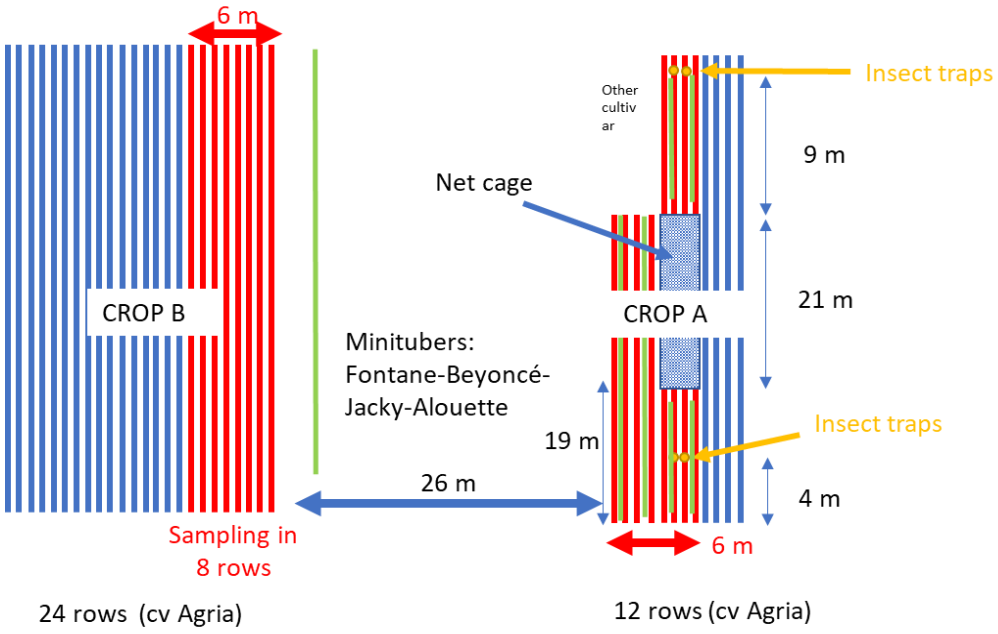

Figure 3 *Situation at Farm D (Zuidschermer, NL) in 2022: CROP A is a PB1 (cv. Agria) crop, CROP B is a PB2 (cv. Agria) crop. Red lines indicate the rows in which sampling was conducted. Sampling was done in 8 rows, alternating taking plants on the left and right side of the row. Blue lines indicate plants of CROP A and B not sampled. Between CROP A and CROP (26 m space), PB1 tubers of the following cultivars were growing: cv. Fontane, cv. Beyoncé, cv. Jacky, and cv. Alouette.*

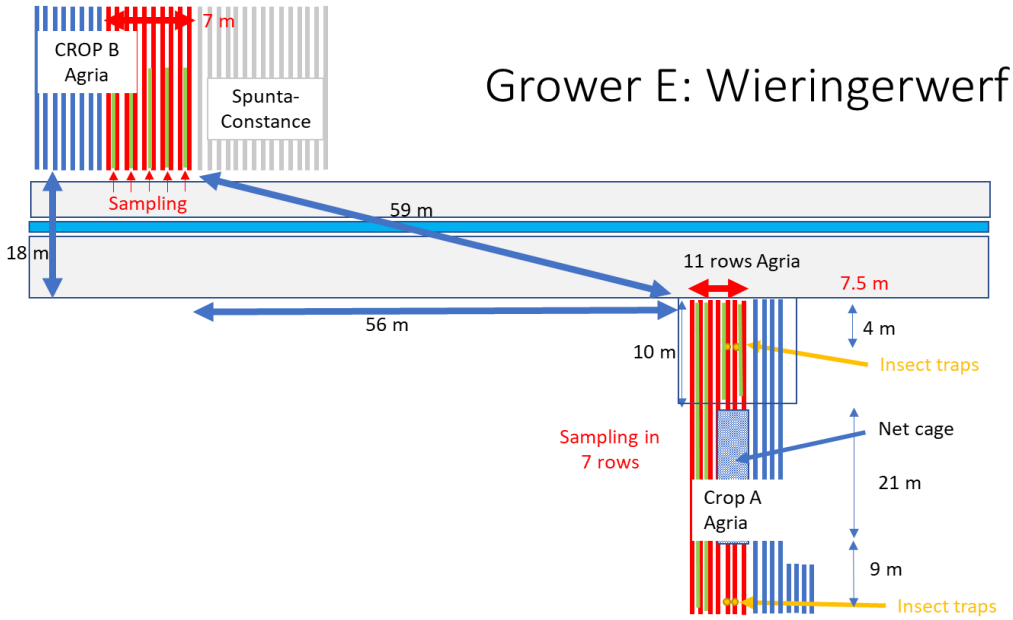

Figure 4 *Situation at Farm E (Wieringerwerf, NL) in 2022: CROP A is a PB1 (cv. Agria) crop, CROP B is a PB2 (cv. Agria) crop. Red lines indicate the rows in which sampling was conducted. Sampling was done in 7 or 8 rows, alternating taking plants on the left and right side of the row. Blue lines indicate plants of CROP A and B not sampled. Grey lines are of crops (Spunta and Constance, respectively) growing adjacent to CROP B (not sampled).*

Coverage of plants with a netting cage

To prevent infections of the PB1 crop surveyed in 2022 by insects, plants were covered with a netting cage with a mesh size of 0.8*0.8 mm and with a size of (21*3*1 m). The cages were placed on 30 May 2022. At grower D, the cage was placed just before emergence of the plants. At grower E, the cage was placed when the plants were already in a two-leaf stage.

Survey of rainwater

Rainwater was collected in polystyrene crates (l*w*h, 0.33*0.55*0.32 m) with a lid. To prevent contamination of rainwater with insects, the lid was removed and an insect repellent gauze (mesh size 1 mm) was secured on top of the crate using an elastic band. The crates were placed on an aluminum standard (Tupola, WUR, Wageningen, UR) at a height of 1.5 m and secured in the open field at a minimum distance of 50 meter from buildings to avoid contamination of splashing water from roofs. Crates remained closed with a lid as long as they were stored. In total 10 standards with crates were made available. In 2021, they were distributed over 10 farmers located at different geographic regions in the Netherlands and in 2022 at 8 farms. Four to eight hours before at least 4 mm precipitation was expected, the standards with the sterilized crates were brought in the open field and the lid was removed. Four to 24 hours after rain fall, the water was collected and concentrated by filtrations through a 0.45 mm filter. Bacteria collected on the filter were resuspended in 5 ml of Ringer solution. Samples were spread-plated undiluted and 10 times diluted on in total four DL-CVP-plates (100 µl per plate). One ml of the concentrate was mixed with 9 ml of pectate enrichment broth in duplicate. Enriched samples were analyzed with subsequently a multiplex TaqMan for detecting all BL-SRP prevailing in the Netherlands and simplex assays to characterize the BL-SRP variant. Cavity forming colonies were analyzed by collecting bacterial slime with a sterile tooth pick followed by TaqMan analyzes of the boiled suspensions (5 min, 100 °C).

Survey on insects

To study the survival on the insects, after trapping the insects in Wageningen, they were taken to the laboratory and spray-inoculated with a suspension of a rifampicin resistant mutant of *P. brasiliense* of ca. 10^8 cells/ml after which traps were placed back in the field. The bacteria were collected immediately after spraying and analyzed (T=0), or one (T=1) or two days (T=2) later, using an enrichment TaqMan assay (T=0). During each time point also a sample of the sticky trap without an insect was analyzed.

Survival in soil

In April 2022, soil samples were collected at a depth of 5-15 cm from a field plot at Wageningen from which the previous year symptomatic potato plants were harvested. These potato plants were grown from tubers vacuum-infiltrated with the rif-strain of *P. brasiliense*. In total 50 soil samples were evenly collected from an area of 880m^2. In case volunteer potato plants were found, samples were collected from the potato plant. DNA was extracted from soil with the MagAttract Power Soil DNA KF kit according to the manufacturer's instructions and analyzed with the TaqMan assay for *P. brasiliense*.

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, and n is the number of individuals per subsample [24]. For analyzing the incidence of infected and symptomatic plants, analysis of variance was performed on the number of diseased plants 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 post hoc test (P=0.05).

3 Results

VBNC experiments
Only inoculation with 0.1 or 1 mM $CuSO_4$ resulted the inability to cultivate *P. brasiliense* on LB-medium within 2 days. Cells subjected to the other treatments still showed colonies on LB-medium after several weeks of incubation.

Live-dead staining of cells incubated with CuSO4 after 2 days showed a large number of living cells, both with Syto9 and with cFDA, which indicates that the cells were alive, but not cultivable. However, cultivability could not be restored with any of the resuscitation treatments.

Field experiments
In 2021, a field experiment was conducted to compare the virulence of the rifampicin resistant *P. brasiliense* strain IPO 4211 with the parental wild type strain IPO3649 using vacuum infiltrated tubers. The disease incidence of rifampicin resistant strain was 62%, significantly less than the wild type strain (88%) (P=0.05). The mock-inoculated tubers did not yield symptomatic plants.

Dose response studies
In 2021 and 2022, field experiments were conducted in Wageningen (NL) to determine which densities of *P. brasiliense* were required to establish infections in case potato haulms were spray-inoculated with *P. brasiliense*. The growing season of 2021 was relatively cool and wet, whereas the growing season of 2022 was warm and dry (Figure 5). The year 2022 was ranked as the year with the highest number of sun hours in the Netherlands since 1901 (Table 2).

Table 2 *Weather conditions in 2021 and 2022 between 1 April and 31 August (source KNMI). Between brackets: ranking from highest to lowest for the years between 1901 and 2022.*

	Avg Temp. (°C)	Sun hours	Precipitation (mm)
2021	14.2 (42)	976 (44)	386 (23)
2022	15.8 (6)	1267 (1)	284 (77)

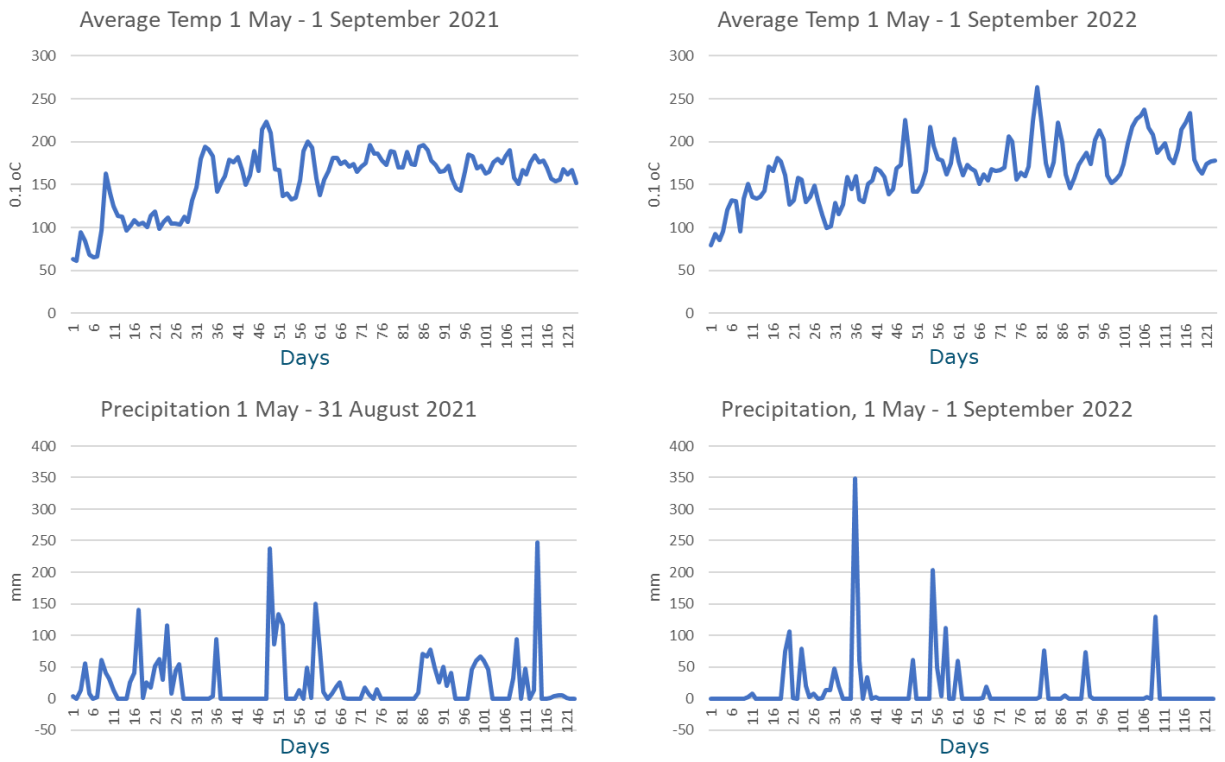

Figure 5 *Data on temperature and precipitation in the growing season of 2021 and 2022 (NL), collected from a weather station located between farms D and E (Berkhout, data source KNMI).*

In 2021, using the enrichment TaqMan assay, a dose – response effect was found. At the highest inoculum densities of 10^8 or 10^5 cells/ml, leaf infections were found in all plants (Figure 6B). At an inoculum density of 10^1 cells/ml the incidence dropped significantly to 70%, but even in the water control around 50% of plants leaves were infected. Also using the direct TaqMan assay a dose response effect was found, with 87% positive samples positive using an inoculum density of 10^8 cells/ml and 43% at 10^1 cells/ml; in this analysis 40% of the mock-inoculated plants were positive (Figure 6A). Almost all tuber samples were positive in the enrichment TaqMan assays; even in the mock-inoculated tubers, 100% of the samples were positive (Figure 6C). Symptomatic plants showing reduced growth, wilting, upward folded top leaves with a dark grey discoloration were only found at the two highest inoculum densities of 10^8 and 10^5 cells per ml, with incidences of respectively 18 and 8% (Figure 6D). After soil inoculation, 20% of the plants became symptomatic. As expected, 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) (Figure 7). In plants spray-inoculated with the highest density of 10^8 cfu/ml, also high densities were found in soil one day after inoculation (data not shown).

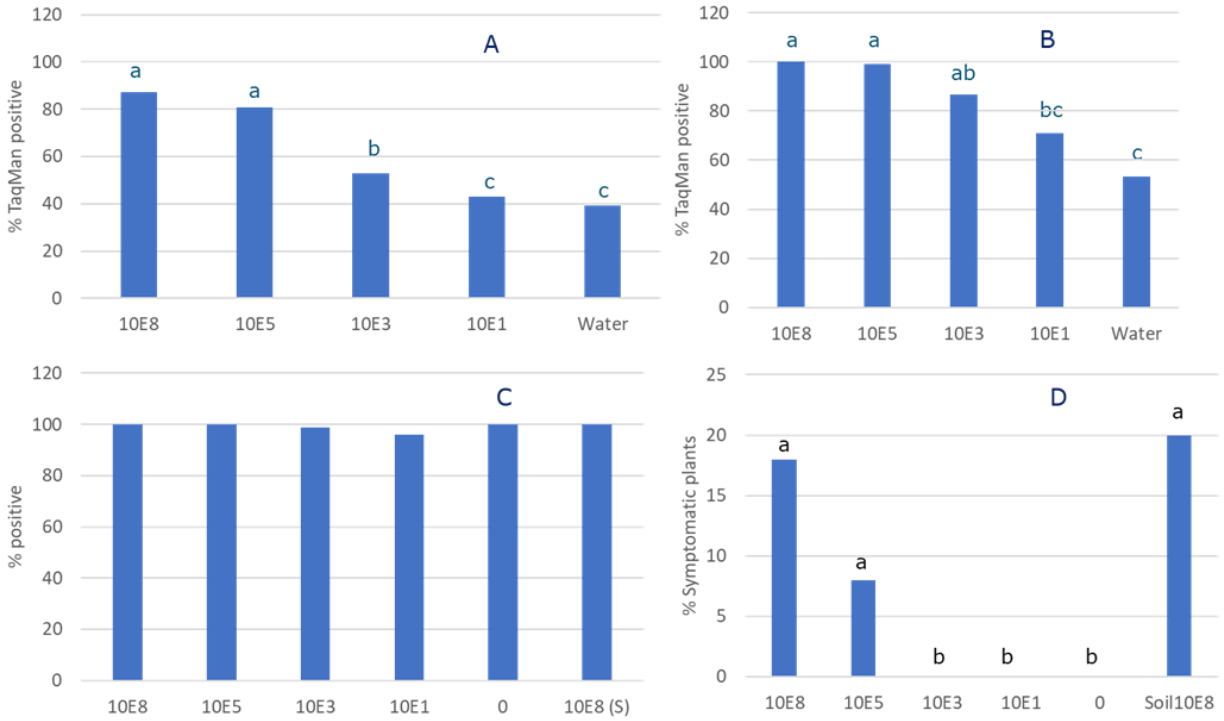

Figure 6 *Dose-response studies in 2021. Haulms of young plants growing in the field in Wageningen (NL) were spray-inoculated with different densities of a rifampicin-resistant mutant of Pectobacterium brasiliense or soil was inoculated around plants with a density of 10^8 cells/ml (10E8 (S)). A. Percentage of haulms positive in a direct TaqMan assay, B. Percentage of haulms positive in an enrichment TaqMan assay, C. Percentage of daughter tubers in an enrichment TaqMan assay (values were not significantly different), D. Percentage of symptomatic plants based on visual observations. Bars with an identical letter were not significantly different (P=0.05).*

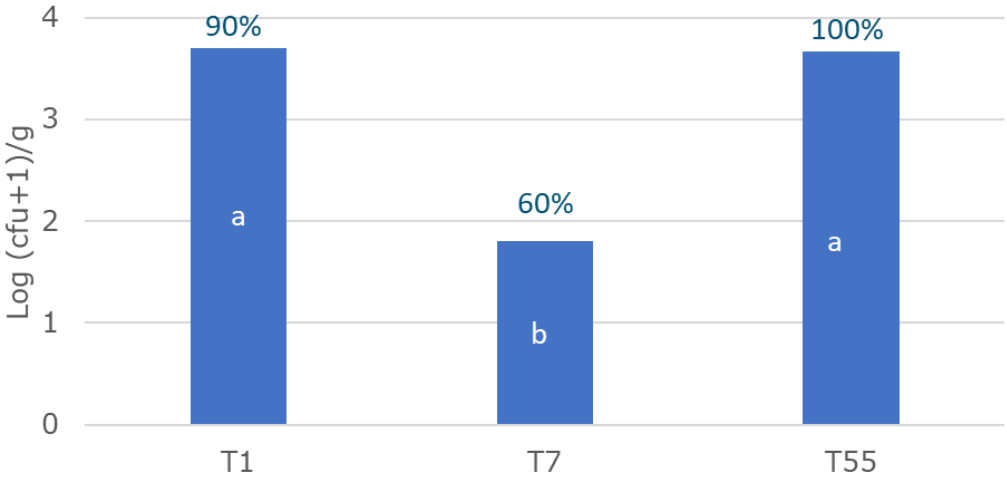

Figure 7 *Densities of a rifampicin resistant Pectobacterium brasiliense strain in soil after soil inoculation in the field experiment of 2021 at 1, 7 and 55 days post inoculation. Above the bars the percentages of positive samples are provided (N=10). Densities were determined by dilution plating. Bars with an identical letter did not significantly differ in densities (P=0.05).*

In 2022, unintentionally, seed potatoes were planted in border rows heavily infected with *P. brasiliense* which caused a high level of blackleg diseased plants in our target crop due to cross-contamination. Blackleg diseased plants from the border crop were removed with their seed potatoes on the 4th of June, but at that time the cross-contamination already happened. Based on the direct TaqMan assay results, from the spray-inoculated plants, only those inoculated with 10^8 cells/ml showed a significantly higher infection incidence

(85%) than all other treatments including the water control and the soil inoculated treatment (Figure 8A). The infection incidence of the other treatments was not significantly different and varied in disease incidence 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 direct assay, except for the treatment with 10^8 cells/ml (Figure 8B). This can be explained by the enrichment procedure, which was done in the presence of rifampicin. The lower disease incidences found in the enrichment TaqMan than in the direct TaqMan assay indicated that samples were infected with the wild strain of *P. brasiliense* disseminated from the border rows, as they will not grow in the presence of rifampicin. Spray-inoculation of plants with 10^8 cells/ml resulted in 100% infected tuber samples (Figure 8C). The incidence decreased significantly to 60% for the inoculation with 10 cells/ml, whereas only 18% of the mock-inoculated tuber samples were positive. All tuber samples were positive after the soil treatment with 10^8 cells/ml. This year, none of the spray-inoculated plants became symptomatic, whereas 4% of the plants of the soil-inoculated treatment showed blackleg.

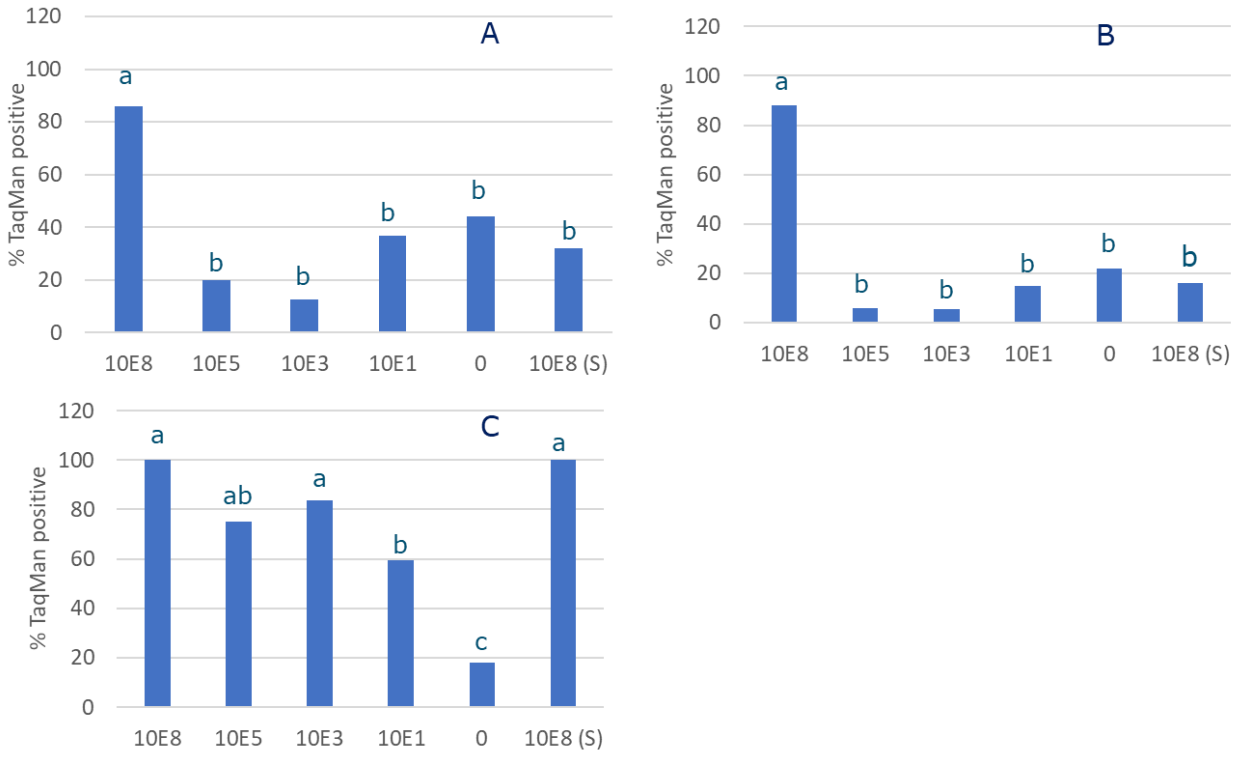

Figure 8 *Dose-response studies in 2022. Haulms of young plants growing in the field in Wageningen (NL) were spray-inoculated with different densities of a rifampicin-resistant mutant of Pectobacterium brasiliense or soil was inoculated around plants with a density of 10^8 cells/ml (10E8 (S)). A. Percentage of haulms positive in a direct TaqMan assay, B. Percentage of haulms positive in an enrichment TaqMan assay, C. Percentage of daughter tubers in an enrichment TaqMan assay (values were not significantly different), Only 4% of plants became symptomatic after soil-inoculation. Bars with an identical letter were not significantly different (P=0.05).*

Surveys of PB1 and PB2 crops

In 2021, 46% of the leaf samples in the PB1 crop at farm D were positive in the enrichment multiplex TaqMan assay, whereas 30% were positive in the direct assay. At farm E these values were 21 and 5%, respectively (Figure 9). Ct-values of the leaf samples in the direct TaqMan assay were as low as 22 for farm D and 19 for farm E, indicating high pathogen densities (Figure 10). At farm D, the virulent group of *P. brasiliense* was detected in almost all BL-SRP positive samples, whereas *P. parmentieri* was detected in 7% of the samples (Figure 11). At farm D, in 16% of the BL-SRP-positive samples the virulent clade of *P. brasiliense* was detected, and in 5% of the samples *P. brasiliense* belonging to other haplotypes was detected.

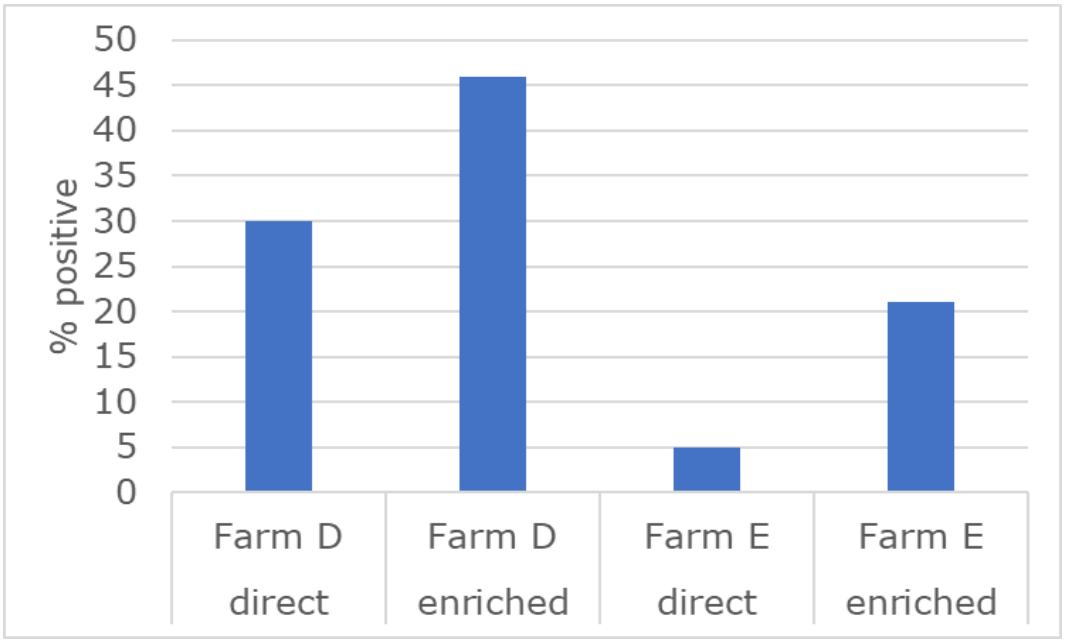

Figure 9 *Infection incidence of ageing leaves in two PB1 crops at farms D and E each in 2021 based on the results with a direct (quantitative) multiplex TaqMan assay for blackleg causing soft rot Pectobacteriaceae or with the multiplex TaqMan assay after enrichment. Hundred leaf samples per crop were analyzed.*

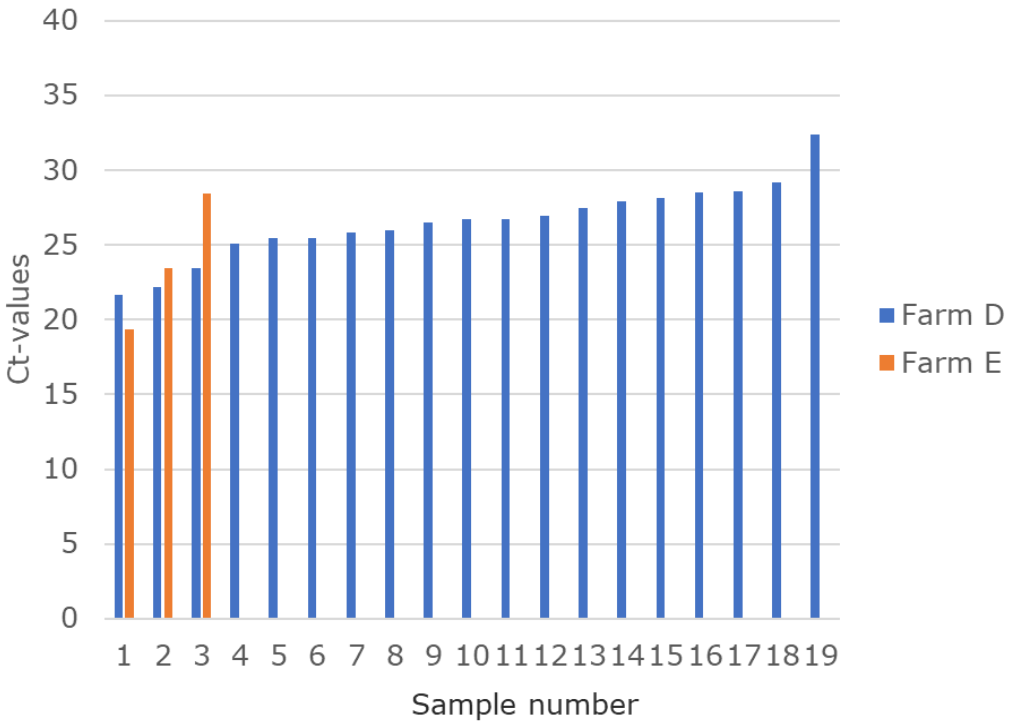

Figure 10 *Leaf samples positive in the direct multiplex TaqMan assay for blackleg causing soft rot Pectobacteriaceae collected at Farm D and farm E in 2021 In total 40 samples were tested. Ct-values below 35 were considered positive.*

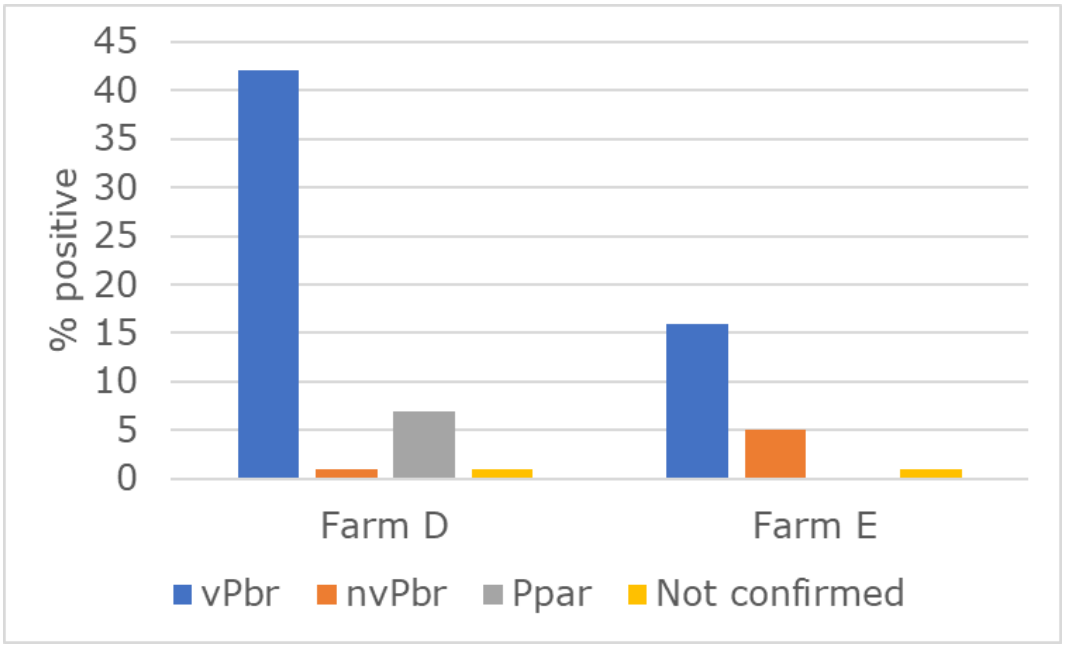

Figure 11 *Survey of two PB1 crops, one at Farm D and one at farm E in 2021. Percentages of leaf samples positive in TaqMan assays for a virulent (vPbr) and a non-virulent (nvPbr), respectively, variant of P. brasiliense and for P. parmentieri (Ppar) are provided. Hundred leaf samples per grower were analyzed. Some leaf samples positive in the multiplex TaqMan assay for blackleg causing bacteria could not be confirmed in the simplex TaqMan assay for the individual variants.*

At farm D, eight collected rotten stems were all strongly positive in the direct (quantitative) TaqMan assay for BL-SRP with Ct-values between 10 and 27 (Figure 12). At Farm E, seven rotten stems were (strongly) positive with Ct-values between 12 and 17, whereas one sample was negative. At farm D, all eight samples were infected with the virulent clade of *P. brasiliense*, whereas 37.5% was infected with *P. parmentieri* (Figure 13). At farm E, 75% of the stems were infected with the virulent clade of *P. brasiliense* and in 25% of the stems *P. brasiliense* belonging to other haplotypes were detected.

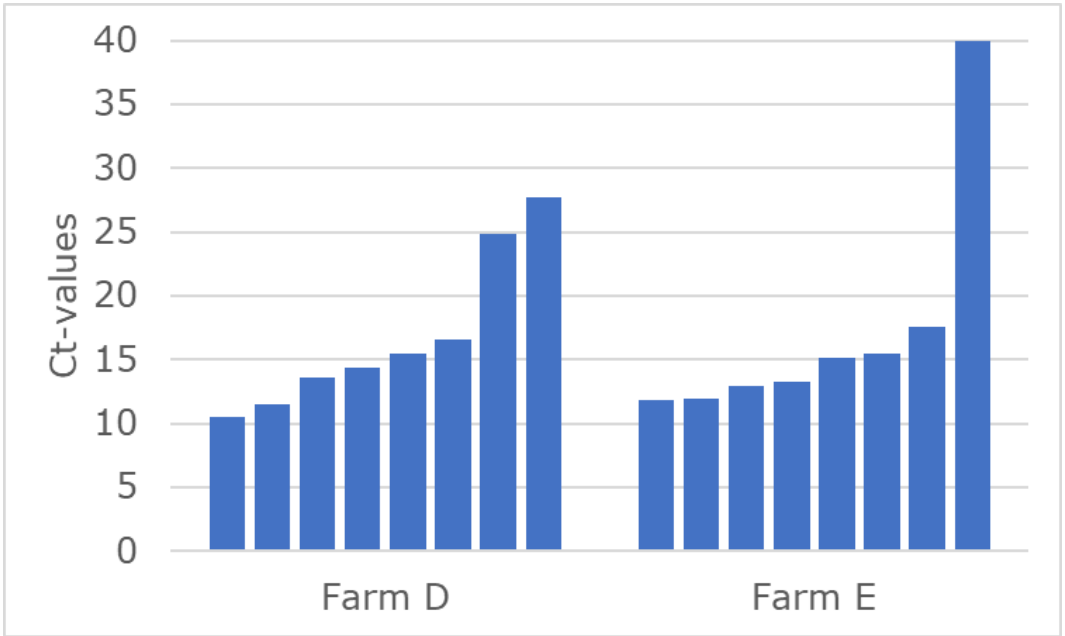

Figure 12 *Ct-values of 8 rotten stem samples collected in 2021 at Farm D and farm E using a direct multiplex TaqMan assay for blackleg causing soft rot Pectobacteriaceae. Ct Values below 35 are considered positive.*

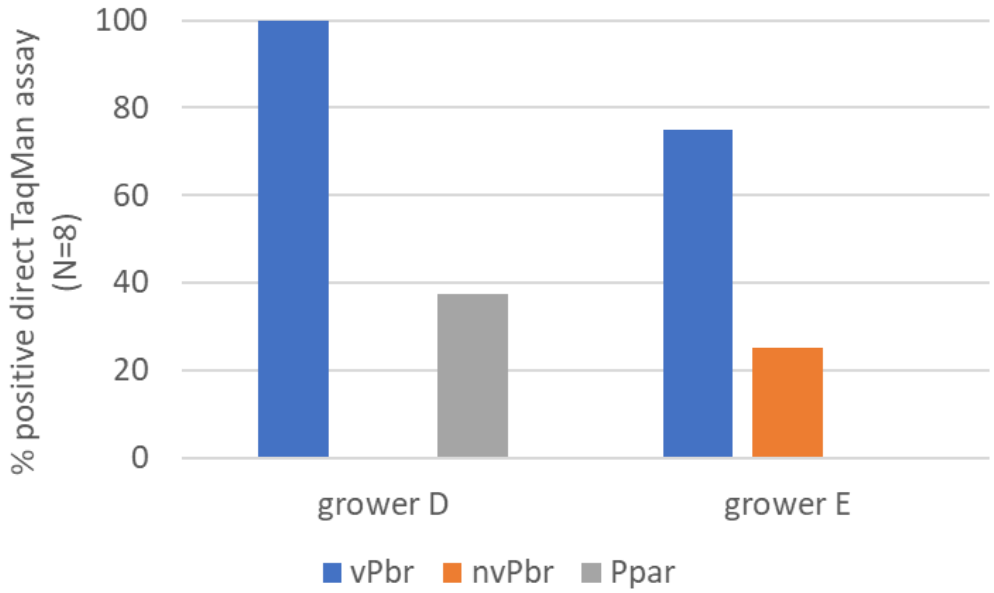

Figure 13 *Survey of a PB1 crop at Farm D and E in 2021. Percentages of rotten stems positive in the direct TaqMan assays for a virulent (vPbr) and a non-virulent (nvPbr) variant of P. brasiliense, respectively,and for P. parmentieri (Ppar) are provided (N=8).*

In 2022, the percentage of infected leaf samples in the PB1 crop was determined during the growing season. Per sampling date, 50 ageing leaf samples per farm were collected from a PB1 cv. Agria crop and a PB2 cv. Agria crop located nearby the PB1 crop. In Figures 3 and 4, situational diagrams are provided for both farms. We hypothesized that insects are important carriers of BL-SRP. Therefore, additionally, 200 PB1 plants were grown under a net cage to prevent plants from visiting insects. We expected a lower level of BL-SRP infections in these plants.

Results are summarized in Table 3. In general, the level of infection with BL-SRP was very low. At grower D, in the PB1 crop, leaf samples of in total 300 unprotected plants were tested from which 2 were positive in the enrichment TaqMan. In the net cage protected plants, no infections were found. At grower D, in the PB2 crop 4 out of 300 leaf samples were positive. At grower E, 5 out of 300 leaf samples of the unprotected PB1 plants were positive in the enrichment TaqMan assay and 1 in the net cage protected plants. In the PB2 plants, 3 out of 300 leaf samples were positive. We did not found an increase in the percentage positive samples during the season. From 15 positive leaf samples, 14 were positive for the virulent clade of *P. brasiliense* and 1 for strains belonging to other haplotypes of *P. brasiliense*.

Table 3 *Percentage of leaf samples of PB1 and PB2 plants positive in a multiplex assay for blackleg causing pathogens during the growing season in 2022.*

Sampling date	Cage[1]	Farm D		Farm E	
		PB1	PB2	PB1	PB2
7-6-2022	no	0	0	0	0
13-6-2022	no	0	2[2]	2	0
20-6-2022	no	0	1	1	0
27-6-2022	no	0	0	0	0
5-7-2022	no	1	1	1	0
11-7-2022	no	1	0	1	3
11-7-2022	yes	0	nd	1	nd

[1] Some leaf samples were sampled from plants shielded from visiting insects by a net cage.

[2] Black marked values: Ct values: 31.4-34.7, red marked: low Ct-value (23.8).

Figure 14 *Locations of water sampling in 2021 (left) and 2022 (right).*

Rain water as infection source

In 2021, 20 water samples were collected from 10 different locations and in 2022, 12 water samples from 8 locations (Figure 14, Tables 4 and 5). Samples were collected in 2021 between the 4th of June and the 20th of June and in 2022 between 5 June and 24 June. The water volume per sampling date and per location varied between 0.3 and 2 liters. All sampled water was concentrated by filtration, except for 1 sample in 2022 which was heavily contaminated with sand and from which only 100 ml could be filtrated due to cloaking. In a number of cases, the concentrated bacteria from the rainwater formed cavities on CVP. In 2022, in total 12 colonies were tested with the multiplex TaqMan assay for BL-SRP, but none of them were positive. The samples with concentrated bacteria were also tested in the enrichment TaqMan assay. Only one sample, collected in 2022 at Bant, the one 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 the virulent clade.

Table 4 *Rain water collected from various locations in the Netherlands in 2021.*

Location	Collection date	Processing date	Volume collected (L)	Contamination
1	4 Jun	6-jun	1,9	
1	22 Jun	23-jun	1	
2	4-jun	6-jun	1,9	
2	18-jun	19-jun	2	
3	17-jun	19-jun	1,2	
3	29-jun	30-jun	1,5	
4	4-jun	5-jun	2	
4	17-jun	19-jun	2	
5	4-jun	5-jun	0,57	
5	17-jun	19-jun	2	
6	17-jun	19-jun	1	pigeon droppings
7	18-jun	19-jun	2	
7	29-jun	30-jun	1,3	
8	18-jun	19-jun	2	sand/insects
8	29-jun	30-jun	2	
9	4-jun	5-jun	2	
9	18-jun	19-jun	2	insects
10	4-jun	5-jun	2	
10	17-jun	18-jun	2	
10	20-jun	21-jun	2	

Table 5 *Rainwater collected on various locations in the Netherlands in 2022.*

Nr	Location	Collection date	Processing date	Volume collected (L)	Contamination
1	Dreischor	5 June	8 June	1	
1	Dreischor	21 June	24 June	0.3	
2	Zeewolde	6 June	8 June	1.5	
3	Bant	6 June	8 June	2	
3	Bant	21 June	24 June	0.75	
3	Bant	21 June	24 June	0.9	sand
4	Mensingeweer	28 June	29 June	0.85	
5	Wieringerwerf	27 June	29 June	1	
6	Metslawier	28 June	29 June	0.95	
7	Zuidschermer	28 June	29 June	2	
8	Wageningen	8 June	10 June	1	
8	Wageningen	21 June	24 June	>2	

Insects as infection source

In the period between 13 June and 8 July 2022, at farm D, seven times insects were collected from traps and at grower E, six times (Table 6, Figure 15). The number of insects sampled and analyzed per time point ranged between 16 and 92. At farm D, in total 361 insects were analyzed, and at farm E, 304 insects. At farm D, the percentage of positive insects in the enrichment multiplex TaqMan assay was 3% and at farm E 2.3% (Ct-value <35). At farm D from 11 insects positive in the multiplex assay, 4 were positive in the simplex TaqMan assay for the virulent clade of *P. brasiliense* and 11 in the TaqMan assay for *P. parmentieri* (Table 7). At grower E, from 7 insects positive in the multiplex assay, 3 were positive in the simplex TaqMan assay for the virulent clade of *P. brasiliense* and 1 in the TaqMan assay for *P. parmentieri*, while for 3 insects the positive results in the multiplex assay could not be confirmed with the simplex assays. On the basis of the results of the simplex assays, overall, 2.3% of the insects were contaminated with BL-SRP. In our study, the insects were winged, but not further identified. The reactions of the positive samples in the enrichment multiplex TaqMan assay were rather weak and the Ct-values ranged between 30.8 and 34.7.

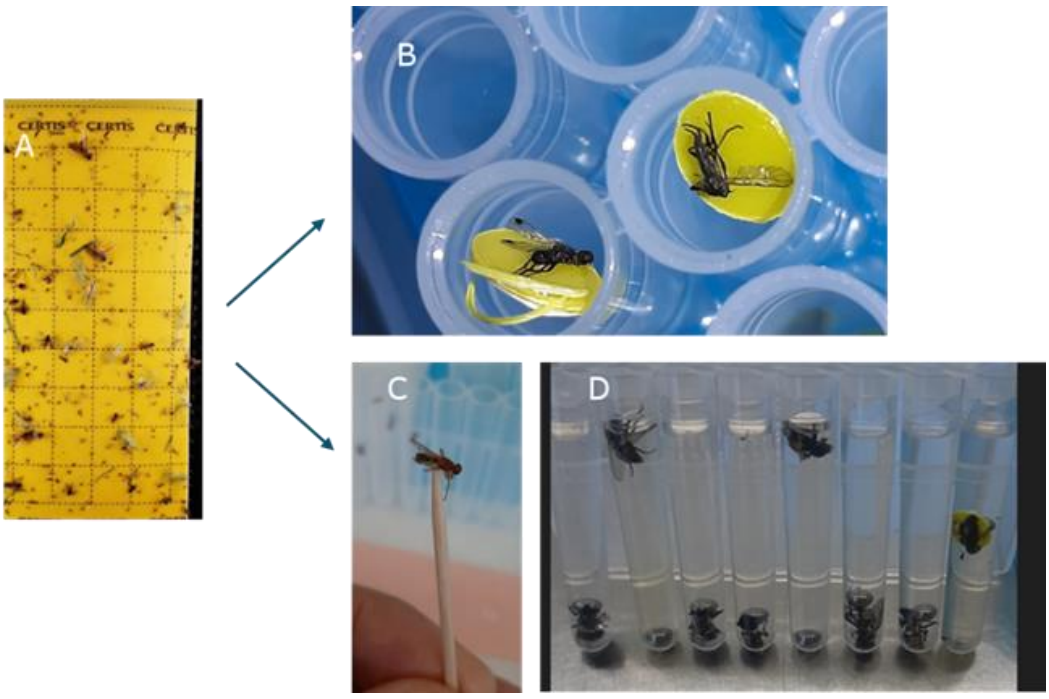

Figure 15 *Collection of insects from sticky traps in 2022. A. Sticky traps with trapped insects. B. Collection of punches with insects solidly fixed on the traps in wells of a 24-wells plate. C. In case insects could be easily removed from the trap, they were collected with a toothpick and transferred to tubes (D) with a buffer for further processing.*

Table 6 *Number of insects collected in June and July 2022 at growers D and E positive in the multiplex TaqMan assay (Ct-values < 35) detecting blackleg-causing soft rot Pectobacteriaceae. Nd = not determined.*

	Grower D		Grower E	
Date	**N**	**positive**	**N**	**positive**
13-6-2022	69	1	64	4
17-6-2022	32	2	nd	nd
20-6-2022	92	5	76	1
24-6-2022	nd	nd	48	2
27-6-2022	64	3	72	0
1-7-2022	56	0	nd	nd
4-7-2022	32	1	28	0
8-7-2022	16	0	16	0
Subtotal	361	11	304	7
Percentage positive		3.0		2.3

Table 7 *Characterization of individual insects positive in the multiplex TaqMan assay at growers D and E with individual assays for respectively Pectobacterium atrosepticum (Patr), P. parmentieri (Ppar), P. brasiliense (for two different groups, one comprising mainly non-virulent strains (nvPbr) and one comprising mainly virulent strains (vPbr)) and Dickeya sp.*

Grower	**Total nr**	**Patr**	**Ppar**	**nvPbr**	**vPbr**	***Dickeya***	**Unknown**
D	11	0	7	0	4	0	0
E	7	0	1	0	3	0	3

In addition, on the 5th of July, traps were placed in the field and insects collected on 13th of July 2022. In total 24 insects were collected in Wageningen from traps in a severely blackleg diseased crop raised from vacuum-infiltrated tubers and analyzed in an enrichment TaqMan assay. No contaminated insects were detected.

We also studied the survival of SRP on trapped insect in the field. For this, traps with insects were taken to the laboratory and sprayed with a suspension of *P. brasiliense* and traps were placed back in the field. The bacteria on the trap were immediately analyzed after spraying (T=0), one day after spraying (T=1) or two days (T=2) for presence of the bacteria, using an enrichment TaqMan assay. During each time point also a sample of the same sticky trap without an insect was analyzed. *P. brasiliense* could still be detected on the insects, 2 days after placing the traps in the field. *P. brasiliense* also survived on the sticky traps outside the insects (Table 8). The average Ct values of the enrichment TaqMan assay increased in 48 hours; it seemed that in time the population of living cells decreased.

Table 8 *Survival SRP on insect traps. Insects were trapped in the field, traps with insects were taken to the laboratory and sprayed with a suspension of Pectobacterium brasiliense after which traps were placed back in the field The bacteria were collected immediately after spraying and analyzed (T=0), or one (T=1) or two days (T=2) later for the presence of the pathogen, using an enrichment TaqMan assay (T=0). During each time point also a sample of the sticky trap without an insect was analyzed.*

Time (days)	**Insects**	**N**	**Nr positive**	**% positive**	**Avg Ct-value**	**Range Ct-values**
T=0	Yes	2	2	100	16.9	16.5-17.3
	No	2	2	100	18.2	16.5-17.9
T=1	Yes	14	14	100	25.5	17.1-31.7
	No	2	2	100	24	17.6-30.4
T=2	Yees	20	20	100	26.5	16.1-34.3
	N	4	4	100	28.7	27.3-30.3

Survival in soil

None of the 50 soil samples, collected in a field from which in a previous year a heavily blackleg diseased potato crop was harvested, was positive in the multiplex TaqMan assay for BL-SRP.

4 Discussion

Detection methods

An enrichment multiplex TaqMan assay was developed able to specifically detect all BL-SRP in the Netherlands at a low detection threshold of 10-100 cells/g of plant tissue. The assay, however, does not discriminate between the different variants. Positive results need to be analyzed further as the blackleg causing capacity of the various SRP variants differ substantially under Dutch weather conditions, with *P. atrosepticum* and a clade of *P. brasiliense* being highly aggressive, and *D. solani* and *P. parmentieri* being moderately aggressive [25]. For *P. brasiliense*, a further analysis is required, as many strains cannot cause blackleg [7] [26]. The multiplex assay was subsequently successfully used in these studies on initial infection. In Canada, *Pectobacterium* strains isolated from blackleg diseased plants also differed considerably in the disease prevalence after seed tuber inoculation, with *P. atrosepticum* as highly aggressive, *P. parmentieri* as moderately aggressive, and *P. carotovorum* and *P. brasiliense* weakly aggressive [27]. Obviously, in Canada *P. brasiliense* strains do not belonging to the virulent clade present in the Netherlands. This becomes also clear from a comparison of genome sequences of strains from Canada and the virulent clade found in the Netherlands (unpublished data).

A spontaneous rifampicin resistant mutant of a virulent *P. brasiliense* strain was selected to discriminate inoculum from wild-type strains of *P. brasiliense*, which seem to be ubiquitous present in agroecosystems. Growth rate in and on media and the soft rot inducing capacity on potato tuber disk assays were similar to that of the wild type strain, but a small significant difference in its blackleg inducing capacity in comparison with the wild type strain (62 t.o.v. 88%) was noticed. For other bacteria, mutations were found in the β subunit of the RNA polymerase gene (*rpoB*) of spontaneous rifampicin resistant mutants. Also, in other pathosystems the virulence of rif mutants could be affected, just as growth, stress response and motility [28-30]. Nevertheless, the blackleg inducing capacity of the rifampicin mutant of *P. brasiliense* was still high and sufficient for successful use in field experiments aimed to determine the risks for infections after haulm inoculations.

Viable but not culturable cells (VBNC)

We found indications that *P. brasiliense* is able to form cells in a VBNC state, but no indications that this is relevant in studies on epidemiology and management. For many plant pathogenic bacteria the forming of cells in a VBNC state has been proven [2-5]. Under specific conditions, VBNC's can resuscitate and cause diseases. The VBNC state has also been demonstrated for *P. atrosepticum* [6]. In this study with *P. brasiliense* it was shown that induction of cells in a VBNC state was possible by treatment with copper ($CuSO_4$), but not with a high salt concentration, with chlorine or by freezing and thawing. Both a direct viable staining method with propidium iodide (PI) and Syto9 indicating cell wall integrity as a staining with PI and carboxy fluorescein diacetate, indicating metabolic activity, showed that cells were viable after the copper treatment. However, we were unable to resuscitate the cells, even if VBNC cells were inoculated in axenically grown potato plants or on potato tuber slices.

Surveys of PB1 crops

A high percentage of plants of a PB1 crop raised from minitubers, assumed to be free of BL-SRP, can already become infected with BL-SRP during the growing season as shown in four-years surveys. From 2019- 2022, these surveys were conducted in PB1 crops of cv Agria, a blackleg susceptible cultivar. In 2019 and 2020 the same five farms were surveyed (Van der Wolf et al., 2022) and in 2021 and 2022 two of these farms (farm D and E). In 2019 and 2022, a relatively low level of precipitation and a high temperature were recorded in combination with a low infection incidence, whereas in the relatively wet and cool growing seasons of 2020 and 2021 a high haulm infection incidence was found. Up to 40% of the haulms of individual plants can already become infected with BL-SRP before haulm destruction. In 2019 and 2020 top leaves of the plants were sampled to reduce the risk that leaf infections were caused by inoculum from soil disseminated via splashing water. Results in 2020 indicated that haulm infections were not caused by soil-borne inoculum as plants with infected haulms did not yield infected tubers. In 2021 and 2022, older leaves already

deteriorating were sampled. It was hypothesized that a higher infection incidence was detected in older leaves growing in a closed canopy under humid conditions than top leaves. Results of the direct TaqMan assay indicated that in a high percentage of the samples relatively high densities (≥ 10^4 cells/g) were present in infected leaves.

During the surveys, mainly BL-SRP were found in the PB1 crop, that were known for their capacity to cause blackleg in the Netherlands, i.e. *P. brasiliense* and *P. parmentieri*. In 2019, mainly *P. parmentieri* was found, whereas in the other years the virulent clade of *P. brasiliense* strains dominated [26] [7]. This suggests that infected potato crops (of a lower grade) growing nearby the PB1 crop are the most important infection sources. In case other host plants than potato would be a source of infection, a broader set of BL-SRP would have been expected

Infections in a PB1 crop prior to haulm destruction have been found previously, although at a lower incidence, whereas the pathogen was never isolated [1]. Between 2015 and 2017, mother tubers collected of in total 88 PB1 crops grown in the Netherlands were tested for the presence of BL-SRP. From each crop, 8 tubers were collected in July and individually tested with an enrichment TaqMan assay. In 2015, 1.9% of the tubers was infected with *D. solani* and 1.1% of the tubers with *P. parmentieri*, in 2016, 0.1% with *D. solani* and 0.1% with *P. parmentieri* and in 2017, 3.4% with *P. brasiliense*. It seems that *P. brasiliense* has become the dominant BL-SRP species in all prebasic seed potatoes ([8]; Van Duivenbode, NAK, Emmeloord, pers. comm.), including PB1 crops.

Rain water

We did not find indications that initial infection of a PB1 crop was due to contact with contaminated rain, as we did not find any contamination of BL-SRP in 32 collected rainwater samples of minimally 0.3 and maximally 2 liter per sample in a two years survey conducted during the growing season. Almost the entire sampled volume was analyzed after concentration on a filter. Part of the sample was directly plated on the semi-selective medium DL-CVP, and the other part analyzed via an enrichment-TaqMan. The single finding of a non-virulent SRP can be due to the presence of dust in this particular sample.

P. carotovorum has been recorded in 80% of the samples collected from ocean water, in rain water and aerosols in the United States, while incidentally also *P. atrosepticum* was found [31]. The pathogen has also been found in 5% of the snow samples in the Rocky Mountains, usually originating from the Pacific ocean [32]. It was speculated that the presence of *P. carotovorum* in snow and rain is due to aerosolization of contaminated ocean water and transport within cloud systems.

The average amount of rain between April and July in the NL is 240 mm which is equivalent to 2400000 liter per ha (KNMI, years 1991- 2020). We sampled in total 45 liter, which is only ca. 0.002% of the total precipitation on a ha. In any case, with a sample volume of 45 liter being negative, the density in rainwater will be less than 0.02 cell per liter. If we assume the presence of 40.000 plants per ha, each plant will receive around 60 liters of rain water during the growing season. The maximum deposit of cells via contaminated rainwater will be less than 1.2 cell per plant. Taking into account the threshold level to initiate an infection, weconsider it unlikely that contaminated rain water plays a role in the epidemiology of SRP in the Netherlands.

Insects

Our study points to a role of insects in the occurrence of initial infections. During the entire growing season contaminated insects were found and the percentage was high (on average 2.3%). In studies in Norway, percentages between 4 and 39% were found with an average of 19%, but in their study all soft rot Pectobacteriaceae were detected, not only those that can cause blackleg [18]. Predominantly *P. parmentieri* and a virulent clade of *P. brasiliense* were found, the variants also detected in potato plants of the PB1 crop, and the variants mainly responsible for blackleg in the Netherlands. However, the reactions of the insect samples in the enrichment TaqMan assay were weak (data not shown) indicating that (most) bacteria were dead at the time they were sampled. At least 10^4 cells per insect are required to obtain a positive TaqMan result [18]. We collected the insects from the traps, 7 or 3 days after they were placed in the field. As evidenced in experimental research in Wageningen, SRP populations on the trapped insects in the field were

still partly alive but the population of living cells declined probably due to desiccation and under the influence of UV light.

No TaqMan positive insect samples for *P. brasiliense* were found in an experimental field in Wageningen, in which 24 insects were analyzed. If the percentage of contamination is near to 2%, this sample size may have been too low to find BL-SRP positive insects.

Already for decades, insects, have been a suspect as carrier of SRP (summarized by Toth et al, 2021 [9]). In the USA, many genera of flies were found contaminated with SRP [17]. In Norway in particular,*Delia* species have been found in association with the transmission of SRP [18]. A high percentage of the *Delia* flies were contaminated with *P. atrosepticum*, the most prevalent BL-SRP. *Delia* species have a wide overlap in host spectrum with SRP and may transmit the pathogen from one host to the other. It was shown that *Delia platura*, but also *Drosophila melanogaster* can transmit *P. carotovorum* from rotten tubers to wounded potato plants [33, 34]. SRP can survive both internally and externally on *Drosophila* species for at least 72 h [35, 36]. SRP present in the insect guts may even support the insect by digestion of plant tissue via the release of cell wall degrading enzymes [37]. In general, flies can migrate over large distances. For hover flies a distance of 70 km has been found and for Drosophila at least 6 km [38, 39]. No specific data on the migration distance for Delia species was found.

The detection of several SRP species, not previously found in a Dutch seed potato crop also indicates a role of the insects in the transmission of the pathogen. In 2019, *D. chrysanthemi* and *D. zeae* were detected in leaf samples of a PB1 crop and in 2020, and *D. fangzhongdai* in tubers. Therefore, it is hypothesized that the insects feed on hosts of these SRP-species, other than potato, after which transmission to the potato crop occurs. The blackleg causing ability of *D. chrysanthemi* and *D. zeae* detected in the PB1 crop has not been proven, as we were not able to isolate the pathogen. In field experiments with vacuum-infiltrated tubers, *D. fangzhongdai* was able to cause blackleg, but it has never been detected in blackleg diseased plants. It can however not be excluded that new variants of BL-SRP are introduced in potato by insects visiting other infected hosts. In this way, *D. solani* may have been transmitted to seed potatoes from infected hyacinth or muscari [8, 9]. *P. brasiliense* has a broad host range, including *Beta vulgaris, Brassica oleracea, Capsicum annum, Cucumis sativus and Solanum persicum*, and aggressive variants may have been transmitted from these crop into potato [9].

The use of aphid nets to cover a potato crop (Info: www.dutchfarmtrading.com) may not only avoid the transmission of viruses by aphids to potato plants, but also of BL-SRP by a broad range of insects. Unfortunately, in a first trial at two farms, we have not been able to proof this. Due to the dry weather conditions in 2022, the infection pressure outside the netted cage was too low to draw any conclusion. It cannot be excluded that BL-SRP survive in insects, larvae, nematodes, slugs or other animals for longer periods in soil. In that case, aphid nets may only partially protect the plants against introduction of the pathogen via insects.

Dose – response studies

Results of the dose-response studies in 2021 and 2022 were quite different. In both years, experiments were located in a similar field with sandy soil in Wageningen, using the same cultivar, the same rifampicin resistant bacterial strain and applied in the same densities. The temperature and precipitation during the growing season were, however, different. In 2021, the temperature was relatively low and the precipitation high, while in 2022 the opposite situation dominated. In 2021, spray-inoculation of haulms resulted in symptomatic plants at densities of 10^5 and 10^8 cells/ml, whereas in 2022 no symptoms were seen after haulm inoculation. In 2021, haulm infections due to the haulm inoculation occurred even at a density of 10^3 cells per ml, but in 2022 only at 10^8 cells per ml. In 2021, all plants yielded infected tubers. In 2022, the percentage of plants yielding infected tubers was density dependent, although even at a density of 10 cells/ml, plants with infected tubers were found as a result of spray inoculation.

A positive correlation between inoculum dose and the incidence of symptomatic plants was also found in field studies using seed tubers inoculated with *P. brasiliense* or *D. solani*. Using vacuum-infiltrated tubers, even a low dose (10^3 cells/ml) resulted in a high infection incidence, indicating the aggressiveness of both pathogens [25].

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, thus causing systemic infections [14]. During rain showers, bacteria may also leak from infected haulms, in particular from symptomatic plants to underground parts, but this has never been proven.

Contamination of soil with the bacteria could not be entirely excluded during spray-inoculation of haulms. Consequently, tuber infections may have occurred directly via contact with soil contaminated during inoculation. In parallel, haulm infections may have occurred via root inoculation, although *P. brasiliense* seems to be less effective as root colonizer than *D. solani* (unpublished results J. Vossen & V. Kurm, WUR). We found that after soil-inoculation, populations dropped 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 those that were symptomatic. The dissemination of inoculum from symptomatic plants to neighboring plants via water in soil may explain the high incidence of tuber infections in 2021.

We conclude that in a warm and dry season, the risks for haulm infections is low, but tuber infections can even occur at a low inoculum load on haulms. We speculate that the inoculum is washed off during a rain shower after which bacterial cells are capable of migration via the soil to the progeny tubers. During a wet season with relatively low temperatures the risks for both haulm and tuber infections are high, even at a low inoculum dose.

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5 Deliverables project

Scientific Publications

- Kastelein, P, Förch, M.G., Krijger, M.C., Van der Zouwen, P.S., Van den Berg, W. & Van der Wolf, J.M. 2020. Systemic colonization of potato plants resulting from potato haulm inoculation with *Dickeya solani* or *Pectobacterium parmentieri*. Canadian Journal of Plant Pathology. https://doi.org/10.1080/07060661.2020.1777465.
- Van der Wolf, J., Krijger, M., Mendes, O., Kurm, V., & Gros, J. (2022). Natural Infections of potato plants grown from minitubers with blackleg-causing soft rot Pectobacteriaceae. Microorganisms, 10(12), 2504. https://doi.org/10.3390/microorganisms10122504.

Reports

- Stevens, L.H. & Van der Wolf, J. 2020. Potentiële fysische behandelingen van aardappelpootgoed voor de eliminatie van plaagorganismen en ziekteverwekkers Rapport Wageningen Plant Research, Nr 958. 40 pages. DOI.org/10.18174/519998.

Presentations

- Van der Wolf, J. 2019. Bacterieziek: biologie en management. NAO cursus pootaardappelteelt. Wageningen, 23 januari 2019.
- Van der Wolf, J. 2019. Bacterieziek veroorzaakt door Erwinia's: biologie en beheersing. 31 januari 2019. De Landelijke Pootaardappeldag Emmeloord.
- Van der Wolf, J. 2019. Biologie en beheersing van bacterieziekten in de pootgoedteelt. Studiebijeenkomst voor pootgoedtelers, CZAV, 7 maart 2019, Heinkenszand.
- Van der Wolf, J.M. 2020. Bacterieziek in de aardappel, biologie en management. NAO cursistendag, Wageningen, 15 January 2020.
- J.M. van der Wolf 2021. Euphresco project 'Dickeya species in potato and management strategies (Dickeya spp.). Soft rot Pectobacteriaceae, diversity and biology. GoToMeeting meeting. 17 March 2021.
- J.M. van der Wolf 2021. Diversity and biology of potato blackleg causing Pectobacteriaceae. Telersdag Adama. Emmeloord. 1 oktober 2021.
- Van der Wolf, J. 2021. Management of potato blackleg. Potato grower's workshop at the Crop & Environment Innovation of the Danish Agriculture & Food Council. Aarhus.7 December 2021.

Related deliverables (partly an outcome of this project)

Scientific publications

- Rossmann, S., Wiken Dees, M., Torp, T., Hong Le, V., Skogen, M., Glorvigen, B., Van der Wolf, J., and Brurberg, M.B. 2019. Field-scale molecular testing of virulent potato soft rot *Pectobacteriaceae* in Norway. European Journal of Plant Pathology DOI 10.1007/s10658-019-01901-0.
- Miia Pasanen, Malgorzata Waleron, Thomas Schott, Ilse Cleenwerck, Agnieszka Misztak, Krzysztof Waleron, Leighton Pritchard, Ramadan Bakr, Yeshitila Degefu, Jan van der Wolf, Peter Vandamme and Minna Pirhonen. *Pectobacterium parvum* sp. nov., having a Salmonella SPI-1-like Type III secretion system and low virulence. International Journal of Systemic and Evolutionary Microbiology. ijsem004057.
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- Van Der Wolf JM, Acuña I, De Boer SH, et al., 2021a. Diseases Caused by Pectobacterium and Dickeya Species Around the World. In. Plant Diseases Caused by Dickeya and Pectobacterium Species. Springer, 215-61. https://doi.org/10.1007/978-3-030-61459-1_7.
- Van Der Wolf JM, Cahill G, Van Gijsegem F, et al., 2021b. Isolation, Detection and Characterization of Pectobacterium and Dickeya Species. In. Plant Diseases Caused by Dickeya and Pectobacterium Species. Springer, 149-73. https://doi.org/10.1007/978-3-030-61459-1_5.
- Van Der Wolf JM, De Boer SH, Czajkowski R, et al., 2021c. Management of Diseases Caused by Pectobacterium and Dickeya Species. In. Plant Diseases Caused by Dickeya and Pectobacterium Species. Springer, 175-214. https://doi.org/10.1007/978-3-030-61459-1_6.
- Van Gijsegem F, Toth IK, Van Der Wolf JM, 2021a. Outlook—Challenges and Perspectives for Management of Diseases Caused by Pectobacterium and Dickeya Species. In. Plant Diseases Caused by Dickeya and Pectobacterium Species. Springer, 283-9. https://doi.org/10.1007/978-3-030-61459-1_9.
- Van Gijsegem F, Toth IK, Van Der Wolf JM, 2021b. Soft Rot Pectobacteriaceae: A Brief Overview. Plant Diseases Caused by Dickeya and Pectobacterium Species, 1-11. https://doi.org/10.1007/978-3-030-61459-1_1.

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- Van der Wolf, J., Bergsma-Vlami, M., Saddler, G., Hélias, V., Tsror, L., Yedida, Iris, Pirhonen, M., Degefu, Y., Tuomisto, J., Lojkowska, E., Li, S. (2020). Report of the Euphresco Project 2015-D-135 "Assessment of Dickeya and Pectobacterium spp. on vegetables and ornamentals (Soft rot Dickeya and Pectobacterium)". http://doi.org/10.5281/zenodo.3603096.

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- Van der Wolf, J. 2019. Weerbaarheid van de aardappelplant tegen bacterieziekten veroorzaakt door Dickeya en Pectobacterium soorten. Wageningen Potato Centre, 13 maart 2019, Wageningen.
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Bijlage 1 Dataonderzoek NAK 2019-2022

Dataonderzoek SRP NAK 2019-2022

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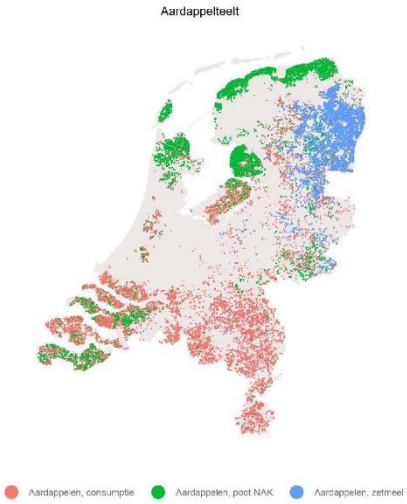
In 2015-2018 heeft de NAK een trial gedaan waarin de partijen die aangeboden zijn voor nacontrole ook getoetst zijn op soft rot pectobacteriaceae (SRP). Deze toets-resultaten zijn gebruikt om de correlatie te bepalen tussen (omgevings-) kenmerken en de aanwezigheid van SRP in het materiaal. Raskenmerken, klasse van het materiaal en de kwaliteit van het uitgangsmateriaal hebben een sterke correlatie met het al-dan-niet vinden van SRP in de nacontrole. Qua omgevingskenmerken wordt er vooral een sterke correlatie gevonden met neerslag, grondverzadiging en luchtvochtigheid in het voor- (april/mei) en naseizoen (september/oktober). In dit artikel wordt de data-analyse beschreven en worden de resultaten toegelicht.

Introductie

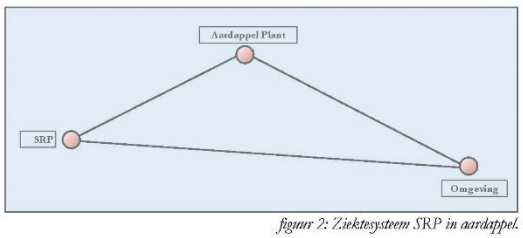
Pootaardappelteelt vindt in Nederland voornamelijk plaats in Zeeland, Noord-Holland, Flevoland, Noord-Friesland en Noord-Groningen. De hogere klassen PB1-S worden voornamelijk geteeld in Flevoland, Noord-Friesland en Noord-Groningen. De percelen worden door de telers aangegeven bij de NAK en, afhankelijk van klasse en ras, door de NAK in de veldkeuring beoordeeld op symptomen van 'Erwinia' (*soft rot pectobacteriaceae* afgekort als SRP[1]). Voor de jaren 2015-2018 is de verplichte nacontrole voor PVY aangevuld met toetsen op *Pectobacterium atrosepticum*, *P. brasiliense*, *P. parmentieri* en Dickeya *spp.* voor partijen met de klassen PB1-S.

In figuur 1 is weergegeven waar (poot)aardappelen geteeld zijn in 2020.



Figuur 1: locatie van aardappelteelt in Nederland (RVO, 2022). Met verschillende kleuren is aangegeven of het aardappelteelt voor consumptie, pootgoed of zetmeel betreft.

In figuur 2 is het ziektesysteem weergegeven. Dit bestaat uit drie onderdelen (SRP bacterie, Aardappelplant en Omgeving) die sterke onderlinge interacties hebben.



figuur 2: Ziektesysteem SRP in aardappel.

Omgevingsfactoren zoals weersomstandigheden, bodemgesteldheid, beschikbare voedingsstoffen en beschikbaarheid van water bepalen in hoeverre een plant zich gezond kan ontwikkelen en weerstand kan bieden tegen ziekten. Daarnaast kunnen ook handelingen door de teler (zoals bijvoorbeeld selectie) het besmettingsrisico doen verkleinen of vergroten.

Omgevingsfactoren hebben ook invloed op het pathogeen. Bijvoorbeeld op de mate waarin een pathogeen zich kan verspreiden tussen waardplanten of vanuit reservoirs buiten de waardplant naar de waardplant. We verwachten dat water (boven of onder de grond) als transportmedium, machines met aanhangend besmet organisch materiaal, en van vectoren (insecten?) die het pathogeen kunnen verspreiden, een belangrijke rol zouden kunnen spelen. (van Doorn, J. & van der Wolf, J., 2005)

Onderzoek

Op basis van hypotheses over epidemiologie en verspreiding van bacterie-ziekten is data-onderzoek gedaan naar correlaties tussen (omgevings-)factoren en de aanwezigheid van Erwinia-bacteriën in monsters aangeboden voor de nacontrole. Hierdoor ligt de focus op latente besmetting in de knollen. De keuze hiervoor is ingegeven doordat er meer partijen latent besmet zijn (ca. 60% met positief toets-resultaat op SRP) dan er symptomen in het veld vertonen (in ca. 20% van de percelen word tijdens de veldcontrole één of meer zieke planten gevonden). Hierdoor, en ook omdat de nacontrole uniformer van aard is (gewastoestand, mate waarin rassen symptomen tonen en weers-omstandigheden spelen geen storende rol), verwachten we dat omgevings-invloeden op het risico van besmetting beter aantoonbaar zijn voor latente besmetting. Doordat *P. brasiliense* in de gehele analyseperiode in ongeveer dezelfde mate en dominant aanwezig was, zijn de resultaten grotendeels bepaald door deze soort. Wanneer algemene uitspraken worden gedaan, is dit onder aanname dat de verschillende soorten SRP bacteriën, waaronder pathogene en niet-pathogene soorten, zich volgens eenzelfde mechanisme verspreiden.

Gebruikte gegevensbronnen

De data die gebruikt is bij dit onderzoek is afkomstig uit de keuringsgegevens van de NAK uit de periode 2015-2018. Reden hiervoor is dat er die periode een trial plaatsvond waarbij alle verhandelbare partijen PB1-S zijn getoetst op aanwezigheid van SRP in het nacontrolemonster. Een pootgoedpartij werd als besmet aangemerkt op het moment dat één of meer reacties in de nacontrole positief waren voor in ieder geval één van de SRP-soorten (NAK). Algemene kenmerken van de partij zijn afkomstig uit de aangifte. Omgevingskenmerken zoals weerskenmerken (KNMI, 2022) (bijvoorbeeld omgevingstemperatuur, relatieve luchtvochtigheid en zonne-instraling), grondkenmerken (RVO, 2022) (Wösten, J. H. M., de Vries, F., & Wesseling, J. G, 2016) (bijvoorbeeld grondsoort en doorlatendheid), raskenmerken (EUROPotato, 2022) en omgevingsgewassen (RVO, 2022) zijn aan een partij gekoppeld op basis van de locatie van het perceel. Indien beschikbaar is gebruik gemaakt van lokale gegevens zoals de neerslagradar in plaats van gegevens van weerstations.

In totaal zijn op basis van deze bronnen 2.200 kenmerken bepaald. Denk bijvoorbeeld aan:

- gemiddelde temperatuur in mei
- totale neerslag in juni
- aantal vorstdagen in februari
- grondsoort
- kwaliteit uitgangsmateriaal

Analyse

Om te bepalen hoe goed een bepaalde kenmerk correleert met de aanwezigheid van SRP in de nacontrole is een logistisch regressiemodel gebruikt. Omdat ras, klasse en generatie van de partij al veel verklaren worden deze kenmerken altijd meegenomen (basiskenmerken) in het model en wordt steeds alleen het te testen kenmerk toegevoegd aan de basiskenmerken. Het model kan dan beschreven worden als in vergelijking 1.

[1] *Pectobacterium atrosepticum, P. brasiliense, P. parmentieri en Dickeya spp.*

$$SRP \sim 1 + basiskenmerken + kenmerk$$ vergelijking 1

Het deel van de correlatie dat verklaard wordt door de basiskenmerken wordt bepaald door een model te genereren me alleen deze kenmerken. Dit heeft dan de vorm van vergelijking 2.

$$SRP \sim 1 + basiskenmerken$$ vergelijking 2

De aanwezigheid van SRP in de nacontrole is een binaire afhankelijke variabele (besmet of niet besmet). De onafhankelijke variabelen zijn categorisch (bijv. zand/klei/leem) of numeriek (bijv. 13,6 °C).

Om de correlatie te bepalen is gekeken naar verschillende kwaliteitsmaten, maar is uiteindelijk gebruik gemaakt van *Mutual Information* (MI). Dit is een maat voor de mate waarin een kenmerk informatie voorspellende waarde heeft voor het label. Omdat de MI een vergelijking maakt tussen 2 categorische variabelen en de voorspelde waarde en kans tussen 0 (negatieve uitslag) en 1 (positieve uitslag) betreft, wordt een treshold waarde gekozen die leidt tot een maximale MI opleverde.

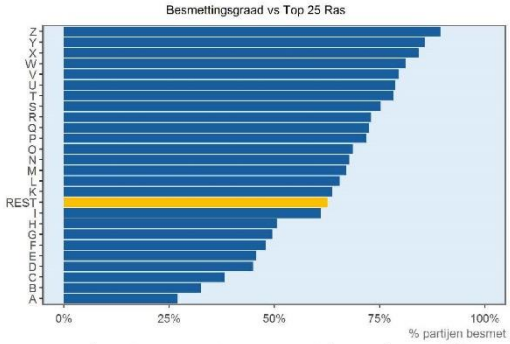
De MI van het model dat bepaald is voor het te testen kenmerk (vergelijking 1) wordt vergeleken met de MI van het basismodel (vergelijking 2). Het verschil wordt toegekend aan het te testen kenmerk.

Omgevingskenmerken wordt alleen als relevant gezien, wanneer deze zowel voor de verschillende klassen als voor de verschillende jaren in de dataset een vergelijkbare effect laten zien. Daarnaast moet de p-score van het betreffende kenmerk in het model lager dan 0,001 zijn en moet het kenmerk over de gehele range van mogelijke waarden consistent zijn (zie figuur 11 voor een voorbeeld waarbij de waarden niet consistent zijn).

Correlatie en causaliteit
Belangrijk om te onthouden is dat correlatie tussen twee kenmerken niet perse causaliteit inhoudt. Kenmerken die samenhangen met iets dat wel een echte invloed heeft, zullen meestal ook correleren met de responsvariabele zonder dat er sprake is van een oorzakelijk verband.

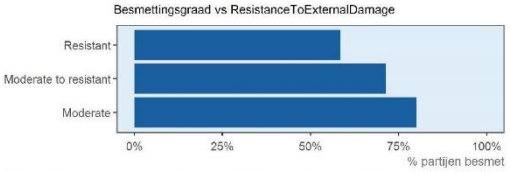
Rasgevoeligheid

Het ras van de aardappelplant correleert sterk met de gevoeligheid van de plant voor SRP-besmetting. In figuur is voor de 25 meest-geteelde rassen in Nederland (50% van alle percelen) plus een restcategorie (REST) weergegeven welk percentage van de partijen positief getoetst heeft op een van de vier SRP bacteriën.

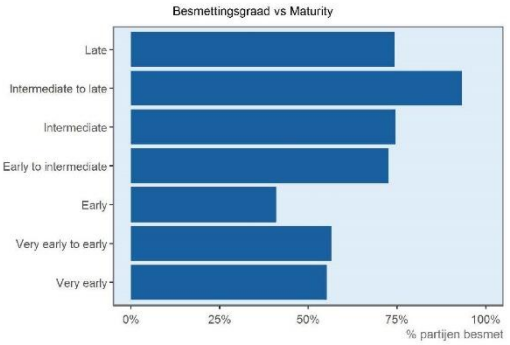


figuur 3 percentage partijen met een positief toets-resultaat voor SRP per ras.

Wanneer de verschillende rassen uitgesplitst worden naar hun kenmerken zien we dat rassen die minder gevoelig zijn voor beschadiging (figuur 4) en vroege rassen over het algemeen minder SRP-besmetting vertonen (figuur 5).



figuur 4 percentage partijen met een positief toets-resultaat voor SRP naar gevoeligheid voor beschadigingen.



figuur 5 percentage partijen met een positief toets-resultaat voor SRP naar maturity.

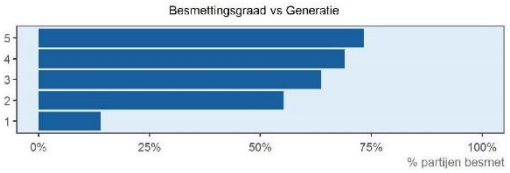
Hierbij moet wel aangetekend worden dat deze gegeven voor veel rassen niet bekend zijn.

Besmettings-mechanismen

Omdat de aardappel een gewas is dat clonaal vermeerderd wordt, zijn er grofweg twee manieren waarop SRP overgedragen kan worden tussen planten en/of knollen. De eerste is verticale besmetting, waarbij een plant of knol besmet raakt, doordat de moederknol reeds besmet was. De besmetting gaat over van generatie op generatie. De tweede is horizontale besmetting, waarbij een plant of knol besmet raakt met een SRP bacterie, doordat de plant of knol de bacterie overgedragen krijgt van een andere plant of knol.

In deze analyse wordt gesproken over verticale besmetting van een partij wanneer een partij besmet is en de moederpartij ook besmet is. Hierbij wordt over het feit heengestapt dat er verschil is tussen *besmetting van een partij* en *besmetting van een individuele knol*. Een knol kan horizontaal besmet raken (bijvoorbeeld als gevolg van contact met een andere knol), terwijl dit in deze analyse nog steeds gezien wordt als een verticale besmetting van de partij.

In figuur 6 is voor de verschillende generaties (1 5) weergegeven welk percentage van de partijen positief getoetst heeft in het nacontrolemonster.

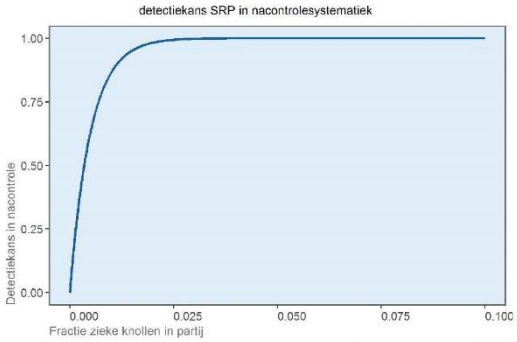


figuur 6 percentage partijen met een positief toets-resultaat voor SRP per generatie.

Dit beeld dat voor elke latere generatie er relatief meer partijen positief getoetst hebben voor SRP is erg stabiel over de jaren. Vanuit plant-pathologisch perspectief is dit een sterke indicator van verticale overdracht van de bacterie, hoewel horizontale overdracht niet volledig kan worden uitgesloten.

Wat opvalt is vooral dat er in generatie 1 (klasse PB1) erg weinig SRP gevonden wordt in de nacontrole en dat daarna een sprong gemaakt wordt naar een veel hoger percentage in generatie 2.

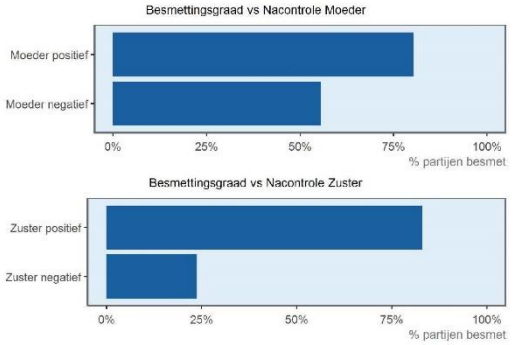
Dit zou veroorzaakt kunnen worden door bias in de selectie van de PB1 partijen of doordat de detectiekans voor hele lage besmettingspercentages in het nacontrole-systeem (4 x 50 knollen) lager is dan voor hogere percentages. De detectiekans voor een gegeven fractie zieke knollen is weergegeven in figuur 7.



figuur 7 detectiekans in nacontrole versus fractie zieke knollen in partij (NAK Nacontrolesystematiek, 2022).

Uit figuur 7 blijkt duidelijk dat partijen die maar licht besmet zijn een duidelijk lagere detectiekans hebben dan partijen die zwaarder besmet zijn.

Wanneer de moeder of zuster van een partij positief getoetst heeft voor SRP, dan is de partij zelf dat vaak ook (figuur 8).



figuur 8 percentage partijen met een positief toets-resultaat voor SRP versus toets-resultaat moeder/ zusterpartij.

Omgevingskenmerken

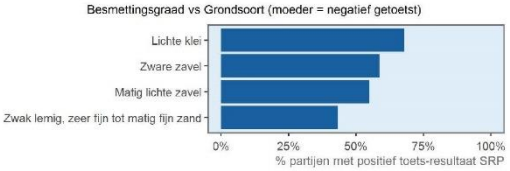
Risicofactoren voor horizontale besmetting zijn lastiger inzichtelijk te krijgen. Er zijn vele effecten die bij besmetting van knollen een rol kunnen spelen zoals bijvoorbeeld overdracht door grondwater, besmetting in de winteropslag, transporthandelingen, weersomstandigheden, etc. (Velvis, H., Kristelijn, K., & van der Wolf, J. M., 2013) (van Doorn, J. & van der Wolf, J., 2005)

Voor veel van deze overdrachtsmechanismen is geen data voorhanden. In deze analyse wordt gefocust op omgevingskenmerken.

Tijdskenmerken
Soms komt uit de analyse dat een kenmerk in een bepaalde maand een grote correlatie vertoont met de aanwezigheid van SRP in een partij. In dat geval moet altijd in gedachten gehouden worden dat het niet specifiek over die maand zelf hoeft te gaan, maar ook om een periode in de teeltcyclus kan gaan. Dit wordt veroorzaakt door grote onderlinge correlatie van de verschillende maanden. September staat bijvoorbeeld voor de periode dat van veel percelen het loof reeds gedood is, maar de knollen nog niet gerooid zijn.

Grondsoort

Pootaardappelen worden voornamelijk geteeld op zavel (78%), zand (11%) en klei (8%). Er is een groot verschil in het aantal partijen waarin SRP gevonden wordt tussen de verschillende grondsoorten (figuur 9).



figuur 9 percentage partijen met een positief toets-resultaat voor SRP per grondsoort. Voor alle partijen geldt dat het uitgangsmateriaal negatief getoetst heeft. De groepen betreffen minimaal 100 partijen.

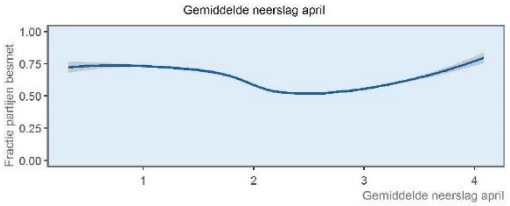
Het lijkt erop dat er in de nacontrole minder SRP besmettingen aangetoond worden in partijen die afkomstig is van grondsoorten die gemakkelijk water afvoeren. Ook zou het kunnen zijn dat besmettingen op lichte gronden sneller tot symptomen leiden, waardoor selectie door de teler beter mogelijk is. Zowel selectie als verlaging in de veldkeuring door de NAK kunnen voor een verschil in besmetting tussen partijen (PB1-S) van verschillende grondsoorten zorgen.

Neerslag

In figuur 10 is de MI weergegeven wanneer gekeken wordt naar de neerslag in de verschillende maanden. De MI is in de grafiek opgesplitst naar de bijdrage van de basiskenmerken (vergelijking 2) die is weergegeven met de blauwe balk en de toegevoegde bijdrage van de neerslag (vergelijking 1) die is weergegeven met de groene balk.

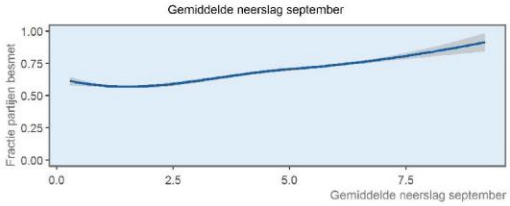
figuur 10 toename van MI door toevoegen van neerslag (groen) aan logistisch model ten opzichte van het basismodel (blauw).

De hoeveelheid neerslag in het voor- (april, mei) en naseizoen (september, oktober, november) vertoont een sterke relatie met de aanwezigheid van SRP in de geoogste partij. De hoeveelheid neerslag in de maanden april (M04), mei (M05) en november (M11) laten geen eenduidige relatie met besmettingskans zien, en zijn waarschijnlijk slechts een toevallige correlatie. Ter illustratie is dit in figuur 11 getoond voor de maand april.



figuur 11 relatie van neerslag [mm/ dag] in april (blauwe lijn) met fractie SRP-positief getoetste partijen. De grijze ribbon toont het 95% confidence interval voor de blauwe lijn. Deze figuur is bedoeld ter illustratie dat er kenmerken zijn die wel een hoge MI kunnen hebben, maar toch niet echt relevant lijken voor de praktijk

De hoeveelheid neerslag in september en oktober toont wel een eenduidige relatie met besmettingskans. In figuur 12 wordt ingezoomd op de neerslag in de maand september.

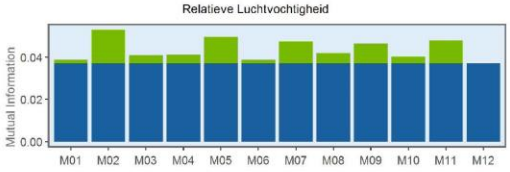


figuur 12 relatie van neerslag [mm/ dag] in september (blauwe lijn) met fractie SRP-positief getoetste partijen. De grijze ribbon toont het 95% confidence interval voor de blauwe lijn.

Te zien is dat een toename van de neerslag van 2 naar 7.5 mm/dag overeenkomt met een toename van 50 naar 75% partijen met een positief toets-resultaat.

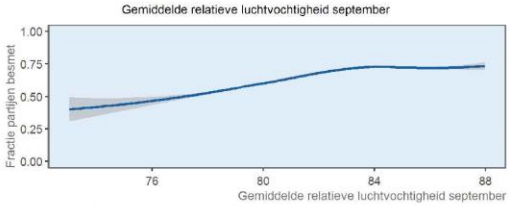
Relatieve luchtvochtigheid

In figuur 13 is het effect op de MI weergegeven van de Relatieve lucht-vochtigheid.



figuur 13 toename van MI door toevoegen van relatieve luchtvochtigheid (groen) aan logistisch model ten opzichte van het basismodel (blauw).

De relatieve luchtvochtigheid in mei (M05), september (M09) en november (M11) vertonen een grote correlatie met de aanwezigheid van SRP in een partij. De luchtvochtigheid in mei en november laat geen eenduidige relatie met besmettingskans zien, en zijn waarschijnlijk slechts een toevallige correlatie. In figuur 14 wordt ingezoomd op de relatieve luchtvochtigheid in september.



figuur 14 relatie van relatieve luchtvochtigheid [%] in september (blauwe lijn) met fractie SRP-positief getoetste partijen. De grijze ribbon toont het 95% confidence interval voor de blauwe lijn.

Te zien is dat een toename van de relatieve luchtvochtigheid van 75 naar 85% overeenkomt met een toename van 50 naar 80% partijen met een positief toets-resultaat.

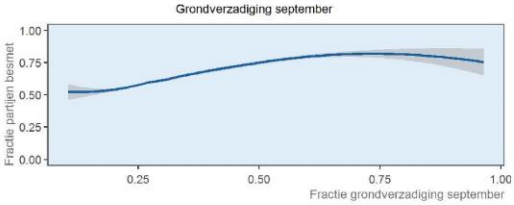
Grondverzadiging

De grondverzadiging is een gecombineerd kenmerk dat vooral bepaald wordt door de grondsoort, gewasverdamping en de neerslag in een bepaalde periode. De grondverzadiging is berekend op basis van een leaky-bucket model dat rekening houdt met zowel de neerslag als de verdamping als de horizontale en verticale afvoer van grondwater. (Evenhuis, A., Kruijne, R., Deneer, J. W., & Schepers, H. T. A. M., 2013) (Massop, H. T. L., Clement, J., & Schuiling, C., 2014) (Wösten, J. H. M., de Vries, F., & Wesseling, J. G, 2016) (Romano, N., Palladino, M., & Chirico, G. B., 2011)

In figuur 15 is het effect op de MI weergegeven van de grondverzadiging. De grondverzadiging wordt alleen berekend voor de periode mei-september (M05-M09), vandaar dat de andere maanden niet getoond worden.

figuur 15 toename van MI door toevoegen van grondverzadiging (groen) aan logistisch model ten opzichte van het basismodel (blauw).

De grootste correlatie met de aanwezigheid van SRP in de nacontrole wordt gevonden met de grondverzadiging in september (M09). In figuur 16 wordt ingezoomd op de neerslag in de maand september.

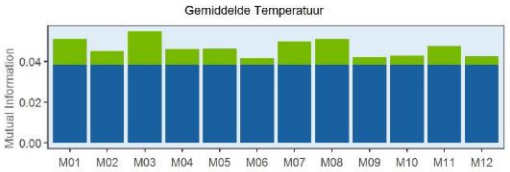


figuur 16 relatie van grondverzadiging [-] in september (blauwe lijn) met fractie SRP-positief getoetste partijen. De grijze ribbon toont het 95% confidence interval voor de blauwe lijn.

Te zien is dat een toename van de grondverzadiging van 0,25 naar 0,65 overeenkomt met een toename van 50 naar 78% partijen met een positief toets-resultaat.

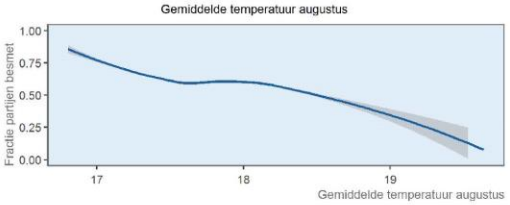
Temperatuur

In figuur 17 is het effect op de MI weergegeven van de temperatuur.

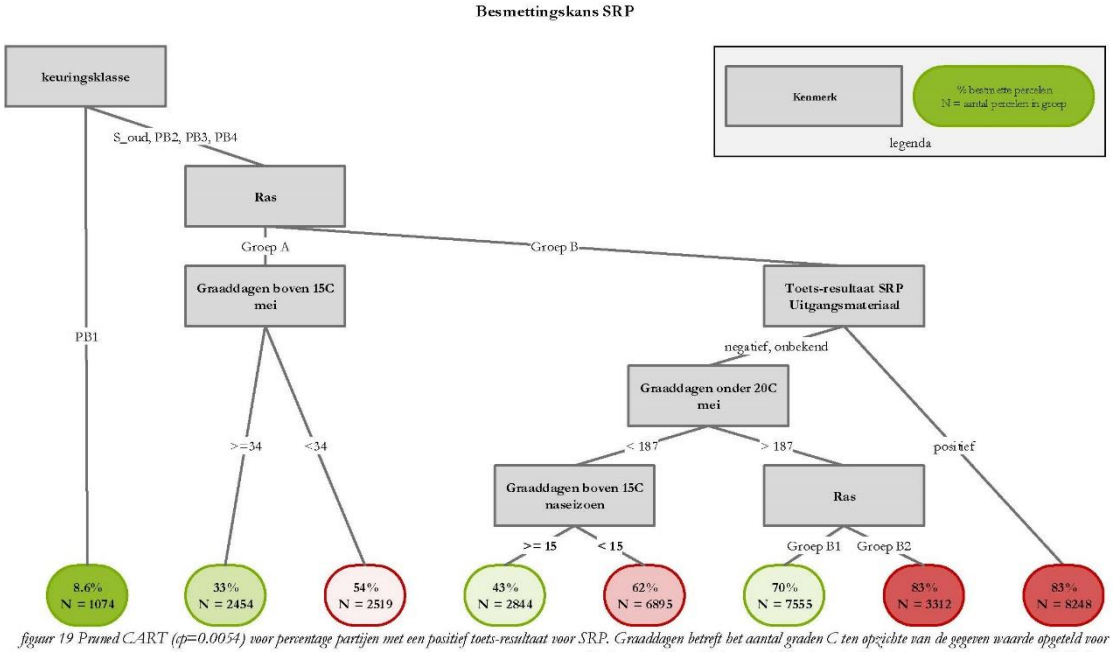


figuur 17 toename van MI door toevoegen van temperatuur (groen) aan logistisch model ten opzichte van het basismodel (blauw).

Vooral de temperatuur tussen maart (M03) en augustus (M08) vertoont een grote correlatie met de aanwezigheid van SRP in een partij. De temperatuur in deze maanden laat niet voor elke maand een eenduidige relatie met besmettingskans zien, en zijn waarschijnlijk slechts een toevallige correlatie. Wat wel opvalt is dat de temperatuur in maart heeft een positieve relatie heeft met het percentage positief getoetste partijen, terwijl de temperatuur vanaf april een negatieve relatie heeft met het percentage positief getoetste partijen. In figuur 18 wordt ingezoomd op de temperatuur in de maand augustus.



figuur 18 relatie van temperatuur [°C] in augustus (blauwe lijn) met fractie SRP-positief getoetste partijen. De grijze ribbon toont het 95% confidence interval voor de blauwe lijn.



figuur 19 Pruned CART (cp=0.0054) voor percentage partijen met een positief toets-resultaat voor SRP. Graaddagen betreft het aantal graden C ten opzichte van de gegeven waarde opgeteld voor alle dagen in de betreffende maand. Naseizoen is de periode vanaf twee maanden na loofdoding.

Diverse

Ook andere kenmerken zoals luchtdruk, gewasverdamping of vectoren laten een correlatie zien tussen het kenmerk en de aanwezigheid van SRP in de percelen. Deze correlaties zijn echter een stuk kleiner dan diegene die hierboven genoemd worden.

In het gehele veldkeuringsseizoen wordt er SRP symptomen gevonden in de veldkeuring. Qua analyse is er over het algemeen weinig verschil of er gekeken wordt naar de nacontrole resultaten of naar de veldkeuringsresultaten, behalve bij die kenmerken die betrekking hebben op de periode na laafdoding.

Interacties met andere kenmerken

De verschillende kenmerken zijn onderling sterk gecorreleerd. Dit is vooral het geval bij weerskenmerken. Een voorbeeld hiervan is dat hoge luchtdruk vaak samenvalt met stabiel droog warm zomerweer. Wanneer naar kenmerken afzonderlijk gekeken wordt, kunnen verschillende kenmerken eenzelfde correlatie laten zien. Het ene kenmerk heeft wellicht een causale relatie met de aanwezigheid van SRP in de nacontrole en het andere kenmerk helemaal niet, maar toevallig wel een grote correlatie met het kenmerk dat dat wel heeft.

In het bepalen welke weerskenmerken een grote correlatie hebben met de aanwezigheid van SRP in de nacontrole is in de analyse deels rekening gehouden. Effecten die verklaard kunnen worden met ras, klasse/generatie of kwaliteit van het uitgangsmateriaal worden door het basismodel reeds verklaard en niet aan het specifieke kenmerk toegekend. Er wordt dan alleen nog gekeken naar de toegevoegde waarde van het specifieke (weers)kenmerken.

Met een classification en regression tree (CART) kan inzicht gegeven worden in interacties van verschillende kenmerken. Deze tree kan extreem groot (en onleesbaar) worden gezien het aantal percelen en het aantal verschillende kenmerken in de analyse. Voor de leesbaarheid is in figuur 19 een pruned CART weergegeven. Door pruning komen kenmerken als grondverzadiging en neerslag niet meer voor in deze tree. Ze spelen wel een rol op een lager niveau.

De kenmerken die geselecteerd worden in de CART kunnen anders zijn dan welke in het regressiemodel geselecteerd worden. Dit wordt veroorzaakt door verschillende manieren waarop kenmerken geselecteerd worden in verschillende modellen en door verschillen in modelstructuur. Daarnaast ligt de relevantie van sommige kenmerken dicht bij elkaar, een klein verschil kan ertoe leiden dat een ander kenmerk geselecteerd wordt. Belangrijk is ook om in gedachten te houden dat de modellen opgebouwd wordt met kenmerken die de **correlatie** met het nacontrole resultaat verbeteren. Dit hoeft niets te zeggen over een **causaal** verband tussen de kenmerken die gebruikt worden in de modellen en het nacontrole resultaat. In een logistische regressie spelen alle kenmerken een rol bij het berekenen van een kans op besmetting, terwijl in een CART alleen die kenmerken een rol spelen die in de tak van de betreffende partij getoond worden.

Binnen de uiteindelijke groepen wordt in de CART geen onderscheid gemaakt tussen de percelen. Dit is een consequentie van de modelleringstechniek. Een regressiemodel zou voor elke partij afzonderlijk een kans-op-besmetting bepalen, maar neemt daarbij voor elke partij dezelfde kenmerken in ogenschouw.

Observaties

Uit de analyse kunnen de volgende observaties gemaakt worden:

- Kenmerken die duiden op verticale besmetting (zoals bijv. klasse/generatie of een besmette moederpartij) vertonen een sterke relatie met de aanwezigheid van SRP in de dochterpartij.
- Er is groot verschil in besmettingsrisico tussen rassen. Sommige rassen worden nauwelijks positief getoetst op SRP in het nacontrolemonster, andere rassen regelmatig.
- Omgevingskenmerken die mogelijk het risico op, of vatbaarheid voor, een horizontale besmetting verhogen vertonen ook een aanzienlijke relatie met de aanwezigheid van SRP in de nacontrole, maar niet zo sterk als kenmerken als klasse/generatie, kwaliteit uitgangsmateriaal of ras.
- Grondsoort, relatieve luchtvochtigheid en neerslag aan het einde van het teeltseizoen en de grondverzadiging die daarmee samenhangt vertonen allemaal een grote positieve correlatie met de aanwezigheid van SRP in de nacontrole.
- Temperatuur in het seizoen vertoont een negatieve correlatie met de aanwezigheid van SRP in de nacontrole.

Interpretatie en discussie

Keuringsklasse, Ras en kwaliteit van het uitgangsmateriaal correleren sterker met de aanwezigheid van SRP in de nacontrole dan omgevingskenmerken. Vooral het belang van de keuringsklasse en de kwaliteit van het uitgangsmateriaal lijken te duiden op verticale transmissie. Raskenmerken (bijvoorbeeld gevoeligheid voor schade) en enkele omgevingsfactoren (temperatuur, neerslag en bodemvocht) geven mogelijk hints over risicofactoren voor horizontale transmissie. Gezien het verspreidingsmodel van bacteriën en de gedachte dat deze in grond en water een tijdlang kunnen overleven lijkt het logisch te concluderen dat de kenmerken die te maken hebben met vocht in de lucht en grond een grote rol spelen in de transmissie van SRP tussen planten onderling en knollen onderling.

De nacontrole resultaten zijn gelimiteerd tot 1 meetmoment, namelijk aan het einde van het seizoen. Daardoor kan er met deze data geen verdere analyse gedaan worden naar het exacte moment van besmetting en is er ook geen onderscheid te maken tussen een besmetting in de bewaring of een veld-besmetting in het groeiseizoen. Afhankelijk van het kenmerk is het slechts mogelijk een aanwijzing te krijgen welk effect op welk moment een grote rol zou kunnen spelen.

In de analyse is alleen gekeken naar kenmerken die beschikbaar zijn door de NAK aangifte en vrij beschikbare data als grondsoorten, KNMI weersgegeven en RVO gewaspercelen. Of een partij al dan niet besmet raakt met SRP zal mede veroorzaakt worden door lokale omstandigheden of interventies door de teler. Deze gegevens zijn niet openbaar beschikbaar maar zouden wel veel inzicht kunnen geven.

Op basis van deze analyse kunnen geen causale verbanden gelegd worden, maar wanneer analyseresultaten in lijn zijn met bepaalde verwachtingen over de verspreiding van SRP, geeft het deze verwachting extra gewicht. Sommige kenmerken vertonen in de periode na het veldseizoen een grote correlatie met de aanwezigheid van SRP in de nacontrole. Deze duiden waarschijnlijk niet op een causaal verband, maar slechts op een correlatie.

Het gebruik van keuringsdata voor het doen van deze analyse heeft verschillende consequenties. Ondanks dat gekozen is om alleen PB1-S materiaal in de periode 2015-2018 mee te nemen in de analyse blijft het risico van een bias bestaan, aangezien ook deze partijen geen willekeurige steekproef van alle partijen betreffen. Daarnaast is de afhankelijke variabele (besmet/niet-besmet) omgeven met enige onzekerheid, doordat het bemonsteringsregime ertoe leidt dat niet voor elk besmettingspercentage (knollen) een even grote kans is op detectie in de nacontrole.

Voor de leesbaarheid zijn in deze tekst alleen plaatjes getoond van eenvoudige omgevingskenmerken zoals relatieve luchtvochtigheid, neerslag en temperatuur. In de analyse is de MI bepaald voor alle beschikbare 2.200 kenmerken. Sommige van deze kenmerken geven een net wat hoger MI, maar zijn lastiger te interpreteren en correleren vaak sterk met de getoonde eenvoudiger kenmerken.

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Tooling

- R-studio met R-versie 4.0.2
- Packages ggplot2 versie 3.3.3, rpart versie 4.1.15, raster versie 3.5.9, ROCR versie 1.0.11, DBI versie 1.1.2

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