



Euphresco

Final Report

Project title (Acronym)

Taxonomy and epidemiology of <i>Pectobacterium</i> and <i>Dickeya</i> spp. in Europe, North America and South Africa

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2. Short project report

2.1. Executive summary

The blackleg-soft rot-aerial stem rot disease complex causes serious losses to the potato industry. It is caused by species of the genera *Pectobacterium* and *Dickeya*, collectively known as the soft rot Pectobacteriaceae. These soft rot Pectobacteriaceae also cause damage in a wide range of other host plants. *Pectobacterium brasiliense* has been the most prevalent in potato and pathogenic species in Europe and South Africa for the past decade, although the species composition is in constant flux due to the introduction of new species and taxonomic reclassification of current ones.

Updated information on the current species composition is required, as well as knowledge of possible differences in symptom expression between species. Such information would aid certification and diagnostic services in testing for the correct species and making accurate diagnoses.

Findings indicate that *Pectobacterium brasiliense* remains the most prevalent and widely distributed species in potato production areas. Other species that were identified included e.g. *Pectobacterium carotovorum*, *Pectobacterium parmentieri*, *Dickeya chrysanthemi* and *Pectobacterium versatile*. *Pectobacterium brasiliense* was also the most pathogenic species on potato. When looking at other host plants a wide variety of *Pectobacterium* and *Dickeya* species with large genetic variation occurs.

MALDI-TOF MS can only be used to identify *Pectobacterium* and *Dickeya* isolates at the genus level but preliminary results after improving the reference library look promising.

Given that there is also a large group of nonvirulent *P. brasiliense* isolates, a specific PCR which can differentiate between virulent and nonvirulent isolates of this species is being developed.

2.2. Project aims

Pectobacterium and *Dickeya* are important bacteria that can cause soft rot on a wide range of host plants. Besides soft rot they are associated with other plant diseases as well (e.g. blackleg of potato plants). Although these bacterial species are not regulated within the European Union, some third countries do have specific requirements regarding the absence of these bacteria on plant material.

Symptoms caused by different *Pectobacterium* and *Dickeya* spp. are mostly indistinguishable. Surveys performed in potato in many different countries revealed changing dominant species in time and place. Together with recent changes in the taxonomic position of many *Pectobacterium* and *Dickeya* strains, these factors make it hard to get a good understanding of the epidemiology of these soft rot pathogens. This is further complicated by the lack of specific tests that can accurately identify each individual member of the large and diverse group of *Pectobacterium* and *Dickeya* genera down to their species or subspecies level.

The goal of this project was to develop and validate tools that allow quick and proper characterisation of *Pectobacterium* and *Dickeya* species at the (sub)species level and to use these tools to get a better understanding of the taxonomy and epidemiology of these bacteria. Accurate taxonomic determination is necessary to perform good epidemiological studies and to take the right measures to restrict pathogen spread. One prerequisite to performing a good taxonomic study is to obtain a collection that reflects the biological diversity that can be found in the field. Therefore, the first objective of this project was to gather a collection of *Pectobacterium* and *Dickeya* species collected from different sites throughout Europe, North America and South Africa in the last decade. This included the collection of new strains during 2021 and 2022.



The second objective of the project was the genomic characterization of the collected strains. This was done using available PCR based techniques but also included multi locus sequence typing. Sequences were collected using multi locus sanger sequencing and/or whole genome sequencing of selected strains. Additionally, sequences already available in public databases were included. The results of these analyses were used to evaluate the current taxonomy of the *Pectobacterium* and *Dickeya* genus. Further, results of the sequence based identification were used to assess the specificity of the available PCR tests.

Besides DNA based bacterial strain identification techniques other options for species identification are available as well. One of these is MALDI-TOF MS analysis. This technique creates mass spectral patterns of whole bacterial cells and allows for rapid identification of bacteria at the species and in some cases subspecies level. Like sequencing based techniques, identification of bacteria using MALDI-TOF MS depends on the availability of high quality databases containing large numbers of different species of bacteria. Although some spectra of *Pectobacterium* and *Dickeya* species are available, more strains needed to be added to be able to use this technique in routine diagnostics of these species. Therefore, the third objective of this project was the analysis of well characterised strains of *Pectobacterium* and *Dickeya* spp., from this and other studies, with MALDI-TOF MS to obtain a large collection of spectral data, which will allow the use of this technique for future diagnostic purposes.

The fourth objective of this project is to get a better understanding of the epidemiology of the members of the *Pectobacterium* and *Dickeya* genus. Therefore, the virulence of different strains that have been collected during surveys in and outside Europe, and that have been characterized as part of this study, were assessed using infection assays. For this, strains were selected based on their phylogenetic relationship and their presence throughout the investigated areas. The selected strains were tested in greenhouse and small field tests as well as *in vitro* assays. This was done on a variety of host plants.

The fifth and last objective of the project focussed on prediction of compatible hosts for a given strain based on the presence and/or absence of specific (variants of) genes in the strains genome. Currently the only way to investigate the virulence of a specific strain on a specific host is by performing disease assays. These experiments are laboursome and often take a lot of time. Since *Pectobacterium* and *Dickeya* species appear to use different genetic tools to overcome plant host barriers, sequencing information and data on virulence on specific hosts will be combined to search for (a set of) genes that were linked to the ability of different strains to infect these specific hosts. This will hopefully allow for better prediction of possible hosts for strains that will be isolated in the future.

Altogether the focus of this project was on the characterization of a diverse set of strains of *Pectobacterium* and *Dickeya* species collected throughout Europe, North America and South Africa. The results of this project will lead to a better understanding of the taxonomy and epidemiology of these bacteria and will aid diagnostics and the development of preventive measures to control disease outbreaks.

2.3. Main activities

University of Pretoria

Throughout 2020 and 2021, samples of diseased potato tubers and haulms were collected from 10 of the 16 potato production regions in South Africa. Primary isolations were made on CVP single-layer agar containing pectin. Individual pits were then sub-cultured onto Nutrient agar to obtain pure colonies for DNA isolation and storage in collection. For identification each DNA sample was subjected to two PCR reactions, the first, for the detection of *Pectobacterium brasiliense*, and the second, a multiplex, for the detection of *Pectobacterium atrosepticum*, *Pectobacterium carotovorum*/*Pectobacterium parmentieri*, and *Dickeya* species. Positive samples were further characterised using multi-locus sequencing.

The identified isolates, together with reference strains obtained from the CMEG culture collection, were evaluated for their maceration ability on tuber slices. The *P. brasiliense* isolate



SRP 1 had the largest average lesion diameter and was selected, along with the *P. carotovorum* and *P. atrosepticum* strains from CMEG, for subsequent pot trials and tuber slice assays. The pot trials consisted of eight different treatments, comprising inoculation with the three species separately, and in all possible combinations. For these pot trials sprouted disease-free minitubers of cv. Mondial, were vacuum infiltrated with bacterial inoculum. After vacuum infiltration, the tubers were allowed to air-dry overnight. One tuber was planted per 1 L pot in sandy loam soil at a depth of 10 cm. Symptoms were recorded from their first appearance, around two weeks post-plant, until the plants started to die. This amounted to a period of 5 days in trial 1 and 8 days in trial 2. Symptoms assessed were, stem rot (blackleg), wilting, chlorosis, stunting, damping-off, non-emergence, and atypical blackleg symptoms. A tuber slice assay was performed as well to compare the same treatments as in the pot trials for their maceration ability.

University of Copenhagen

UCPH has contributed to objective 1, strain collection, and objective 2, genomic characterization. Together with Danish plant consultants and potato producers, we have collected symptomatic potato plants and tubers in 2021, 2022 and are currently collecting for 2023. In 2021 we isolated 46 *Pectobacterium* isolates and 1 *Dickeya* isolate from potato plants and tubers showing symptoms of blackleg and soft-rot. Isolates from 2022 have not yet been sequenced and isolates from 2023 are still collected. For all isolates, we have metadata describing; sampling date, sampling area and tuber variety.

NAK

The main objectives were to collect different isolates of *Pectobacterium* and *Dickeya* spp. and perform field experiments to determine how pathogenic the different species are in potato.

NIB

A retrospective survey of diagnostic samples obtained from 2017 to 2021 was conducted. Two relevant environmental niches were included in the survey: potato plants and tubers with soft rot symptoms and surface water samples. The survey focused on the general detection of pectinolytic bacteria of the genus *Dickeya* and specifically on the detection of *Dickeya fangzhongdai*. The survey was based on molecular testing. We used the general real-time PCR assay for *Dickeya* spp. by Pritchard et al. (2013) and a specific real-time PCR assay for the detection of *D. fangzhongdai* that was developed and validated in-house.

ILVO

Over the past decades, numerous *Pectobacterium* and *Dickeya* isolates have been obtained in diagnostic research from a variety of plant species (GBBC acronym) or from other laboratory/official collections. In particular over the project period (2021-2022), several *P. aroidearum* and *P. zantedeschiae* isolates from Calla nurseries in BE/NL were added to the collection.

The performance of conventional PCR assays for several DNA barcodes was evaluated for the type strains of 19 *Pectobacterium* species and for *P. atrosepticum* LMG 6677. Furthermore, the results of two generic TaqMan real-time PCRs were compared. Also, exploratory steps have been taken at ILVO to characterise *Pectobacterium* strains with MALDI-TOF MS.

Several virulence tests were done at ILVO, using the 19 *Pectobacterium* type strains. Strains were first screened in laboratory assays for their ability to cause soft rot in chicory leaves, in tomato fruits, and on cucumber and potato slices. Stems of potato seedlings were then inoculated using a pin-prick inoculation method under greenhouse conditions. Lastly, in May 2022, a field trial was set up with vacuum-infiltrated seed tubers of cv. Agria (pre-basic seed class) to study the virulence on potato and the transmission to progeny tubers of the 18 *Pectobacterium* type strains and LMG 6677 under outdoor conditions.



NVWA

Surveys performed in the last two decades on various host plants in the Netherlands resulted in a high number of representatives of these genera. Their accurate identification in the past was rather complicated due to lack of specific tests. This, in combination with recent taxonomic changes made it hard to get insight in the epidemiology of these soft rot pathogens. We focussed on the characterization of 70 of these isolates. Using Illumina sequencing, whole genome sequences were generated and the assembled genomes were compared with the genomes of the type strains that were available at NCBI or were generated as part of this project. Although none of the selected isolates originated from potato, several of them belong to species that are known potato pathogens. Based on their phylogenetic relationship, isolates have been selected and tested for their virulence on potato. Additionally, the use of MALDI-TOF MS for the reliable identification of isolates belonging to *Pectobacterium* and *Dickeya* species has been investigated.

2.4. Main results

University of Pretoria

This project aimed at addressing these questions by collecting and identifying isolates from symptomatic plants and tubers across South Africa and subsequently, comparing their pathogenicity and symptom expression in a tuber slice assay and greenhouse pot trial. Findings indicate that *P. brasiliense* remains the most prevalent and widely distributed species in the country by a substantial margin. Other species that were identified included *P. carotovorum* (14%), *P. parmentieri* (4%), *Dickeya chrysanthemi* (3%) and *Pectobacterium versatile* (3%). The latter is a first report in South Africa, and pathogenicity of the *P. versatile* isolates was confirmed through Koch's postulates. *P. brasiliense* was also the most pathogenic species, with a significantly higher incidence and severity of symptoms compared to the others.

University of Copenhagen

In 2021 47 isolates were collected and all these isolates were sequenced using Illumina (short reads) and Nanopore (long reads) methods. Hybrid assembly was performed using Unicycler. Based on whole genome-based taxon identification 46 *Pectobacterium* isolates and 1 *Dickeya* isolate were identified. Hybrid assembly generated high quality assembled genomes. Two major abundant isolates *P. atrosepticum* and *P. brasiliense* formed two distinct clusters, with *P. brasiliense* dividing in 2 subclusters. Members of these subclusters have high ANI similarity among themselves ranging from 94% to 100%.

NAK

During the project we obtained 16 *Dickeya* and 167 *Pectobacterium* isolates from potatoes and water surveys. We performed one field trial where we vacuum infiltrated each isolate, using a 10^6 concentration, into 96 minitubers and monitored their development during the growing season for blackleg symptoms. Potato plants inoculated with *D. chrysanthemi* and *D. dieffenbachiae*, isolated from water, did not develop any symptoms in the field trial. Also no symptomatic plants were observed for plants inoculated with *P. polaris*, *P. versatile* and *P. punjabense*, originally obtained from potato. In contrast, the results obtained from different *P. brasiliense* isolates, originating from potato, were divided in 3 groups: (1) none to only sporadic symptomatic plants, (2) 8 to 18 % symptomatic plants, and (3) 61 to 91% of the plants showing blackleg symptoms. The inoculation with *P. parmentieri*, *P. atrosepticum* and *D. solani* resulted in respectively 4, 30 and 14% symptomatic plants.

The PCR used by the NAK to detect *Pectobacterium* and *Dickeya*, detects all four blackleg causing species. Given that there is also a large group of nonvirulent *P. brasiliense* isolates, we are developing a specific PCR which can also differentiate between virulent and nonvirulent isolates.



NIB

Bacteria of the genus *Dickeya* were found in both potato plants and surface water samples. The most common *Dickeya* spp. detected in potato plants was *D. solani*, however the most prevalent soft rot pathogen was *Pectobacterium brasiliense*. Almost a quarter of all test potato samples contained mixed infections of *Pectobacterium* spp. and *Dickeya* spp. However, the presence of *D. fangzhongdai* was not confirmed in any potato sample. A high prevalence of *Dickeya* spp. was detected in surface water. Of 53 samples tested, 55% were positive for the general presence of *Dickeya* spp. In addition, 11% of the surface water samples were positive for the presence of *D. fangzhongdai*. However, the bacteria were present at lower concentrations in the water samples compared to plant samples.

ILVO

The performance of four conventional PCR assays for DNA barcoding was evaluated for the type strains of 19 *Pectobacterium* species and for *P. atrosepticum* LMG 6677: (1) *pelY* (based on Darrasse *et al.*, 1994), (2) *leuS* (Portier *et al.*, 2019), (3) *dnaX* (Sławiak *et al.*, 2009), and (4) *recA* (Waleron *et al.*, 2002). Under common PCR conditions at the ILVO lab, an amplicon was not obtained for four, one, zero and two of the tested strains, respectively. This should be taken into account for future identifications. Furthermore, the results of two generic TaqMan real-time PCRs were compared: (1) *Pectobacterium* & *Dickeya* TaqMan assay as described in Humphris *et al.* (2015), and (2) *Pectobacterium* TaqMan test as described in Arizala *et al.* (2022). Based on the quality of the amplification curves, the Arizala TaqMan performs better and is hence preferred at ILVO if only *Pectobacterium* is to be detected. Also first steps to test the use of MALDI-TOF MS for the characterization of *Pectobacterium* strains were taken. Initial results indicate reliable resolution at genus level.

For virulence testing the strains were first screened in laboratory assays for their ability to cause soft rot in chicory leaves, in tomato fruits, and on cucumber and potato slices. Except for *P. fontis*, all strains caused symptoms in most of the assays. Also, no pectolytic activity was observed for *P. atrosepticum* type strain LMG 2386, it was therefore replaced by *P. atrosepticum* strain LMG 6677 for subsequent tests in greenhouse and field.

Stems of potato seedlings were then inoculated using a pin-prick inoculation method under greenhouse conditions. After 3 weeks, there were no symptoms visible on the exterior of the stem. When stems were cut longitudinally, limited browning of the inner tissue could be observed. Symptoms were mildest for LMG 17936 (*P. cacticida*), CFBP 6051 (*P. versatile*), and CFBP 800 (*P. zantedeschia*).

For the field trial twenty vacuum-infiltrated seed tubers were planted per *Pectobacterium* strain at a distance of 30 cm between tubers, with alternating planted and unplanted ridges. At the end of the growing season, stems were inspected for blackleg symptoms and serial dilutions of stem extracts were plated. The obtained *Pectobacterium* isolates were found to be the introduced strain in only a few cases, indicating the omnipresence of indigenous environmental strains (especially *brasiliense* and *polaris* strains). Additionally, to assess the transmission capacity from mother to daughter tubers, composite stolon end samples were taken, after which extracts were anaerobically enriched in pectate medium and then checked in Arizala or species-specific TaqMan tests for the presence of *Pectobacterium*. In case of a positive signal in real-time PCR, amplification and sequencing of the *pelY* or *leuS* gene fragment was performed to accurately identify the *Pectobacterium* strains residing in the daughter tubers. In this way, transmission to progeny tubers could be confirmed for *P. actinidiae* (10 out of the 19 plants analysed), *P. aroidearum* (5/19), *P. atrosepticum* (2/17), *P. brasiliense* (6/20), *P. odoriferum* (2/20), *P. parmentieri* (7/19), *P. polonicum* (2/20) and *P. zantedeschia* (3/20).

NVWA

The analyses of whole genome sequences revealed that many *Pectobacterium* and *Dickeya* species do occur in the Netherlands and that for some species high intra-species genetic



variation exists. Also some isolates that, based on their genome sequences, do not fit very well with any of the existing type strains were observed. Lastly it was shown that several *Dickeya* species cluster together with the *Musicola* species rather than with the other *Dickeya* species. Although none of these isolates originated from potato, several of them belong to species that are known potato pathogens. Twenty isolates were selected to use for two different disease assays on potato. One assay was done on tubers that were stored in a stove after inoculation while in the second assay inoculated tubers were planted and placed in a greenhouse. A high variability in virulence was observed in both assays with some isolates being highly virulent while other isolates were not virulent at all. Unexpectedly in most cases the virulent isolates were either virulent in the stove assay or in the greenhouse assay. Only one tested isolate, closely related to *P. polaris* and *P. parvum* was highly virulent in both assays.

Also at the NVWA MALDI-TOF MS was tested for identifying *Pectobacterium* and *Dickeya* isolates. When using the Bruker standard library only identification at the genus level was possible. For *Dickeya* new reference spectra (MSPs) were created to test if this would improve the identification. Preliminary results suggest that indeed the identification of *Dickeya* isolates at the species level might be possible.

2.5. Conclusions and recommendations to policy makers

Many members of the genera *Pectobacterium* and *Dickeya* cause important plant diseases on a large range of host plants. Accurate taxonomic determination is necessary to perform epidemiological studies and to take the right measures to restrict pathogen spread. To be able to perform such an accurate taxonomic determination, knowledge of the genetic variation and phylogeny of these genera and the availability of specific diagnostic tests are important prerequisites.

The first and second objectives focussed on collecting new isolates and genetically characterizing these and previously collected isolates. In South Africa (UP), Denmark (UCPH), the Netherlands (NAK) and Slovenia (NIB) isolates were collected from symptomatic and asymptomatic potato plants and tubers. In Belgium (ILVO) and the Netherlands (NVWA) also isolates from a wide range of host plants other than potato were characterised. Although several species of *Pectobacterium* and *Dickeya* were isolated from potato *P. brasiliense* was the most dominant species in South Africa, Denmark, the Netherlands and Slovenia. In the Netherlands *P. brasiliense* was also isolated from other hosts than potato such as tomato and squash. Within the 70 samples analysed in the Netherlands 17 different species were found, and several isolates could not be assigned to one of the existing species. Also in several cases mixed populations were found on one host plant. In addition, *D. fangzhongdai* appears to be present in some water sources in the Slovenian environment. Together these findings show the high abundance and huge diversity of *Pectobacterium* and *Dickeya* species in Europe and South Africa.

The third objective of this project was investigating the use of MALDI-TOF MS for the identification of *Pectobacterium* and *Dickeya* isolates. When using the standard reference library provided by the manufacturer Bruker at both ILVO and the NVWA identification of *Pectobacterium* and *Dickeya* species was only possible at the genus level. This fits with the results of a previous Euphresco project (2018-A-271). However, the creation of additional reference spectra (MSPs) for *Dickeya* strongly improved the identification of isolates of this species and made it possible to correctly identify almost every tested species. Additional testing is needed to confirm these results and to see if similar improvements can be obtained for the identification of *Pectobacterium* species as well.

The fourth objective was to get a better understanding of the epidemiology of *Pectobacterium* and *Dickeya* species by studying their virulence. Most of the disease assays that have been performed within this project focussed on potato. Although different species and isolates have been used in the different experiments the general picture that arises is that there is a high variability between and even within species when looking at virulence on potato. *P. brasiliense*

was generally found as most virulence but isolates of e.g. *P. parmentieri*, *P. atrosepticum* and *D. solani* were found to be virulent as well. However, also *P. brasiliense* isolates that were non-virulent on potato have been found. Interestingly, for some of the *Pectobacterium* species (such as *P. actinidiae*), transmission to progeny tubers was demonstrated for the first time.

The fifth and last objective of the project focussed on prediction of virulence on specific hosts for a given strain based on the presence and/or absence of specific (variants of) genes in this strain. To find these kind of gene variants large sets of closely related virulent and non-virulent isolates are needed. NAK has been working on developing a specific PCR to differentiate between virulent and nonvirulent isolates but although some potential targets have been found more work is needed to verify the predictive power of these targets.

Altogether, whole genome sequencing gives us an insight into the epidemiology of the disease and is an important tool to get a better understanding of how the disease is evolving and spreading. The updated knowledge gained from this study can aid certification and diagnostic services in testing for the correct pathogens and making accurate diagnoses. Future work should focus on continuing collecting and characterising isolates to further improve our knowledge of *Pectobacterium* and *Dickeya* and to be able to follow changes in the most dominant species. The availability of large sets of isolates will also aid in the development of diagnostic test that can specifically detect virulent isolates for selected host species.

2.6. Benefits from trans-national cooperation

By joining the Europhresco project we have gained the possibility to collaborate with other contributors by sharing isolates and sequence data to get a better overview of the isolates from Europe. Also this trans-national collaboration facilitated knowledge exchange and capacity building, allowing researchers to enhance their skills in the field of potato pathogen diagnosis and management. The collaboration also provided access to advanced research techniques and communication with international scientists.



3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

Theron, E. Bophela, K.N., Bisschoff, J., Shin. G., Coutinho, T.A., van der Waals, J.E. (2022). Survey of soft rot *Pectobacteriaceae* infecting potatoes in South Africa. *Potato Research*, <https://doi.org/10.1007/s11540-022-09598-1>.



4. Open Euphresco data

No data has been deposited in public databases yet. This is expected to be done in the future.