
Euphresco
Phytosanitary ERA-NET

EUPHRESCO Final Report

for Non-Competitive research projects

Please send the final report to all your project partners, to the NC topic coordinators and to the EUPHRESCO Secretariat (euphresco@fera.gsi.gov.uk).

Dickeya spp:
***Dickeya* species in potato and management strategies.**

Project Duration: 2 years

Start date:	01/01/11
End date:	31/12/12

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1. Executive Summary

Dickeya species in potato and management strategies

Introduction:
In 2005, *Erwinia chrysanthemi* was divided in six species inside the genus *Dickeya*. Until recently, it was thought that only the "cold tolerant" species *Dickeya dianthicola* (biovar 1 en 7) was present in Europe, causing the disease potato blackleg. Results acquired from a number of seed potato lots in last 5 years showed that a new variant (biovar 3) of *Dickeya* was isolated and that it did not match any of the described six species of *Dickeya.* This new variant, tentatively called "*Dickeya solani"*, was reported in several European countries (e.g. the Netherlands, Poland, Belgium, Finland, France, UK) and Israel. This new variant is also found in flower bulb crops, such as hyacinth and iris.

Main objectives
Research is needed to address the following objectives:

1. Monitoring (surveys) of *Dickeya* species in potato with a special emphasis on the new *Dickeya*-type ("*D. solani"*)

2. Risk assessment (improved understanding of potential risk and impact) also including

    -aggressiveness

    -host range

    -epidemiology

3. Identification and detection tools

    - new detection and identification tools for the different *Dickeya* spp.

Results: Surveys resulted in knowledge on the incidence of various blackleg and soft rot causing bacteria, indicating the relative risks of infections with the various genetic clades of Dickeya for disease expression in the different climates. It also helped to understand the routes of dissemination of the pathogen. The strains derived from the isolations were the basis for all other activities and have been essential to find new genetic clades. The results showed that *D. solani* was detected in several samples in which *Dickeya* was identified in different European countries and, therefore, *D. solani* is not uncommon in the European potato fields. Additionally, disease incidence varied largely per year and it was in most of the cases strain dependent, as it has been demonstrated in studies performed in the last few years in different countries in Europe with several representatives of Dickeya spp.

Conclusion: New genetic variants among the *Dickeya* spp. have been found in different European countries, including *D. solani,* as demonstrated in the majority of the surveys performed in this EUPHRESCO project. The aggressiveness of these new genetic variants of *Dickeya* spp. depends largely on the climatic conditions present.

Report

The collaboration in this EUPHRESCO project actually included almost all scientists working currently on this phytopathogen. This project has built on existing research and knowledge in Europe and elsewhere. The work was linked to EU-funded projects or other national projects (e.g. the Dutch "Deltaplan Erwinia" and the Brittish "*Dickeya* research plan") to ensure synergies and to prevent duplication.

In Table 2 a summary of the work allocation is provided. For each activity, 1 or more coordinators collected, developed and distributed protocols to harmonize experiments as much as possible.

Table 2. Summary activities Euphresco project Dickeya (2011, 2012)

	Activity	***Coordinator(s)***	***Execution***	***Deliverables***
1.7.1	**Risk assessment**			
	Surveys	V.d. Bovenkamp, Helias	all countries	Knowledge incidence Dickeya and Pectobacterium species
	Compilation of a strain panel	V.d. Wolf	NL	Reference panel of strains
	Genotyping	Lojkowska, Saddler	PL, UK, BE, FI	Protocols for genetic typing. DNA bank of characterized strains. Knowledge on genetic diversity
	Host specificity	V. Vaerenbergh, Lojkowska	BE, NL, UK, PL, CH, IE	Protocols for bioassays. Knowledge on host range
	Survival water	V.d Wolf, Saddler	UK, FI, NL	Protocols for testing in water. Knowledge on risks of use surface water
	Survival materials	Müller	UK, DE, NL	Protocols for testing materials. Knowledge on risks use contaminated machines and equipments
	Disease expression	Tsror, Keiser, Helias	DE, DK, UK, FI, NL, IE	Knowledge on relation between density and symptom expression in various ecosystems
	Spreading from plant to plant	Saddler, V. Vaerenbergh, Keiser	UK, BE, CH	Determination risks for dissemination in infected fields
1.7.2	**Seed testing**			
	Sampling	Keiser, Tsror	FI, DK, UK, IE	Protocol for sampling (number of tubers, tuber sample)
	Isolation	Helias	FR	protocol for isolation
	Enrichment TaqMan PCR	V.d. Wolf, V. Vaerenbergh	NL, UK	Protocols for detection of Dickeya genus and individual species
	Validation	V.d. Bovenkamp	NL, ...	basis for validated protocols in a quality assurance system

Names underlined are the contact persons

The main objectives as defined were:

1. Compilation of a strain panel

Activities. A set of ca. 50 strains was compiled representative for the various genetic clades of *Dickeya* with a relevance for potato. It included a set of *Dickeya* strains used in a previous study to characterize "*Dickeya solani"* (unpublished). It was extended with strains from unclassified genetic clades of *Dickeya* in existing collections, with unclassified strains derived from surveys and with a strains that have been used extensively in genetic studies (*D. dadantii* 3937). Strains were characterized phenotypically and genotypically. Strains were provided to all partners in the Euphresco project and deposited in international collections (NCPPB, CFBP and LMG).

2. Surveys

Monitoring (surveys) of *Dickeya* species in potato with a special emphasis on the new *Dickeya*-type ("*D. solani"*).

Activities. Surveys on the incidence of diseases caused by the various *Dickeya* genetic groups (genomic species) was conducted in several participating countries. The relative

contribution of the different *Dickeya* to the disease was determined using species and genus specific (enrichment) TaqMan PCR assays developed at PRI, SCRI and FERA. Analysis was directed towards both symptomatic plant tissue (stem and tuber tissue) and latently infected plant tissue (tubers) and water. Additionally, isolations via dilution plating were conducted for genetic and phenotypic analysis of strains and for use in ecological studies. Although the work was mainly focussed to assess the risk of *Dickeya* species, during the surveys, specific assays for the different *Pectobacterium* species were used to complete our knowledge on the causative agents of blackleg and soft rot.

Deliverables. Surveys resulted in knowledge on the incidence of various blackleg and soft rot causing bacteria, indicating the relative risks of infections with the various genetic clades of Dickeya for disease expression in the different climates. It also helped to understand the routes of dissemination of the pathogen. The strains derived from the isolations were the basis for all other activities and have been essential to find new genetic clades.

3. Risks of new genetic clades

Risk assessment (improved understanding of potential risk and impact) which including aggressiveness, host range and epidemiology.

3.1. Typing methods

Activities. Protocols were established and/or developed for analysis of strains with biochemical and genetic techniques such as multilocus sequence typing (MLST), rep-PCR techniques and pulsed field gel electrophoresis after digestion of genomic DNA with rare cutting restriction enzymes (PFGE). The strain panel was analysed with at least MLST and optionally with rep-PCR and PFGE. Cluster analysis was done to determine the phylogenetic relationship between strains.

Deliverables. Protocols for analysis of *Dickeya* strains with biochemical techniques, MLST, AFLP, rep-PCR and PFGE. A databank with information on genetic features of a representative panel of *Dickeya* strains. The genotypic analysis of strains gained knowledge on the relationship between strains and allowed the selection of representative strains for studies on ecology, control and plant pathogen interactions.

3.2. Host specificity

Activities. Protocols were established and/or developed for testing *Dickeya* strains on different (host) plants, including weeds and crops grown in rotation with potato. In addition, protocols were established for sampling of plant material from fields on which a *Dickeya* infected potato crop has been grown recently. The host range of various strains was further determined using detached plant parts, entire plants and by testing weeds and crops grown on potentially contaminated fields. This activity was supported by molecular studies on host pathogen interactions to find markers that are involved in host specificity. The availability of markers benefited a fast determination of the potential risks of strains in crop production.

Deliverables. Protocols for testing alternative host plants and for sampling material from potentially contaminated fields. Knowledge on the host range and aggressiveness of various genetic clades of *Dickeya*.

3.3. Disease expression

Activities. Protocols were established for field studies on the effect of *Dickeya* species, potato cultivar and environmental conditions on disease expression. (Naturally) infected seed potatoes were planted in different EU countries and Israel with various *Dickeya* strains and different potato cultivars. The environmental conditions (humidity, temperature, soil type) and the cultivation practices were recorded. The development of other potato diseases and pests was monitored to increase knowledge on interaction with other pathogens. The development of blackleg and stem rot was monitored and progeny

tubers were analysed to study the effect of environmental conditions on disease development and tuber infection.
Deliverables. Protocols to study disease expression under various conditions. Knowledge on the role of *Dickeya* species, potato cultivar, cultivation practices and environmental conditions on disease expression to determine the risks of various *Dickeya* species in different potato growing ecosystems.

3.4. Dissemination in the field.

Activities. During cultivation *Dickeya* can be spread by free soil water from infected plant material and in particular from rotten tubers to neighbouring plants. Protocols were established to study the dissemination in the field. In field experiments, the dissemination of various *Dickeya* species was studied and their ability to infect tubers and roots of plants using different potato cultivars.
Deliverables. Protocols to study dissemination in the field. Knowledge on the distance *Dickeya* can be disseminated with free soil water. Knowledge on risks for infection of tubers and roots by different *Dickeya* species.

3.5. Seed testing

Activities. Control of *Dickeya* is currently based mainly on hygienic and cultivation practices that avoid introduction and dissemination of the pathogen and on the use of pathogen-free seed, which can only be guaranteed if reliable seed testing methods are used. In this project, information on protocols for seed testing was exchanged, and if possible evaluated, including sampling of seed (sampling size, sampled tuber tissue), pre-treatments of tubers, extraction methods to homogenize material, enrichment protocols, DNA-extraction methods and (TaqMan-)PCR procedures. In addition, a protocol for isolation of *Dickeya* from symptomatic and symptomless infected plant tissue by dilution plating was established.
Deliverables. Recommended protocols for seed testing, comprising protocols for sampling and enrichment TaqMan PCR. Protocols for isolation of *Dickeya* from plant tissue.

4. Identification and detection tools

Deliverables. New detection and identification tools for the different *Dickeya* spp. were presented and further discussed. A summarized overview of all the available detection and identification tools for *Dickeya* spp. is provided.

Results per work package

1. Work Package: Compilation of a strain panel

Activities. The work was conducted according to the planning. A set of ca. 50 strains, representative for the different Dickeya clades and characterized with biochemical and genetic techniques is deposited in international collections. This strain panel has been used in the different work packages of this project.

2. Work Package: Surveys

Ewa Lojkowska, IFB UG, Gdansk, Poland

Aim: to establish whether *Dickeya "solani"* is present in Polish seed potato fields.
Number of samples: about 150 samples from about 70 seed potato fields.
Method: experienced staff from potato firms and plant inspection service inspecting seed potato field crops for diseases will collect stems of plants with unusual heavy symptoms of black leg and stem necrosis and also tubers with soft rot symptoms. All samples were sent by mail to Ewa Lojkowska's laboratory. The homogenate tissue was the source of material for the direct real time PCR assay (primers, Laurila et al. 2010). For the positive sample bacteria from the infected tissue isolations on CVP medium were performed and propagated on nutrient agar. Presence of *Dickeya solani* was be confirmed by Multiplex-PCR, PCR amplification of recA, rpoS and gyrA gene fragments.
Additionally, a survey in about 100 water sites was performed. At least 100 ml of water sample was collected and filtered. Bacteria from the filter were isolated on CVP medium and propagated. Presence of *Dickeya solani* was confirmed by Multiplex-PCR, PCR amplification of recA, rpoS and gyrA gene fragments.

Andreas Keiser, SHL – Switzerland

Survey was done by ACW Changins (Santiago Schaerer, Henri Gilliand):

a) Certified seed (Swiss and import) was tested for *Pectobacterium atrosepticum* and *Dickeya* by Elisa + CVP plating and biochemical tests; for *P. carotovorum* by PCR + CVP plating and biochemical tests.
b) About 60-80 samples from plants with "Erwinia" symptoms (seed potato fields, were visually inspected, ware house potatoes and commercial fields) were also tested.

Brice Dupuis and Santiago Schaerer, Agroscope, Switzerland

Each year, a survey was done on 150 Swiss potato seed lots (around 15-20% of the production) and on all imported potato seed lots (150 to 300 lots each year). On each lot, a *Pectobacterium* and *Dickeya* detection was performed on an DPEM-enriched bulked tuber tissue sample (obtained from 200 tuber cones). ELISA was used to detect *Dickeya spp*. and Pca and PCR was used to detect Pcc.
In 2011, Pcc had been detected in 37% of the Swiss lots while only 0,5% of the lots presented Pca and 3% *Dickeya* spp. This ratio was quite different from the imported lots with 19% infected with Pcc, 7% with Pca and 8,5% with *Dickeya spp*. The same survey had been done on the Swiss potato seed production in 2012 and Pcc had been detected in 16,5% of the lots, *Dickeya* in 2,5% of the lots while none of the lots presented Pca. The absence of Pca in the 2012 harvest was surprising because this species had been detected in 19% of the plant samples (black leg) collected in the 2012 "post-harvest testing" fields. This result suggested that no transfer to progeny tubers was found for Pca in potato grown under Swiss conditions even if Pca was able to provoke symptoms in the field during the season consecutive to the import. This phenomenon had already been observed in the past with other similar surveys carried out in Switzerland.

Gerry Saddler, SASA – Scotland
Pre-planting survey: All non-Scottish origin seed was tested for *Dickeya* prior to planting. A 600 tuber sample was taken from each seed lot and tested using the method outlined below. In previous years this amounts to approximately 100 crops.
Growing crop survey: In 2011, it was predicted that Scotland will have approximately 11,000 Ha seed & 15,000 Ha ware. In previous years, approximately 0.1% of the seed area and 2% of the ware area was planted with non-Scottish origin seed, amounting to approximately 100 crops. All of these crops were inspected twice by our agricultural inspectors and where evidence of blackleg was found, stem samples were sent to SASA for testing. In addition to this approximately 500 indigenous crops were selected at random and where blackleg found, stem samples were sent to SASA for testing. To place this in context, in 2010 approximately 1,500 seed crops were recorded as having any sort of amount of blackleg in Scotland. Our survey was therefore designed to cover between 1/4 and 1/3 of all indigenous, symptomatic crops.
River survey: Somewhere between 75-150 rivers were sampled, in most cases between 3-4 sampling sites were visited for each river. Rivers were selected because they had been used previously to irrigate potatoes, are in close proximity to previous *Dickeya* outbreaks, had been found to contain *Dickeya* in previous surveys or because they had not be tested in the previous three preceding years. The method used is outlined below.
Post-harvest survey: In 2011 approximately 500 crops were tested for *Dickeya*, this was similar to 2010. The post-harvest *Dickeya* tuber survey for Scotland was concluded by the end of April 2011. In total, 502 seed and ware stocks had been tested, including testing all seed stocks that had been grown from non-Scottish seed and all irrigated seed crops. With regards ware, one ware crops from every seed farm growing ware, every ware crop grown from non-Scottish seed and a range of other ware crops targeted specifically by this survey (grown on the same farm/in the vicinity of previous findings, etc.) had also been tested. (Note: Just for info., no positives have been found in the 2011 survey).

Maria Bergsma-Vlami, NPPO,The Netherlands

1. Isolation (on CVP) from stem material of a selection of the ± 250 plants showing symptoms, random selected (varieties, locations). The same samples were further tested by PCR in the inspection service NAK (see here onder).
2. Isolation (on CVP) from 49 water (different locations) samples (combined with the water samples for *Ralstonia solanacearum*).

A novel set of approximately 130 pectinolytic bacterial isolates (9 *Dickeya* sp. but mainly *Pectobacterium* sp. isolates) was obtained, at several sampling occasions, from Dutch waterways and symptomatic potato plants. A major issue in such epidemiological work is the correct identification of *Dickeya* sp. and *Pectobacterium* sp. isolates. The identity of the 9 Dickeya sp. isolates was confirmed using the specific Taqman PCR developed at PRI, the Netherlands. From the 9 *Dickeya* sp. isolates, 8 belong to the *Dickeya zeae* and 1 isolate belonged to the new variant of *Dickeya* sp. (*Dickeya "solani").*

Steen Lykke Nielsen, Dept. Integrated Pest Management, Slagelse – Denmark
Aim: to establish whether *D. solani* is present in Danish seed potato fields.
Method: experienced staff from potato firms and plant inspection service inspecting seed potato field crops for diseases collected stems of plants with unusual heavy symptoms of black leg and stem necrosis and mailed the samples to Steen Lykke Nielsen's laboratory. Bacteria from the zone between infected and healthy looking tissue were isolated on CVP medium and propagated on nutrient agar. Presence of *Dickeya solani* was be confirmed by a real time PCR assay.
Number of samples: from up to 20 fields and 1-2 samples per field.
A survey was carried out in 2011 collecting stem pieces with symptoms of blackleg or stem necrosis from 29 potato fields. DNA was extracted directly from the tissue. Presence of *D. dianthicola*/*D. solani* was tested with conventional PCR according to

Nassar et al. (1996). Specific presence of *D. solani* was tested with q-PCR according to Pritchard et al. (2012) with the primer set 'SOL-C'. The results showed that *D. solani* was detected in 25 out of the 29 positive samples in which *Dickeya* was identified. In conclusion *D. solani* is not uncommon in Danish potato fields.

Juliana Spies Perminow, Bioforsk, Norway

Aim: to establish which species of *Dickeya* and *Pectobacterium* are present in seed potato fields in Norway.
Method: experienced staff from the Norwegian agricultural inspection service inspecting seed potato field crops for diseases collected stems of plants with severe symptoms of black leg and stem necrosis and mail the samples to Bioforsk Plantehelse`s laboratory. Interesting samples received at the Institutes plant clinic were also considered. Bacteria from the zone between infected and healthy looking tissue were isolated on CVP medium and propagated on nutrient glucose agar. The presence of blackleg/stem rot-causing bacteria was confirmed by real time PCR assays.
Number of samples: from up to 20 fields and 1-2 samples per field.
All 62 symptomatic samples were tested with real-time PCR using FERA primers. Isolations were performed on MBCVP. Isolates were identified with fatty acid analysis and above mentioned primers. *D.solani* was found in seed potato of foreign origin (one incidence).

Petra Muller, JKI, Germany

Occurrence of *D. solani* in the potato production: In 2010 and 2011, potato plants showing typical symptoms of infection with *D. solani* were examined under the seed certification scheme. For this purpose, CVP medium and real time PCR were used. Those years saw only a low number of infected plants because it was very dry and rain came only late in the potato growing season. *D. solani* was identified. In some samples both *D. solani* and *Pectobacterium atrosepticum* were found.

Kristyna Hromadova Czech Republic

In the State Phytosanitary Administration of the Czech Republic we started with detection surveys of *Dickeya* spp. in potatoes in 2011. Surveys have been focused on samples of potato tubers and stems with symptoms. Results: from 13 samples analysed in Olomouc, 6 were positive for *P. carotovorum* subsp. *carotovorum* and 7 samples were negative. Dickeya spp. was not found.
However, in the survey in 2012 *Dickeya* spp. was detected. From 37 potato samples with symptoms, 6 were positive for "*Dickeya solani*", 19 samples positive for Pcc, 4 samples positive for Pa and 8 samples were negative. The first interception of a *Dickeya* spp. was found in the lab in Havlíčkův Brod from latently infected seed tubers from the Netherlands (var. Carrera) during the detection surveys of Cms and Rsol. In the Netherlands and Scotland the identity of the pathogen as „*Dickeya solani*" was confirmed. Additionally, 5 more positive samples of „*Dickeya solani*" were detected from plants with black leg symptoms and stem necrosis in the Czech Republic.

Yeshitila Degefu, MTT, Finland
Rivers and water courses

Water from rivers and water courses from the municipalities of Liminka and Tyrnävä were collected during June and first week of September 2012. The 2012 growing season was characterized by heavy rains and flooding from fields to rivers and ditches. Water samples were analyzed after concentrating by centrifugation (ca. 300 rpm for 10 min) and enrichment in polypectate enrichment medium (PEM) at 27 °C for 48 hours. The samples from June showed contamination by only *Pectobacterium carotovorum* but not Dickeya spp and *Pectobacterium atrosepticum*. Neither *Dickeya* nor *Pectobacterium* spp. *was* detected by PCR from samples.

Incidence of *Dickeya* and *Pectobacterium* spp in Blackleg of potato : the role of temperature

A total of 65 plants showing typical blackleg symptoms collected from four independent fields with heavy black leg outbreaks were tested for *Dickeya* spp, *P. carotovorum*, *P. atrosepticum* and *Pectobacterium wasabiae*. More than 85% of all the investigated symptomatic plants tested positive for *P. atrosepticum*, only 7 samples (12%) tested positive for *Dickeya* spp., and P*. carotovorum* was detected in 5 (ca. 8%) of the blackleg symptomatic plants. Unlike the last three consecutive seasons where *Dickeya* spp ("Dickeya solani"), (Degefu *et al.*, 2013, doi:10.1111/aab.12020) was the predominant species, in the symptom complex of the observed heavy outbreaks P. *atrosepticum* was the species of the year in Northern Finland. The 2012 summer was characterized by cool and wet conditions compared to the previous three summers (2008, 2010 and 2011**).** In 2012, the national summer mean temperature was 0.4 °C below the long term average. This was the coolest summer since 2008. There were a total of 18 hot days where daily maximum above 25 °C which is well below average. Thus the predominant occurrence of *P. atrosepticum* associated with the blackleg is consistent to its known feature as a cool climate blackleg pathogen. Based on this observation it can be concluded that the prevailing weather condition, especially, temperature dictates the species which plays a major role in the blackleg complex at a given season. In the events of warm spring and summers where daily average temperatures reach 25°C and above, '*Dickeya solani'* dominates (see Degefu *et al*., 2013).

Minna Pirhonen, Asko Hannukkala, Yeshitila Degefu,
University of Helsinki and Agrifood Research Finland

Aim: to study the presence of *Dickeya* in Finland in potato samples and in river water. Inspectors collected samples from suspected *Dickeya* contaminated plants showing symptoms and the samples were kept frozen. When dozens of samples had been collected, a part of the sample was thawed and liquid squeezed out of the symptomatic tissue. Real time PCR was performed from the diluted liquid to detect the presence of *Dickeya* in the sample. Bacteria were isolated on CVP from samples that we found positive in real-time PCR. River water samples were filtered, centrifuged and plated on CVP to isolate bacterial colonies. When latently infected plants were sampled, the bacteria were enriched and plated on CVP. The *Dickeya* species of the isolates were identified with IGS sequencing and phylogeny or with TaqMan PCR where available.

Sampling of diseased plants

Samples of potato plants showing blackleg and soft rot symptoms were collected by the seed potato inspectors throughout the potato growing area. In total 415 samples were collected from 61 lots. Small diseased tissue samples were incubated in water, which was then diluted 100 times and used directly in real-time PCR analysis. The samples showing small amounts of bacteria in the water, equal to liquid culture having 10^2 bacteria/ml, were estimated to be potentially contaminated, and the samples giving lower Ct values were estimated as truly positive. Results of the analysis showed that especially the plants having rot in the upper parts of the stems were scored *Dickeya* positive. The farmers were asked about the background of their seed, if known. The percentage of positive samples was higher when the seed potato was reported to be imported, whereas the samples showing low values were more common when the used seed was of Finnish origin. The truly positive samples were most common in fields where certified potatoes of class E had been used, and also foreign certified seed of class A were highly contaminated. One should keep in mind that the amount of samples in each group was not very high, which means that the results are not statistically meaningful. Furthermore, different amounts of samples were taken from different lots, and some of the lots had been sown with seed having the same origin, which further affects the results. In conclusion, the results suggested that the *Dickeya* contamination is fairly common in the potatoes grown in Finland. Especially the plants showing symptoms in the upper parts of the stems, but also those showing rot that was light in color, were shown to be

contaminated with *Dickeya*. The contamination was more common in the class E seed and in potato lots grown from foreign seed, but the contamination was also evident in the lots with Finnish origin.
The background of all the Dickeya-positive lots was investigated. The truly positive lots could be traced back to 9 lots, one of which was of Finnish origin whereas the others had been imported to Finland at some point in the past. Two of the positive lots had been imported directly, whereas the other ones had been cultivated at least once in Finland, either by a ware potato producer or a small seed potato producer. These lots were of 9 different cultivars, were sold by 5 seed potato companies and were either ware potato cultivars or starch potato cultivars. One of the imported lots had been tested negative by the MTT seed testing laboratory. The suspected *Dickeya*-positive lots with low CT-values in real-time test were sold by 3 seed potato companies and corresponded to 8 cultivars of which some were the same cultivars as the lots estimated as truly Dickeya positive. Even among the suspected positive lots, one was truly of Finnish origin, whereas the other ones had been imported and then cultivated at least once in Finland. All the interesting samples, either having Finnish origin or tested previously negative by MTT, were taken out of the freezer and plated on CVP, but no positive colonies were obtained. After that the samples were enriched in polypectate enrichment medium (PEM) at 27 °C for 48 hours and then plated, but even this time no colonies were obtained.

In conclusion, *Dickeya* strains were more common in seed potato that had been imported to Finland, but even some seed lots with Finnish origin were contaminated. Because almost all the seed lots had been multiplied at least once by the farmer himself or by a small seed potato producer, the origin of the contamination is not clear. The contamination was evident when samples were tested with real-time PCR, but in spite of this, no bacteria could be isolated from the samples on CVP plates.

Survival in volunteer potato
According to relevant literature, blackleg causing bacteria do not overwinter well in soil without plant remnants. However, the volunteer potatoes are likely source of contamination in fields where potatoes are grown in consecutive years. To study the possibility of contamination carryover from one year to the next in Finnish conditions, the overwintering of *Dickeya* spp. in volunteer tubers was investigated from four fields in 2011 and one field in 2012. The plots were inspected and volunteer potato plants and tubers were collected and analyzed. Stems, mother tubers and daughter tubers were subjected to enrichment and then real-time PCR detection. In two lots positive samples showing low Ct values were observed, but no cavities were observed on CVP plates.
In conclusion, the results suggest that *Dickeya* can overwinter in volunteer tubers and could thus contaminate the lot that will be cultivated in the same area the following year. However, to be scientifically sound, we would need to isolate live *Dickeya* cells from the overwintered volunteer potatoes, but so far this has not been successful.

Detection of *Dickeya* from water samples
In total, 65 water samples were collected 2011 from rivers throughout the costal regions of Finland, from Oulujoki to Kymijoki and the bacteria were isolated from the water samples either with or without enrichment. Samples were taken with a 3 liter bottle from 1-2 m away from the riverbank. Samples were stored overnight at +6 °C and then 250 ml was filtered and centrifuged (12310 RCF, 30 min). Most of supernatant was removed and the pellet was suspended on about 200 µl of the supernatant. 100 µl of the suspension was plated on CVP-S2 plates and the other half was enriched. After 48 h in 28 °C the bacteria from cavities in the plates were transferred on new CPV-S2 plates or LB agar. The bacterial strains were then tested for anaerobic growth and indole production. In these analyses, large amounts of *Pectobacteria* were identified from the samples, whereas only in few water samples *Dickeya* were isolated. When the ITS sequences were analyzed, it was obvious that strains from only one river, Seinäjoki, were identical with *D. solani*, and from Keravanjoki *D. zeae* was isolated. Some of the strains

showed biochemical characteristics of *Dickeya* but the ITS sequence resembled that of *Pectobacterium*. Similar strains have been isolated also elsewhere in the past, but in each case it could be a result of contamination or mix-up between strains.
In late September 2011, we repeated the sampling in the two positive rivers to see if the contamination was persistent. Many *Pectobacterium* strains were obtained but no *Dickeya* were isolated. Furthermore, the positive samples collected during the summer were reanalyzed after two months of storage in +15 centigrade, but none of the previously positive samples were positive after the incubation in storage.
Rivers were also sampled at 17 sites in August 2012. The sampling locations were in the west coast of Finland from North-Ostrobothnia (Pyhäjoki) to South-Ostrobothnia (Jalasjärvi). The isolations from river water showed that only in one river (Seinäjoki) *Dickeya* bacteria were identified. A second sample was obtained a month later and shown to be positive. The same river was the only one in 2011 river survey where *Dickeya solani* was identified. This river runs through the main area used for cultivation of ware potatoes.
In conclusion, *Dickeya* was identified from two rivers in 2011 and from one river in 2012. It is a mystery why so many *Dickeya* isolates were obtained in our previous survey 2004-2005, but in the present survey mostly *Pectobacterium* was identified from the rivers. One explanation could be the weather. At least in 2012, the majority of the infections in potato fields were caused by *Pectobacterium*, not *Dickeya*, which may be reflected in the bacteria present in the river water.

Table 1: Summary of incidences in seed potato as found in the surveys

Country	Situation	Source
SE	D. solani found, but in low incidences in seed	SREAS/ SUAS
UK (Scotland)	No D. solani in potato and surface water in last years	SASA
FI	High incidences with D. solani, in warmer years much damage	UH, MTT Oulu
UK (England)	Low incidences with Ds, more Dd. Pa dominant pathogen	FERA
PL	Increasing incidences in potato. Increasing number of regions	UG
CH	For a long time much Dickeya. Incidences are not increasing	Agroscope
FR	Slight reduction incidences with D. solani	INRA
NL	Incidences with Dickeya decreasing from 70 – 30%. Pectobacterium more important in 2012	NAK
CZ	D. solani found in imported seed	Hromadova
BE	Incidences Dickeya decreasing, different Pectobacterium species found	ILVO
DE	D. solani found. Damage can be high	JKI
IE	D. solani found in imports. Damage can be high	ARO
NO	D.solani found in seed potato of foreign origin, summer 2012	BIOFORSK

Table 2: Summary of incidences in water as found in the surveys

Country	Situation	Source
Scotland	D. Solani found several years in water ways, but not in 2011, 2012	SASA
Fl	D. solani (1x) and D. zeae(1x). Not found in high grade seed areas	UH, MTT Oulu
UK (England)	ca. 12% of the rivers positive for *Dickeya*. *D. zeae, Dickeya* sp., *D. dianthicola* (2x) and *D. solani* (1x)	FERA
Pl	3500 samples tested (lakes, rivers, waste water): 25 positive, all *D. zeae*	UG
NL	One finding D. solani. D. zeae often present in high densities	NPPO

Table 3: Summary of incidences in weeds as found in the surveys

Country	Situation	Source
IE	In on and in roots of Cyperus rotundus	ARO
FI	In Finland detected by PCR on different weeds (Brassicaceae)	MTT Oulu
Eng.	Not found in weeds (S. nigrum, C. album)	FERA
FR	Found in weeds during the growing season, not thereafter	INRA
Scotl.	Found on weeds in greenhouses after artificial inoculation with high densities	JHI
Engl.	Soil from infected crop: 2 months after harvest positive; next spring negative	FERA

Work package 3 Risks of new genetic clades

3.1 Typing methods

This is discussed in the results of work package 4 on Identification and detection tools.

3.2 Host specificity

Brice Dupuis and Santiago Schaerer, Agroscope, Switzerland

Three cultivar susceptibility trials were carried out on four potato cultivars (Agria, Innovator, Victoria and Charlotte). The first trial was performed on inoculated tuber slices, the second on inoculated tubers planted in pots and cultivated in the greenhouse and the last trial on infected tubers planted in the field. Each trial was repeated two times. The field trial offered the most reproducible results. The main conclusions of those trials are that differences in susceptibility to *Dickeya* were observed and that Agria seems to be the most susceptible of the four varieties.

3.3 Disease expression

Van der Wolf, PRI, NL.

Studies were conducted to explain the relative success of '*D. solani*'. *In vitro* experiments showed that both species, '*D. solani*' and *D. dianthicola,* were motile, had comparable siderophore production and pectinolytic activity, and that there was no antagonism between them when growing. Both *'D. solani'* and biovar 1 and biovar 7 of *D.*

dianthicola rotted tuber tissue when inoculated at a low density of 10^3 cfu ml^{-1}. In an agar overlay assay, *D. dianthicola* was susceptible to 80% of saprophytic bacteria isolated from tuber extracts whereas *'D. solani'* to only 31%, suggesting that *'D. solani'* could be a stronger competitor in the potato ecosystem. In greenhouse experiments at high temperatures (28 °C), roots were more rapidly colonized by *'D. solani'* than by biovar 1 or 7 *D. dianthicola* and at 30 days after inoculation higher densities of '*D. solani'* were found in stolons and progeny tubers. In co-inoculated plants, GFP- or DsRed-tagged *'D. solani'* out-competed *D. dianthicola* in plants grown from vacuum-infiltrated tubers. In three years of field studies in The Netherlands with *D. dianthicola* and *'D. solani'*, disease incidence varied largely annually and it was strain dependent. In summary, *'D. solani'* possesses features which allow more efficient plant colonization than *D. dianthicola* at high temperatures. In temperate climates, however, tuber infections with *'D. solani'* will not necessarily result in a higher disease incidence than infections with *D. dianthicola* but latent seed infection could be more prevalent.

Van Gijsegem, INRA, France

1 - Development of a protocol for bacterial transcriptomic studies *in planta* during the early phases of infection
The challenge for studying transcriptomes of pathogens inside host tissues is that the amount of pathogen RNA often becomes vanishingly small compared to host RNA. We developed a reliable and rapid method for bacterial cell purification from infected plant tissues, efficient even when bacterial amounts are very low as regard to plant material. This is achieved by differential filtrations and centrifugations followed by a density gradient purification step to drastically lower plant material contamination. The whole purification protocol is performed in the presence of a RNA stabilizing reagent to freeze the bacterial gene expression profile as in the *in planta* state. The efficiency of this method for transcriptomic analysis of the early steps of plant colonization, before symptom occurrence, was validated on microarrays.

2 - Determination of the complete genome sequence of a *D. solani* French isolate (in collaboration with Denis Faure, ISV, France)

The sequence of the complete genome of *D. solani* was determined by combining Illumina sequencing of two DNA libraries, one of 300 nt pair-ended and one of 8 kb mate-pair. Comparizon with the genome of the *D. dadantii* model strain 3937 revealed a close relatedness and a high synteny between both genomes. A few hunderds genes are however present in only one of these strains. A great proportion of these genes are clustered and some of these gene clusters contain mobile genetic elements pointing to an acquisition by horizontal transfer. Remarkably, several gene clusters regoup enzymes involved in primary metabolism or in the biosynthesis of secondary metabolites that might take part to signalling events. The complete sequence of a *D. dianthicola* French isolate has also been determined and comparizon studies with *D. solani* and *D. dadantii* genomes are underway.

Brice Dupuis and Santiago Schaerer, Agroscope, Switzerland
Disease expression: for the first time a field trial was carried out with five different *Dickeya* isolates (two *D. solani* and three *D. dianthicola*). Inoculated tubers were planted and the development of blackleg (aerial stem rot) symptoms were observed. The most and the least aggressive isolates were two different *D. dianthicola* isolates. This is suggestive of some genetic diversity within this bacterial species.

3.4 Dissemination in the field

Steen Lykke Nielsen, Dept. Integrated Pest Management, Slagelse – Denmark
The activity comprised two years of field trials. The field trial lay out was both years block design with 4 replicates and 3 treatments: tubers infected with *D. solani, D. dianthicola* and water control. The potato cv was Bintje. Each replicate was 20 row m with 3 plants per m. Tubers were infected by DIP inoculation (accord. to V. Hélias, personal communication). Top symptoms were assessed weekly. At harvest the average tuber weight in g, numbers of tubers and numbers of rotten tubers of 10 m harvested row per replicate were assessed. The results showed no difference in development of top symptoms, in harvested tuber weight, tuber numbers and rotten tubers between the *D. solani* and the *D. dianthicola* treatments. In conclusion *D. solani* has not been more harmful than *D. dianthicola* under the Danish growth conditions in 2011 and 2012. A detailed report of the results is published in Danish on http://www.kartoffelafgiftsfonden.dk/rappport/Rap12/AU_Dickeya_2011_12.pdf

Brice Dupuis and Santiago Schaerer, Agroscope, Switzerland
Healthy potato plants were inoculated three times in the field by watering (10^6cfu/ml) at 7 day intervals, starting two weeks after emergence. One month after the first inoculation, 4% of black leg symptoms were recorded in the inoculated plots. This result suggests that an early infection at the root level could provoke the development of symptoms in the field.

Yeshitila Degefu, MTT, Finland
1. Overwintering in tubers: Overwintering tubers which have been exposed to surface during land preparation were collected from two fields in Tyrnävä where heavy outbreaks of blackleg were recorded during the previous growing season. The samples included intact and rotten tubers. All the tubers were analyzed for *Dickeya* and *Pectobacterium* species according to the protocol described in Degefu *et al*. (2009) and none of these tubers tested positive in the PCR suggesting that bacteria might not have survived the cold winter temperatures in tubers exposed to the soil surface unlike in the tubers which are deep buried in the ground which were found to harbor the pathogen in overwintering mother tubers and the resulting daughter tubers of the volunteer potato.
2. Survival in Soil: Soil samples were collected from random sites from the same fields where the overwintering tubers were obtained and confirmed cases of blackleg outbreaks were observed. Sampling was done soon after harvest and during early weeks of May 2012 when the snow was melted and before the fields were planted with other crops. Twenty five gram of soil was resuspended in 75 ml of sterile water. Flasks containing the samples were subjected for gentle shaking for about 30 min and aliquots were taken from the samples and enriched for 48 hours in PEM at 27°C shaking at 200 rpm. Diagnostic PCR revealed soil contamination by *Dickeya* soon after harvest but no overwintering Dickeya was detected in soil samples obtained in spring after the snow cover suggesting that overwintering in soil seems unlikely.
3. Volunteer potato: PCR analysis of volunteer potato plants collected from fields where there were heavy outbreaks of blackleg and soft rot during the preceding growing season indicated survival in infected mother tubers and the resulting daughter tubers.
4. Weeds: Preliminary results indicated that *Dickeya* detected by PCR from the roots of a weed which belong to the genus *Brassica*. No disease symptoms have been in the roots of the plant has been observed and the pathogen has not been isolated from the root extract. The investigation on this and other weed species is still carried out in 2013.

Petra Muller, JKI, Germany

1. Survival on different surfaces

The first phase focused on the examination of the methods and tests to be used since there had never been any studies on the survival of *D. solani* on different surfaces. Tests

were made on wood, cotton, rubber, plastic, varnished and corroding iron surfaces. The CVP medium revealed to be most suitable to test the survivability of the pathogen. Furthermore, the fluid medium PEM (Pectate Enrichment medium), but also phosphate buffer are well suitable to recover *D. solani* from the surfaces. After several hours enrichment in PEM, however, saprophytes had multiplied so much that they suppressed *D. solani* and a "false negative" result came out. These negative impacts were avoided through a short incubation period.

After thorough examination infected tuber tissue was used to contaminate the surfaces. As test conditions two temperatures and low and high relative humidity were chosen. So, it has been possible to reisolate living *D. solani* from the contaminated surfaces still after four weeks incubation at 5 °C and high relative humidity without having clearly reduced bacteria density.

Table 4: Summary of risks of new genetic clades

Country	Situation	Source
DK	No difference in symptom expression or disease incidence was found after dip inoculation of tubers with Pa or Ds or Dd. Overall little effect was found due to infections with Ds or Dd on tuber weight and tuber numbers	AU
UK (England)	Inoculation of seed with various densities of Dickeya (low, medium and high) show a strong dosis- response effect	CSL
CH	Watering potato plants three times with 10e6 cells/ml of Ds did not result in 4% symptom expression. The infection rate was not determined.	Agroscope
IE	Dickeya populations can extinct in next generations.	ARO
CH	Dickeya populations can become undetectable in next generations.	Agroscope
	In temperate zones, pathogenicity *D. solani* is similar to other blackleg pathogens. Under high temperature conditions *D. solani* can cause a lot of damage	ARO, PRI

Table 5: Summary of disease expression and biocontrol

Country	Situation	Source
DK	None of the commercial cultivars were really resistant, but Arinda was relatively tolerant	AS
UK (Scotland)	Rating of susceptibility of cultivars is independent on infection with Pa, Dd or Ds	SASA
NL	Tuber treatments with a biocontrol agent may be useful for control of blackleg	PRI

Table 6: Summary of plant pathogen interactions

Country	Situation	Source
Israel	Ds isolates isolated from plants grown in countries with a warm climate show increased expression of some virulence genes	ARO
France	Development of a protocol for bacterial transcriptomic studies *in planta* during the early phases of infection	INRA
France	Determination of the complete genome sequence of a *D. solani* French isolate (in collaboration with Denis Faure, ISV, France)	INRA

Work package 4: Identification and detection tools

Van der Wolf – PRI (in collaboration with Euphresco partners).

The purpose of this work was to develop TaqMan PCR assays for the detection of individual *Dickeya* species, namely *D. dianthicola, D. dadantii, D. paradisiaca, D. chrysanthemi, D. zeae, D. dieffenbachia* and a new genetic clade of biovar 3 *Dickeya* strains, tentatively called *"D. solani".* In addition, the TaqMan PCR assays for *D. dianthicola* and *"D. solani"* were evaluated as part of a method validation procedure. Sequences of the gene coding for *dnaX* were used for the design of primers and probes. The assays were usually specific and only rarely showed weak reactions with non-target *Dickeya* strains, but never with bacteria from other genera. The detection thresholds for DNA extracted from pure cultures of positive strains ranged from 10 to 100 cfu/ml. In the case of *D. dianthicola* added to potato peel extract, enriched in a semi-selective broth, the detection threshold was 100.000 cfu/ml and ranged between 100 and 100.000 cfu/ml for *"D. solani"*, depending on the strain. The coefficients of variation for repeatability and reproducibility were low and results were largely independent of the type of substrate (potato tuber or carnation leaf extracts) used. TaqMan PCR assays have been developed for the detection of seven individual *Dickeya* species, which are useful for rapid strain identification, surveys on population patterns in crops, and in quantitative studies on spatial distribution, dissemination, survival, plant colonization and the effect of control strategies.

Describing *D. solani* as new species: D. solani was found to be Gram-negative, motile, rods shaped. They gave positive reactions in a Dickeya specific PCR assay with *pelADE* primers. They produced phosphatases and were able to produce acids from α-methyl glucoside. Differential physiological assays used previously to differentiate between strains of *E. chrysanthemi*, showed that these isolates belonged to biovar 3. Eight of the isolates, seven from potato and one from hyacinth, were analysed together with 21 reference strains representing all currently recognised taxa within the genus *Dickeya*. The new isolates formed a distinct genetic clade in multilocus sequence analysis (MLSA) using concatenated sequences of the intergeneric spacer (IGS), as well as genes *dnaX*, *recA*, *dnaN*, *fusA*, *gapA*, *purA*, *rplB*, *infB*, *rpoS* and *gyrA.* Characterisation by MALDI-TOF mass spectrometry, pulsed field gel electrophoresis after digestion of whole genome DNA with rare cutting restriction enzymes, average nucleotide identity analysis and DNA-DNA hybridization studies, showed that although related to *D. dadantii*, these isolates represent a novel species within the *Dickeya* genus. for which the name *Dickeya solani* sp. nov. (type strain IPO 2222^T =LMG25993^T=NCPPB4575^T) is proposed.

Table 7:Summary of tools

Country	Situation	Source
IE	A high correlation (85%) between laboratory test (E-PCR) and symptom expression)	ARO
NL	High correlation (85%) between laboratory test (E-multiplex TaqMan and symptom expression	NAK
UK	VNTR analyses *D. solani* strains from different countries (5 markers): 2 different types were found. For D. dianthicola 8 types were found	FERA
UK (Scotland)	New Dickeya species-specific TaqMan assays were developed. Ring test gave poor results	JHI
BE	MLSA schemes for Dickeya and Pectobacterium were developed	ILVO
UK (Schotland)	MLSA schemes for Dickeya were developed	SASA
NL	MSLA schemes for rapid screening were developed	NPPO

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