Chapter 8

Summarizing Discussion
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This thesis focuses on the ecology and pathogenicity of biovar 3 \textit{Dickeya} sp. provisionally called “\textit{D. solani}”, a blackleg and soft rot pathogen, recently introduced into Europe. The aim of this study was to acquire knowledge on the dissemination of the pathogen in the environment and on new infection routes in relation to tuber infection as well as to develop new strategies for controlling the pathogen on the basis of this new knowledge. Substantial research activities were directed towards the application of biological control strategies to eradicate “\textit{D. solani}” from infected tubers.

NEW INFECTION ROUTES IN RELATION TO PATHOGENESIS OF BIOVAR 3 \textit{DICKEYA} SPP.

Potato seed tubers are the main source of blackleg and soft rot inoculum and the predominant route for long-distance spreading of \textit{Dickeya} and \textit{Pectobacterium} species. Production of pathogen-free seed lots and tubers can be regarded as the easiest and most effective approach nowadays to control blackleg and soft rot diseases during (seed) potato production. However, potato seed lots might become infected during virtually all phases of tuber production and the infection can occur both from outside via lenticels and/or from inside via vascular tissue. Knowledge on infection routes and possible entrances used by pectinolytic bacteria to infect plants is limited. Till present important issues concerning bacterial distribution inside tubers, infection routes and the systemic colonization of (seed) tubers were only partially addressed. Knowledge on the distribution of pectinolytic bacteria on and inside tubers is essential for development of new and monitoring existing sanitation methods. Only limited information on the distribution of pectinolytic bacteria on and inside tubers was present prior to this study. Similarly, the importance of the different inocula present on and inside potato tubers for the blackleg and soft rot development was not completely understood.

In order to gain knowledge on the distribution and population structure of blackleg and soft rot bacteria in naturally infected seed potato tubers, we sampled two potato cultivars (i.e. cv. Arcade and cv. Kondor) harvested from blackleg diseased crops cultivated at two different locations in The Netherlands (Chapter 3). Both naturally infected seed lots were latently contaminated with \textit{Dickeya} spp. and \textit{Pectobacterium carotovorum} subsp. \textit{carotovorum} but not with \textit{P. atrosepticum}. The highest pectinolytic inoculum (i.e. \(10^3 - 10^4\) cfu g\(^{-1}\)) was located in the stolon end and its vicinity, but hardly any bacteria were present deeply inside
tubers. Although relatively high densities \((10^3 - 10^4 \text{ cfu g}^{-1})\) of pectinolytic bacteria were also found in peels, these densities calculated per gram of tuber tissue were low (approx. \(10^1 \text{ cfu g}^{-1}\)) compared to the densities in stolon ends \((10^2 - 10^3 \text{ cfu g}^{-1})\). REP-PCR, 16S rDNA sequence analyses and biochemical assays grouped all \(Dickeya\) spp. isolates into the new genetic clade of \(Dickeya\) sp. biovar 3.

The occurrence of the highest bacterial density inside stolon ends indicates that the tubers became infected via transport of bacteria through the vascular tissue from stolon into tuber. This pathway of infection is in agreement with field experiments in which stolon ends of vacuum infiltrated with \(Dickeya\) spp. tubers became infected just when progeny tubers were formed by the bacteria transported via vascular tissue from stems and stolons (Velvis & van der Wolf, 2009).

There are at least two ways by which progeny tubers can be infected with pectinolytic inoculum derived from vascular tissue. Firstly, the bacteria can move from the rotten seed (mother) tuber directly into stems and stolons and colonize progeny tubers. Secondly, bacteria that leaked from the rotten mother tuber to soil, may move with free soil water inside soil and infect potato roots and/or (progeny) tuber periderm. Infection of tuber peel will certainly result in internal infection of vascular tissue of progeny tubers. Infection of roots from soil can, however, result in the vascular systemic infection of the plant but this problem was not assessed in detail till present.

\(Dickeya\) spp. bacteria are not expected to survive in soil during winter. \(Dickeya\) spp. are generally not recognized as a soil-borne pathogens (Perombelon, 2002). Infestation of soil with \(Dickeya\) spp. can, however, occur from a diverse array of sources. The bacteria can be repeatedly introduced into soil when heavily rotten tubers are present. Alternatively, inoculum can also be released directly from symptomatic stems during rainfall. In addition, soil infestation with \(Dickeya\) spp. may occur also from hosts other than potato (alternative hosts), e. g. from weeds.

To investigate the ability of biovar 3 \(Dickeya\) spp. to infect roots from artificially infested soil and to colonize potato plants systematically, we made use of a representative biovar 3 \(Dickeya\) sp. isolate tagged with plasmid based green fluorescent protein (GFP) (Chapter 4).

One day after soil inoculation, we observed adherence of the \(Dickeya\) sp. bacteria on roots and internal colonization of plants being detected using epifluorescence stereomicroscopy and confocal laser scanning microscopy. Two weeks after soil infestation, \(Dickeya\) sp. was detected, on average, inside 42% of the roots, 13% of the stems and 13% of the stolons in plants with intact roots. At two weeks after soil infestation, \(Dickeya\) sp. was found inside 50% of the roots, 25% of the stems and 25% of the stolons of plants with damaged roots. Thirty days
post inoculation, plants expressed true blackleg symptoms. In roots, *Dickeya* sp. was detected in parenchyma cells of the cortex, both inter- and intracellularly. In stems, bacteria were found in xylem vessel protoxylem cells. In addition, we frequently found pectinolytic bacteria in stolon end of progeny tubers which indicates that vascular infections may play an important role in the dissemination of the bacteria within a field.

Clean, blackleg bacteria-free potato tubers can become infected by various practices during cultivation. Contamination of potato plants may result from contaminated machines, insects, water used for crop irrigation, rainfall, aerosols or animals and humans entering potato fields. Infection resulted from these sources occurs more readily in aerial than in underground plant parts. Tubers may be colonized directly after haulm infections if bacteria are able to move downward inside vascular tissue of the stems or indirectly via soil if bacteria are washed off from symptomatically infected stems during rainfalls.

No information until now was available on the ability of *Dickeya* spp. to move downward inside the vascular tissue of potato plants and if haulm infections could result in infection of underground plant parts (including progeny tubers). In order to study the ability of biovar 3 *Dickeya* sp. strains to infect roots, stolons and progeny tubers from inoculated haulms, we artificially inoculated stems or leaves of plants grown in the greenhouse (Chapter 5). Again, we used GFP-tagged biovar 3 *Dickeya* sp. strain and epifluorescence stereo microscopy and confocal laser scanning microscopy techniques to follow the fate of the bacteria. Thirty days after stem inoculation, 90 % of plants showed typical blackleg symptoms at the stem base and 95 % of plants showed discoloration and browning of internal stem tissue. GFP-tagged *Dickeya* sp. was detected in the stem interiors (100%), stem bases (90%), roots (80%), stolons (55%) and progeny tubers (24%). In roots, GFP-tagged *Dickeya* sp. was found inside and between parenchyma cells, whereas in stems and stolons, *Dickeya* sp. was found in the xylem vessels and protoxylem cells. In progeny tubers, the strain was detected in the stolon end. Thirty days after leaf inoculation, *Dickeya* sp. was detected in extracts of 75 % of the leaves, 88 % of the petioles, 63 % of the axils, and inside 25 % of the stems taken 15 cm above the ground level. UV-microscopy confirmed the presence of GFP-tagged *Dickeya* sp. inside petioles and in the main leaf veins. No blackleg or aerial stem rot was observed after leaf inoculation and no translocation of the GFP-tagged *Dickeya* sp. to underground plant parts.

In conclusion, the results suggest that systemic colonization of potato plants after infection of roots or haulms may play a significant role in blackleg epidemiology. The data also indicate that systemic infections of underground plant
parts including progeny tubers readily occur via translocation of bacteria from infected stalks as well as via roots from soil-borne inoculum. The systemic infections may explain the high incidences and the high bacterial densities found in tuber stolon ends of naturally infected seed lots.

This knowledge on the distribution of *Dickeya* and *Pectobacterium* species in potato tubers is important to decide on which part should be sampled in seed tuber testing programmes. This research also points that seed treatments based on the superficial disinfection cannot eliminate the bacterial pathogens because frequently the bacteria are located inside tubers and not on their surfaces. It also indicates that drainage of soil is of large importance to avoid spreading of bacteria from infected to neighboring plants which can result in root colonization. It finally demonstrates the risks for haulm infections in a pathogen-free crop.

**THE EFFECTIVENESS OF BIOLOGICAL CONTROL OF BIOVAR 3 *DICKEYA* SPP. (“*D. SOLANI*”)**

Production of pathogen free seed tubers is one of the most effective strategies in blackleg control. At this moment, hygienic measures and good cultivation practices only result in a partial control of blackleg (Chapter 2). Although research is undertaken to develop control strategies based on tuber treatments with chemical and physical agents and to create (partially) resistant potato cultivars, success is limited so far. Chemical agents and physical treatments lack the ability to eliminate inoculum inside plants and tubers. Biological control seems to be a new and promising alternative. In theory, biocontrol agents might be able to control the bacterial pathogens in the vascular tissue of plants.

In order to isolate potentially effective antagonists, we isolated bacteria from potato rotten tissue as this habitat is rather selective (Chapter 6). In total, we isolated 649 isolates which were screened for antibiosis against biovar 3 *Dickeya* sp. and for the production of siderophores. Forty one strains (6.4%) produced antibiotics and 112 strains (17.3%) produced siderophores. A selection of 41 antibiotic-producing strains and 41 siderophore-producing strains were tested in a potato slice assay for the control of the *Dickeya* sp. *in vitro*. Strains able to reduce rotting of potato tuber tissue by at least 50% of the control were selected for further studies. Strains were characterized by 16S rDNA analysis as belonging to the genera of *Bacillus*, *Pseudomonas*, *Rhodococcus*, *Serratia*, *Obesumbacterium* and *Lysinibacillus*. Twenty three isolates belonging to different species and genera; 13 producing antibiotics and 10 producing siderophores, were further characterized by
testing for quorum quenching, motility, biosurfactant production, growth at low (4.0) and high (10.0) pH, growth at 10 °C under aerobic and anaerobic conditions and for auxin production. In replicated greenhouse experiments, four antagonists selected on the basis of in vitro tests, were tested in planta using wounded or intact mini tubers of cv. Kondor subsequently-inoculated by vacuum infiltration with an antagonist and a GFP-tagged biovar 3 Dickeya sp. strain. A potato endophyte A30, characterized as S. plymuthica protected potato plants by reducing blackleg development by 100% and colonization of stems by Dickeya sp. by 97% (Chapter 6).

Protection of potato plants was found after application of antagonistic bacteria directly to tubers by vacuum infiltration, so, under conditions favorable for blackleg and soft rot development, showing that the S. plymuthica A30 isolate can be valuable biological control agent.

The development of effective biological control strategies requires fundamental knowledge on the ecology and the interaction of the control agent with the pathogen, the host plant and the rhizosphere microbial communities. Therefore we investigated (Chapter 7) the ability of S. plymuthica A30 strain to colonize the potato plant root system and to protect the plant from biovar 3 Dickeya sp. infections. In order to follow the fate of both the antagonist and the pathogen biovar 3 Dickeya sp. strain was tagged with red fluorescent protein (DsRed) and S. plymuthica A30 with green fluorescent protein (GFP).

S. plymuthica A30 was able to control biovar 3 Dickeya sp. in vitro conditions and in planta. In laboratory assays, A30 strain produced antibiotic against biovar 3 Dickeya sp. and was able to stop the growth of the pathogen after incubation for 24 h at 28 °C in overlay plate assay. In potato slice assays S. plymuthica was able to completely reduce potato tissue maceration caused by Dickeya sp. at inoculum sizes 10 and 100 times higher than those of Dickeya sp. Under greenhouse conditions the biocontrol strain was able to colonize systematically potato plants after tuber treatments. The A30 strain was found in the vascular tissue of roots and stems and in the internal tissue of seed tubers seven days post soil inoculation. S. plymuthica A30 populations inside plants were stable during the entire course of the experiment. Application of A30 strain on the tuber just before planting protected potato plants against blackleg and resulted in the eradication of the Dickeya sp. biovar 3 strain from plant tissue such that after 28 days the pathogen was not found in any of the co-inoculated plants.

These results clearly show the great potential of S. plymuthica A30 as antagonist in the biocontrol of Dickeya spp. biovar 3. Although bacterial
antagonists controlling blackleg and soft rot causing pathogens have already been described in literature, in most cases the evaluation of these strains was limited to studies taken only under in vitro conditions. Very limited information is present on the potential of the described biocontrol agents to protect potato tubers and plants under field conditions or during storage.

In principle, the use of biocontrol agents may be further combined with other approaches to control blackleg and soft rot in an integrated strategy (Chapter 2). Biological control of plant pathogenic bacteria is promising for the future, when applied alone or combined with new breeding programmes to obtain lines expressing increased resistance against potato bacterial and fungal pathogens.

**FUTURE PROSPECTS AND OUTLOOK**

This thesis describes aspects of the ecology of *Dickeya* spp. in relation to blackleg pathogenesis (Chapter 3, 4 and 5). It also gives an insight in the possibilities of biocontrol of blackleg causing bacteria with the use of bacterial antagonists (Chapter 6 and 7).

My present findings also create new research questions to be subject for further investigations.

As it was shown in Chapter 3, the majority of pectinolytic bacteria is located in stolon end of the (seed) tubers and rarely in other internal tissues. Pectinolytic bacteria can be also present in and on peel in lenticels and cracks and wounds. It remains unclear, however, which populations present in and/or on tuber are responsible for the initiation of the tuber rotting. In line, it is also unknown, if this process starts in the densely contaminated areas and what conditions influence this primary stage of tuber rotting. It is generally accepted that the infection starts in densely colonized tissues rather than from places where only low inoculum is present. Yet, the contribution of different pectinolytic populations located in peel and/or stolon end of tuber to the blackleg incidence is unknown and should be further investigated in future research projects.

The work presented in Chapter 4 and 5 describing systemic infection of potato plants after root or haulm infections provided insight into the dissemination of the *Dickeya* sp. inside potato plants during infection. However, the role of the soil-borne inoculum in the blackleg incidence and the frequency of root infection directly from soil have not been assessed so far in detail for (biovar 3) *Dickeya* spp. and other pectinolytic bacteria. Similarly, the mechanism by which bacteria move actively inside xylem vessels against water stream (downward translocation of
bacteria from stems to roots, stolons described in Chapter 5) after inoculation of stems and the translocation of the inoculum to the progeny tubers have neither been fully unrevealed. Again, better understanding of these processes is required to enable the development of adequate predictive models to be used in integrated control strategies.

Successful control of biovar 3 *Dickeya* spp. “*D. solani*” by *S. plymuthica* A30 under greenhouse conditions (Chapter 6 and 7) generated questions on the control mechanism(s) involved as well as on the mode of action of *S. plymuthica* A30 during colonization of root, stems and tubers. On top of this, it will be of practical interest to understand the conditions under which *S. plymuthica* is able to colonize potato roots and to establish stable populations in and on potato plants in order to fully use the biocontrol potential of this strain.