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Robert Czajkowski (2011)
Ecology and control of *Dickeya* spp. in potato
Leiden: Leiden University, Institute of Biology, Leiden, 2011
Ph.D. thesis Leiden University, The Netherlands
with references, with summaries in English, Dutch and Polish

ISBN: 978-90-9026234-5

This research was supported by Technologiestichting STW *via* grant 10306

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PROEFSCHRIFT

ter verkrijging van
de graad Doctor aan de Universiteit Leiden,
op gezag van der Rector Magnificus prof. mr. P. F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 7 september 2011
klokke 13.45 uur

door

Robert Czajkowski

geboren te Gdynia, Polen
in 1981

Promotiecommissie

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It may be that the gulfs will wash us down;
It may be that we shall touch the Happy Isles,
We are not now that strength which in old days
Moved earth and heaven,
that which we are, we are
One equal temper of heroic hearts,
Made weak by time and fate, but strong in will
To strive, to seek, to find, and not to yield.

(Ulysses by Alfred, Lord Tennyson)

Moim Rodzicom,

dzięki którym jestem kim jestem

(To My Parents, thank to whom I am who I am)

Contents

Chapter 1

General Introduction

POTATO PRODUCTION

Potato plants originated from the Andes mountains in South America where potato tubers were consumed as early as 8000 years ago. In the late XVI century (around 1570) potato was introduced into Europe by Spanish explorers initially as a decorative plant and then, later also for consumption (Hawkes & Francisco-Ortega, 1993). From there, the potato quickly spread to Italy, England and The Low Countries. By 1650, potatoes were cultivated as a field crop in Flanders, and had spread to the north of The Netherlands; to Zeeland by 1697, to Utrecht by 1731 to Overijssel by 1746 and to Friesland by 1765 (Messer, 2000). By 1800, the potato tubers were accepted as part of Dutch national diet (Davidson, 1992).

Potato is currently produced in 126 countries and the area of potato cultivation is rapidly increasing especially in developing regions (Leff et al., 2004). Today more than 325 million tonnes of potato are produced all over the world but the majority of potato production still occurs in Europe and Asia (approximately 80% of world potato production) (van der Zaag & Horton, 1983). In Europe ware potatoes are mainly cultivated in Russia, Ukraine, Poland, Germany, Belarus, France and in the United Kingdom. Simultaneously, The Netherlands with an export of ca. 700 000 tons per year for more than 40 years is the world leading producer of certified seed potatoes (NAO - Nederlandse Aardappel Organisatie; International Potato Center, Lima, Peru).

Today, in Europe, The Netherlands is among the top 10 potato producers with a harvest of approximately 7.2 million tonnes a year. Almost 25% of the Dutch arable land (around 160 000 ha) is used for potato production with an average yield of 45 tonnes per hectare. Around 50% of the potato crop is grown for food, 30% for starch production and the last 20% are seed tubers. The annual production of seed potatoes in The Netherlands is estimated to be about 1.2 million tonnes, which is 37% of the total seed potato production in the European Union. In 2009, the total area in which seed potatoes were cultivated in The Netherlands was 37.000 hectares. In the season 2008/2009 the export of seed potatoes to the European Union, Asia and America (altogether approximately export to 80 countries) reached 662.000 tonnes, which is 70% of export worldwide (J Gottschall, NAO, personal communication).

The intensive production of (seed) potato tubers increases the risks for spread of potato diseases. Potato plants and tubers are affected by approximately one hundred sixty diseases from which fifty are caused by fungi, ten by bacteria,

forty diseases are caused by viruses and the rest are based on the unknown-yet origin (Arora & Khurana, 2004). Diseases may influence potato production in any stage of crop growth and in storage, and can effect foliage, tubers or both. Potato diseases may reduce heavily crop yield and quality (Hooker, 1981).

From all pathogens infecting potato seed production, bacteria are recognized as the most serious problem (Van der Wolf & De Boer, 2007). Bacteria are able to infect potato plants and tubers from wounds and natural openings like stomata and lenticels and openings that occur during lateral root formation. They are often introduced via infected seed potatoes and are spread easily within a seed lot via the soil, water or/and contaminated machines, in particular during harvesting. If the environmental conditions favor their multiplication, bacteria will easily establish infections in potato tissues and spread through the entire plant. The most serious aspect of bacterial pathogenesis is that there are hardly any possibility to control bacterial pathogens on potato. Frequently, symptomless infections are present, not found during field inspections. Resistance in commercial cultivars is largely absent and chemicals to cure tubers and plants during cultivation are not available, moreover hygienic measures are insufficient to prevent seed infections (Van der Wolf & De Boer, 2007).

BLACKLEG AND SOFT ROT OF POTATO

Most harmful and damaging bacterial diseases of seed potato production in Europe are blackleg and soft rot caused by *Pectobacterium* and *Dickeya* species. The economic losses in seed potato production in The Netherlands due to the blackleg and soft rot diseases are estimated between 15 and 30 million euro annually (Prins & Breukers, 2008).

Blackleg and soft rot are seed-borne diseases. Production of pathogen-free seed tubers is therefore of great economic importance and of major growers' interest (Perombelon, 2002).

Bacterial species belonging to different genera like *Pectobacterium*, *Dickeya*, *Pseudomonas*, *Bacillus*, *Clostridium*, *Aerobacter*, *Flavobacterium* and *Rhodococcus* are able to cause tuber rot. All these bacteria possess the ability to produce plant tissue macerating enzymes. Of these, soft rot and blackleg causing *Pectobacterium* and *Dickeya* spp. are regarded as the most important. *Pectobacterium* and *Dickeya* spp. are primary pathogens whereas species belonging to the other genera are in general only able to enhance decay after rot

has been initiated by *Dickeya* and/or *Pectobacterium* spp. (Perombelon & Lowe, 1975).

Blackleg and soft rot causing *Pectobacterium* and *Dickeya* species are responsible also for diseases in other than potato crops like carrots, onion, cucumber, cabbage and in ornamentals such as hyacinth and cyclamen (Kado, 2006).

Potato blackleg can be caused by *P. atrosepticum*, *P. carotovorum* subsp. carotovorum, *P. carotovorum* subsp. *brasiliensis*, *P. wasabiae* and *Dickeya* spp. wherever potato is cultivated (Pitman et al., 2010, Duarte et al., 2004, Perombelon, 2002, Samson et al., 2005). Disease symptoms caused by these different pathogens are indistinguishable.

All species easily cause soft rot of tubers during storage if environmental conditions favor disease progression, but the bacteria differ in their relative contribution to blackleg incidences (Perombelon, 2002). For a long time, *P. carotovorum* subsp. *carotovorum* was considered to play a minor role in potato blackleg, but recently it has been proven that *P. carotovorum* infections can result in typical blackleg in Europe (De Haan et al., 2008).

In 2004, a first report on a *P. carotovorum* subsp. *brasiliensis* causing severe blackleg symptoms in potato in Brazil was published (Duarte et al., 2004) This pathogen is more virulent than *P. atrosepticum* under warm climate conditions. So far *P. c.* subsp. *brasiliensis* was only isolated in South America and Africa (van der Merwe et al., 2010).

In the past, in temperate climate zones, particularly in Europe and in North America, *P. atrosepticum* was regarded as the dominant causative agent of blackleg. Molina and Harrison (1977) reported that the high blackleg incidences in potato crop in Colorado were dominantly caused by *P. atrosepticum* (Molina & Harrison, 1977). Perombelon (1972) in Scotland showed that up to 80% of progeny tubers were contaminated with *P. atrosepticum* although that plants often did not show any symptoms (Perombelon, 1972).

Dickeya spp. were recognized as pathogens of tropical and subtropical regions, being associated mainly with ornamentals (Perombelon & Salmond, 1995) Nevertheless, strains of *D. dianthicola* were frequently isolated from the blackleg-diseased plants in Northern and Western Europe. These "cold tolerant" *D. dianthicola* strains possessed a lower growth temperature optimum than other *Dickeya* species (Janse & Ruissen, 1988). In the last five years an increase in the blackleg incidences caused by *Dickeya* spp. bacteria in Europe was observed. Since 2005, *Dickeya* spp. was responsible for 50 to 100% of field infections in The Netherlands and France (Van der Wolf et al., 2008). A similar increase in *Dickeya*

spp. caused blackleg incidences were reported in other countries like Finland (Laurila et al., 2010) and in Israel (Tsror et al., 2008).

PRESENCE OF A NEW GENETIC CLADE OF BIOVAR 3 *DICKEYA* SPP. – " *D. SOLANI* "

The increase in blackleg incidences caused by *Dickeya* spp. is related to the occurrence of a new, highly virulent genetic clade of *Dickeya* spp. Since 2005, this new genetic clade, representing probably a new *Dickeya* species has spread all over Europe (Tsror et al., 2008, Slawiak et al., 2009). Strains belonging to this clade were isolated from potatoes in France, Finland, Poland, England, the Netherlands, Germany, Sweden and in Israel (van der Wolf, personal communication). In the Netherlands, in last five years this clade was almost exclusively isolated from seed potatoes.

The strains cannot be classified in any of the six, known *Dickeya* species described till now (Samson et al., 2005). Results from dnaX and 16S rDNA sequence analyses, Rep-PCR and biochemical assays showed that all new *Dickeya* isolates from Europe and Israel that belonged to this new genetic clade were clonal. This points to their common origin and possibly a single introduction event. All isolates belong to biovar 3, a group of biochemically distinct strains which were isolated from crops grown in a warm climate or cultivated in greenhouses. The new unclassified biovar 3 strain is provisionally called "*D. solani*". *D. solani* possesses a higher growth temperature optimum than the European *D. dianthicola* strains previously isolated from potato (Janse & Ruissen, 1988).

OBJECTIVES

The main objectives of this study were to gain knowledge on the ecology of the new genetic clade of biovar 3 *Dickeya* spp. "*D. solani*" currently found in seed potatoes in Europe. and to find an effective biocontrol strategy to cure infected potato tubers from blackleg caused by *D. solani.*

APPROACHES

Direct isolation of viable bacteria cells using (selective) plating techniques combined with molecular (16S rDNA and Rep-PCR), serological (DAS-ELISA) and biochemical (biovar determination) characterization of the isolates were used to evaluate the presence of *Dickeya* spp. and *Pectobacterium* spp. in different plant parts and tissues.

Bacterial population dynamics in planta was studied in greenhouse experiments. For the studies on colonization of potato tissues by biovar 3 *Dickeya* spp. ("*D. solani*"), and *Serratia plymuthica* A30, bacterial strains were transformed with plasmid-based genes coding for red or green fluorescent proteins (DsRed and GFP respectively) that were constitutively expressed in bacterial cells. Epifluorescence stereo microscopy and confocal laser scanning microscopy were used to visualize bacterial presence in different tissues of potato tubers or plants.

For isolation and characterization of bacterial isolates able to decrease and/or eradicate biovar 3 *Dickeya* sp. populations in infected plants, bacterial strains were isolated from rotten potato tissue. Selected isolates were assessed for features that are important in antagonism (i. e. antibiosis, siderophore, auxin and biosurfactant production, motility, quorum quenching) in plate assays, semi in planta potato slice assays and under greenhouse conditions favorable for blackleg disease development. Strains were characterized by partial sequencing of 16S rDNA and classified into risk categories.

Interactions of biocontrol *Serratia plymuthica* A30 and blackleg causing biovar 3 *Dickeya* sp. type strain (IPO2222) were evaluated in potato slice assay and in planta under greenhouse conditions. Population dynamics was assessed by direct bacterial isolation on selective media and by studies with epifluorescence stereomicroscopy and confocal laser scanning microscopy.

OUTLINE OF THE THESIS

Chapter 2 provides a literature review on the possible methods to control blackleg and soft rot causing bacteria. The paper summarizes major characteristics of the methods used in practice and evaluates the development and application of cultivars resistant to blackleg causing pathogens, the use of genetically modified potatoes and chemical, physical and biological treatments. This review provides

also basic information on the major (bacterial) pathogens and on the ecology of *Pectobacterium* and *Dickeya* species in relation to control strategies.

Chapter 3 presents a study on the distribution of blackleg causing bacteria and their population structure in naturally, latently infected tubers of two potato seed lots. Knowledge on the distribution in (seed) tuber tissue, which is largely missing, is required for sampling of tuber tissue in seed testing programmes and for the development of efficient tuber sanitation procedures.

Chapter 4 describes the ability of a strain from the new genetic clade of biovar 3 *Dickeya* sp. to infect roots from infested soil and to further colonize potato plants systemically after root infection. Potato tuber decay during growth results in the release of a large bacterial inoculum and infestation of soil. During wet weather conditions bacteria will migrate with free soil water and may infect neighboring plants and machines used for tubers collecting, which will increase the chances of infection or latent contamination of progeny tubers. These soil-borne bacteria may either directly infect tuber lenticels or, after root infection, may infect progeny tubers systemically.

Chapter 5 describes the ability of a new *Dickeya* sp. strain to latently infect progeny tubers after haulm infection. Pathogen-free potato plants may become contaminated during cultivation and the infection may originate from a variety of sources such as contaminated machines contaminated insects, irrigation water, rain water, aerosols, human activity during field inspections or via animals entering potato fields. For all these routes, contamination will result in infection of haulms rather than of underground plant parts. Aerial stem rot, which is frequently found in the field under wet conditions, may be the result of these introductions but knowledge on the contribution to the contamination and infection of progeny tubers is lacking.

Chapter 6 provides information on the possibilities for the (bio)control of a new clade of biovar 3 *Dickeya* sp. in potato by using antagonistic bacteria isolated from rotten potato tissue. Selection procedures are described to obtain bacterial antagonists able to survive and multiply in (rotting) potato tubers. The chapter also provides information on the characterization of the obtained antagonists for features potentially important in antagonism and for their ability to survive in different environments and different growth conditions. Finally, an antagonist is described that showed a high level of protection against infections caused by biovar 3 *Dickeya* sp. under greenhouse conditions.

Chapter 7 describes the study on the control of the biovar 3 *Dickeya* sp. ("*D. solani*") by *Serratia plymuthica* A30 in planta under greenhouse conditions. The *S. plymuthica* A30 was isolated and characterized in the study presented in

Chapter 6. The strain showed good potential in control of *Dickeya* sp. in in vitro and in planta tests. This chapter also provides information of *Dickeya* sp. and S. plymuthica A30 interactions inside roots and shoots of potato plants and survival of biocontrol agent in this environment. Information is also provided on the ability of *S. plymuthica* A30 to colonize potato roots and stems from soil-borne inoculum and how this will contribute to the control of *Dickeya* sp. in potato.

Chapter 8, gives a summarizing discussion on the implications of the results for the control of biovar 3 *Dickeya* sp. ("*D. solani*")

Chapter 2

Control of blackleg and tuber soft rot of potato caused by *Pectobacterium* and *Dickeya* species: a review

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Plant Pathology (2011) (DOI: 10.1111/j.1365-3059.2011.02470.x)

ABSTRACT

Potato blackleg and soft rot caused by pectinolytic *Pectobacterium* and *Dickeya* species are among the most severe bacterial diseases of potato which affect seed and ware potato production globally. The pathogens are mainly seed-borne and are readily spread via latently infected tubers. Despite the introduction of pathogen-free minitubers, the problems with blackleg and soft rot pathogens remain unresolved and even appear to be increasing. This paper briefly summarizes the causative agents of blackleg and soft rot diseases and the disease syndrome including epidemiological and etiological aspects. It critically evaluates control methods used in practice based on the avoidance of the contamination of plants, in particular the use of seed testing programs and the application of hygienic procedures during crop production. It describes the perspective for resistance breeding and genetic modification to introduce resistance. It also evaluates the application of physical and chemical tuber treatments to reduce inoculum load and examines the possibility for biocontrol using antagonistic bacteria and bacteriophages.

INTRODUCTION

Potato (*Solanum tuberosum*, L.) is a worldwide cultivated tuber bearing plant which is the fourth main food crop in the world after rice (*Oryza sativa*, L.), maize (*Zea mays*, L.) and wheat (*Triticum aestivum*, L.) both in area cultivated and total production (Douches et al., 1996). Potato does not require special growth conditions; it has been for a long time a major field crop in temperate and increasingly in warmer regions (Haverkort, 1990).

Commercial cultivars are derived from a restricted number of potato clones introduced in Europe in the 16th century following the exploration of South America. It consequently has resulted in a narrow genetic base hence with a limited range of resistance to many diseases that lower yields and tuber quality (Hooker, 1981). An estimated 22% of potato crops is lost per year due to viral, bacterial, fungal diseases and pests which is equivalent to an annual loss of over 65 million tonnes (Ross, 1986, International Potato Center, Lima, Peru http://www.cipotato.org/; Food and Agriculture Organization, United Nations http://www.fao.org/).

Bacteria belonging to the *Pectobacterium* and *Dickeya* genera are causal agents of blackleg and tuber soft of potato (Perombelon & Kelman, 1980, Perombelon, 2002). In seed potato production these diseases are next in economic importance to bacterial wilt caused by *Ralstonia solanacearum* and before ring rot and common scab caused by *Clavibacter michiganensis* subsp. *sepedonicus* or *Streptomyces scabies* respectively (Van der Wolf & De Boer, 2007). During the last forty years different aspects of blackleg and tuber soft rot and their pathogens have been reviewed. Attention has focused on taxonomy (Dye, 1969, Graham, 1964, Hauben et al., 1998, Samson et al., 2005), ecological and epidemiological characteristics (Charkowsky, 2006, Perombelon, 2002, Starr & Chatterjee, 1972, Perombelon & Kelman, 1980, Perombelon & Salmond, 1995, Toth et al., accepted to Plant Pathology), pathogenesis and regulation of extracellular enzyme synthesis (Barras et al., 1994, Hugouviex-Cotte-Pattat et al., 1996, Perombelon, 2002, De Boer, 2003, Stanghellini, 1982, Perombelon, 1982), genetics and molecular biology (Chatterjee, 1980, Robert-Baudouy, 1991), comparative genomics of pectinolytic bacteria (Toth et al., 2003, Toth et al., 2006) and biochemical basis of resistance of potato to blackleg and soft rot (Lyon, 1989). In contrast, there is no comprehensive review of disease control to date.

This publication is a critical evaluation of past and current attempts to control blackleg and tuber soft rot mainly from a European perspective. Disease control measures examined are: avoidance of contamination by the blackleg pathogen, role of fertilization, application of classical breeding and genetic modification to introduce resistance, the use of physical and chemical tuber treatments and research on biocontrol of blackleg and soft rot pathogens. The measures refer to both seed and ware crops to different extent; in the case of the former the objectives are primarily to produce healthy crops by avoiding/reducing tuber contamination, whereas with the latter, the aim is to minimize yield loses by avoiding/preventing disease in the field and in storage.

DISEASE SYNDROME

Bacteria

The main bacteria causing blackleg, which affects the growing plant, and tuber soft rot of potato are the soft rot bacteria *Pectobacterium atrosepticum* (Pa), *P. carotovorum* subsp. *carotovorum* (Pcc) and *Dickeya* species (Van der Wolf &

De Boer, 2007), formerly belonging to the genus *Erwinia* (*E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi*) (Dye, 1969). They are pectinolytic Gram-negative, facultative anaerobic, non-sporing, motile, straight rods with peritrichous flagellae (Charkowsky, 2006). They belong to the γ-*Proteobacteria* subdivision and are clustered in the *Enterobacteriaceae* family (Charkowsky, 2006). They characteristically produce a variety of cell wall degrading enzymes that allow infiltration and maceration of plant tissues on which they feed (Barras et al., 1994).

Whereas Pcc has a broad host range worldwide, Pa is restricted only to potato principally in temperate regions. In contrast, *Dickeya* spp. affect a restricted number of host species in both temperate, sub-tropical and tropical regions (Ma et al., 2007, Toth et al., accepted to Plant Pathology).

The three species of bacteria can cause tuber soft rot but for a time only Pa was believed to cause blackleg in temperate and *Dickeya* spp. in warmer regions (Perombelon & Salmond, 1995). However, recently Pcc has been shown to infect potato plants causing typical blackleg symptoms (De Haan et al., 2008). This may reflect the warmer summers prevailing lately or that new adapted forms might have arisen. It is notable that in Colorado and Arizona in the USA with hot summers, Pcc was regarded for a long time as a true blackleg pathogen together with Pa (Molina & Harrison, 1977).

Although *Dickeya* spp. have long been associated with blackleg in tropical and subtropical regions, only strains of *Dickeya dianthicola* were isolated from blackleg-diseased plants in Western Europe in the past two/three decades. These, so-called "cold tolerant" strains showed a lower growth temperature optimum (Janse & Ruissen, 1988). However, since 2005, a new genetic clade, representing probably a highly virulent *Dickeya* species belonging to biovar 3 has been detected in Europe (Tsror et al., 2008, Slawiak et al., 2009) . Strains belonging to this clade were isolated from seed potatoes in France, Finland, Poland, The Netherlands and Israel. In many of these countries the pathogen was introduced via the international trade of seed potatoes. All isolates were clonal, which suggests a common origin and possibly a single introduction event. The same genetic clade has been found in hyacinth . One might speculate that in the recent past this genetic clade was introduced from hyacinth into potato, possibly via the use of contaminated irrigation water (Slawiak et al., 2009), however the role of flower bulbs in the dissemination of the pathogen is still unknown.

Recently, two new subspecies of Pcc were described as potato blackleg causing organisms. *P. c.* subsp. *brasiliensis*, a highly aggressive bacterium, is

responsible for the majority of blackleg incidences in Brazil (Duarte et al., 2004) and South Africa (van der Merwe et al., 2010). *P.c*. subsp. *wasabiae* has been described as a new potato pathogen in New Zealand responsible for high blackleg levels (Pitman et al., 2010). Until then, *P.c. wasabiae* was found only in association with soft rot on Japanese horseradish (Gardan et al., 2003, Pitman et al., 2010).

Symptoms

The most characteristic symptom of potato blackleg caused by both *Pectobacterium* and *Dickeya* species is a slimy, wet, black rot lesion spreading from the rotting mother tuber up the stems especially under wet conditions. However, when conditions are dry, symptoms tend to be stunting, yellowing, wilting and desiccation of stems and leaves (Perombelon & Kelman, 1980). Late in summer, under persistent rainy conditions, extensive stem rot, starting from the top and progressing downwards to the base, can develop and can be confused with those of blackleg. They are usually caused only by Pcc (Perombelon & Kelman, 1980).

Tuber soft rot is initiated at lenticels, stolon end and/or in wounds under wet conditions. The lesion can spread to the whole tuber and thence to neighbouring tubers in storage. Tuber tissue is macerated to a creamy consistence which turns black in the presence of air, developing an evil smell when invaded by secondary organisms. When seed tubers start rotting in the field before emergence, blanking occurs. In inadequately ventilated cool stores, rotting can spread to adjoining tubers as liquid from the rotting tubers percolate onto others, leading sometimes to massive rotting pockets in the stored tuber lot.

Epidemiology

Our knowledge on this topic is derived from studies done usually in temperate countries involving mainly Pcc and Pa and rarely *Dickeya* spp. which only recently have become more economically important. One has to be careful when extrapolating past results to *Dickeya* spp.

The soft rot bacteria do not overwinter in soil. Survival in soil is restricted to one week to six months, depending on environmental conditions, such as soil temperature, moisture and pH. Survival can be longer in association with plant material, including volunteers. In any event, the bacteria cannot survive in the soil

in a crop rotation system of 3 to 8 years (Anilkumar & Chakravarti, 1970, Lim, 1975, Rangarajan & Chakravarti, 1970, Perombelon & Hyman, 1988).
It is now generally accepted that the major source for blackleg infection is the latently infected seed (mother) tubers (Perombelon, 1974). When the mother tuber rots the bacteria are liberated in the soil and are transmitted by soil water to contaminate neighboring progeny tubers. Czajkowski and co-workers (2010) showed that the bacteria in soil can colonize also potato roots and subsequently move via the vascular system into progeny tubers. Once in the stems, the bacteria do not necessarily cause stem rot (blackleg) and can survive in latent form.

Crop contamination can also occur from air borne sources (Perombelon, 1992, Harrison et al., 1987, Perombelon et al., 1979, Graham, 1976). Soft rot bacteria can be carried from diseased plants by air-borne insects over long distances to contaminate other potato crops. Also, they can be present in aerosols produced by rain impaction on blackleg plants and by haulm pulverization prior to harvest. Air sampled in Scotland, even away from potato crops, contained both Pcc and Pa, more on rainy than dry days. Although they remain viable for 5-10 min only, they can be blown away for several hundred meters before deposition mainly by the scrubbing action of rain.

Surface water in the USA and Scotland was found to be contaminated with Pcc and to a lesser extent with Pa (McCarter-Zorner et al., 1984, Harrison et al., 1987). Recently, in Europe, a biovar 3 *Dickeya* spp. genetic clade, not previously described, has been found in river water and at the same time in seed potatoes in Finland (Laurila et al., 2008, Laurila et al., 2010). Hence, surface water when used for irrigation purposes is likely to be a source for the pathogen, and may also be a source of new variants of the pathogen.

Contamination of crops can also occur during mechanical flailing (Perombelon et al., 1979). Most importantly, contamination of tubers can occur during harvesting and handling (grading) in store from the ubiquitous rotting tubers which disintegrate and spread the rotting tissue on the machinery, thence in wounds inflicted during handling (Elphinstone & Perombelon, 1986, Perombelon & Van der Wolf, 2002). Therefore, there is a high risk that one or several soft rot bacteria contaminate the commercially produced tubers, on which bacteria can survive from one generation to the next (Perombelon, 1974, Perombelon & Kelman, 1980, Perombelon, 1992, van Vuurde & de Vries, 1994).

Aetiology

Blackleg develops after rotting of seed (mother) tubers, however, it is not a necessary sequel to mother tuber rotting. Conditions which favor decay also favor blackleg development (Perombelon, 1992). An important environmental factor for blackleg development is soil water level. Presence of a water film on the tuber surface induces development of anaerobic conditions in the mother tubers, thereby favoring bacterial multiplication and initiation of rotting (Perombelon et al., 1989b). Anaerobiosis affects oxygen-dependent host resistance, allowing unhindered bacterial multiplication and cell wall degrading enzyme production resulting into a rotting lesion (Fuqua et al., 2001).

Another critical condition in disease development is the level of seed contamination as shown in the case of Pa. The higher the bacterial density is, the more likely the pathogen will predominate in the incipient lesion and the sooner rotting is initiated (Bain et al., 1990). Progeny tuber contamination is related to seed tuber contamination as well as blackleg disease (Toth et al., 2003). Although only limited data concerning *Dickeya* spp. are available, it seems that level of seed contamination is less important for blackleg development possibly because of their higher virulence (Velvis & van der Wolf, 2009).

Competition within rotting mother tubers, modulated by environmental conditions, temperature especially, determines which pathogen will predominate if more than one is present (Perombelon, 2002). The soft rot bacteria can also interact with other pathogens, especially vascular ones, such as *Ralstonia solanaecearum*, *Fusarium* spp., *Verticillium* spp. and *Rhizoctonia solani* (Tsror et al., 1990, Perombelon, 2002) Weakening of the host resistance by one pathogen may favour the development of another.

CONTROL STRATEGIES

Several approaches have been studied to control blackleg and tuber soft rot, but the degree of success has been variable. Methods based on avoiding contamination and reliance on seed certification schemes are widely used and have been partially successful. Improved store management can reduce bacterial load on tubers and tuber rotting . Both physical, especially hot water treatment, and chemical methods have been explored but with limited success. The use of

biological control has been and is still being attempted, but it is too soon to say how successful it will be. Finally, breeding for resistance has so far failed but the use of GM techniques is promising if politically feasible.

Avoiding contamination

When it was realized that blackleg is not a soil-borne disease, the blackleg plant was thought to be the main source of the pathogen. Therefore, disease control would be achieved by an indexing (certification) system of seed obtained from disease-free crops. In Europe, plant inspection services under national jurisdiction are responsible for certification of seed potatoes. The European Plant Protection Organization (www.eppo.org) provides standardized protocols and guidance for certification of plant material. Inside the European Union, the Phytosanitary Directives (2000/29/EG) describes general regulation on the crop production requirements and guidance for the member states in respect of good cultivation practices (for example in The Netherlands, NAK provides guidance for certification).

For more than half a century seed-potato certification has been the traditional approach to ensure that this objective is achieved. However, the degree of control achieved is erratic and heavily dependent on the weather prevailing during growth of the seed crop. The greatly improved understanding of blackleg epidemiology and aetiology can now explain why this approach has met with little consistent success. These measures cannot detect widespread latent infection of progeny tubers (the next generation seed) from symptomless plants as discussed above. Moreover, depending on weather conditions, heavily contaminated seed can give rise to little or no disease and the converse is also true. Despite this, the measures can do some good, for example, roguing at an early stage of crop growth which entails the removal of diseased plants including daughter tubers, no doubt contribute in reducing an important source of the pathogen. Rotting progeny tubers are common on symptomatic plants, from which bacteria can spread during mechanical handling at harvest and post-harvest.

Seed potato crops are classified into different seed grades according to several criteria including the level of roguing and blackleg, from nil to a given percentage depending on national certification scheme criteria. Seed crops are subjected to field inspections usually twice during the growing season in most seed producing countries in Europe. Infected crops can be downgraded to a lower seed category or rejected from the market. Harvested progeny tubers (future generation

seed) can be tested also with molecular and serological methods to detect latent infections. This, however, is not an obligatory part of testing programs yet. As tubers from disease-free crops are frequently contaminated, laboratory testing can help to detect latently infected tubers. In contrast to other bacterial diseases of potato where there is often zero tolerance, some contamination of seed tubers can be allowed, in particular in low grade seed. Therefore the use of a detection procedure is desirable which allows estimation of both the density of bacteria and the incidence of contaminated tubers.

The relationship between seed health status and its contamination level has not been fully evaluated. It involves several steps, namely collection of representative tuber samples from large quantities of seed lots, preparation of tuber tissue for testing, quantification method to be used and, last but not least, interpretation of the results in terms of blackleg risk assessment (Perombelon, 2000). An additional compounding factor is the high cost involved in testing for contamination level. One possibility is to restrict testing to the highest seed grades where tolerance level could be zero. Testing of tubers should include the peel to detect lenticel and wound infections and the stolon end including the vascular tissue.

When it was demonstrated that the seed (mother) tuber is an important source of the soft rot bacteria, attempts were made to produce pathogen free progeny (next generation) tubers. Initially, potato stem cuttings were used (Graham & Harper, 1967) , later by axenically produced micro plants and currently in vitro produced minitubers which should produce bacteria-free progeny tubers (Stead, 1999). Minitubers are grown in a controlled pathogen-free environment, using aeroponic and hydroponic cultures or in artificial soil systems in order to prevent contamination with soft rot bacteria (Ranalli et al., 1994, Rolot & Seutin, 1999, Farran & Mingo-Castel, 2006, Ali et al., 1995). Testing of approximately 100 seed lots of minitubers per year during four consecutive years showed that minitubers were consistently free of blackleg causing bacteria (Velvis & van der Wolf, 2009).

In large scale seed potato production, multiplication of the initial minitubers in the field is necessary for economic reasons. However, this has led, even after only two or three field generations, to ca. 30%, contaminated with *Dickeya* spp. and 10% with Pa (Velvis & van der Wolf, 2009). Similar results were found in previous studies in Scotland, which showed that contamination of an initially bacteria-free potato stock became progressively more contaminated after the third year in the field. Interestingly contamination occurred at the time mechanical handling at harvest and grading in store became necessary (Perombelon et al., 1980). Therefore it is likely, that initial contamination came from machines

already contaminated although contamination by air-borne bacteria cannot be ignored. In an attempt to overcome this problem, the number of generations from bacteria-free initial propagative material is restricted to a set number before loss of seed status in order to reduce buildup of contamination during seed stock multiplication.

Studies carried out in the eighties on the ecology of the bacteria have identified several sources of the bacteria to contaminate seed crops before and after harvest (Perombelon, 1992). This knowledge has allowed a more focused approach to reduce risks of introducing the bacteria at different stages of seed production. For example, it is desirable that crops should be dedicated as seed or ware since the tolerance levels for blackleg are different as well as harvesting time. However, for economic reasons, dual purpose crops are sometimes grown, harvested late to maximize yield and the seed size fraction separated later from the ware. It is unavoidable that quality would suffer (van der Zaag & Horton, 1983). Use of well drained fields reduces the risk of tubers being surrounded by a water film that can result in anaerobiosis and consequently tuber decay in the field (Perombelon, 1992). Late harvesting allows bacterial multiplication on leaves and in debris left on the ground following haulm flailing. This may result in contamination of progeny tubers underground during wet weather conditions (Burgess et al., 1994).

Monitoring tuber contamination during bulking in crops derived from stem cuttings over five years on five seed producing farms in Scotland showed that farms which regularly applied hygienic measures consistently produced cleaner seed than the others (Perombelon et al., 1980). Washing and disinfection of machines used when planting, spraying, haulm flailing, harvesting and grading in store no doubt help in reducing risks of introducing soft rot bacteria in a pathogen-free crop (Perombelon & Kelman, 1980, Perombelon, 2002). Spreading and smearing of the bacteria in a seed lot can be reduced by removal of rotten tubers during harvesting and grading. Avoidance of wounding by correct machinery adjustment during harvesting and grading is important to reduce the risks of wounding in which bacteria can survive after wound healing (Perombelon, 1992, Van Vuurde & De Vries, 1994). Use of mature tubers with a well-developed periderm will also reduce risks on wounding.

Storage in bulk or preferably in one tonne boxes in the case of seed in well-ventilated stores at low temperatures will avoid condensation on tuber surface which in turn will prevent multiplication of the blackleg pathogen. If the tubers remain wet long enough tuber decay can ensure that result in further spread of the bacteria when tubers are graded and sometimes massive tuber decay (Perombelon, 2000). It is critical to dry rapidly the tubers by forced ventilation with warm air to

favour wound healing followed by cooler air for controlling sprouting and long term storage (Wale et al., 1986, Wale & Robinson, 1986). Good storage management is of importance not only to prevent tuber decay but also avoid increasing the tuber inoculum load which would result subsequently in greater disease risks.

Reduction in the incidence of tuber infections with blackleg and soft rot bacteria can be achieved by using true botanical seed instead of seed tubers. Such seed derived from sexual crosses are believed to be free from blackleg and soft rot bacteria (Perombelon & Kelman, 1980). Although soft rot bacteria are not easily transferred to botanical seed via vascular tissue, some reports suggest that true seed may also become externally contaminated with low populations of the bacteria (Colyer & Mount, 1983). However, this externally sited inoculum can be removed by hot water or chlorine treatments (Colyer & Mount, 1983).

For the majority of developing countries, seed potato classification schemes have failed or are not available at the moment whereas imported seed potatoes are often too expensive (van der Zaag & Horton, 1983, Chujoy & Cabello, 2007). In particular in these countries, the use of true botanical seeds may be an attractive and a low cost alternative for the use of seed tubers. The main advantage of using true seed is that they can be easily produced in large numbers (Chujoy & Cabello, 2007). Above all, botanical seed do not require cold storage facilities. They can be kept in simple stores for a long time, which is of considerable importance in hot regions in developing countries (Wiersema, 1986). However, its major drawback is the genetic diversity of the produce which requires that every generation has to be carefully selected for desirable traits to ensure a stable and as uniform as possible crop (Chujoy & Cabello, 2007).

Effect of nutrition on plant resistance to blackleg and soft rot

Plant nutrition is believed to be an important component of natural disease resistance. Nutrition affects growth of plants, the interaction with pathogens as well as other plant associated microorganisms and in general is important for plant fitness status (McGovern et al., 1985). Deficiency of essential elements will often result in an increased susceptibility to diseases.

Calcium plays an important role in the resistance of plants against bacterial pathogens (Bateman & Miller, 1966, Berry et al., 1988). A high calcium content in crops is often positively related with an increased resistance against bacterial diseases including potato blackleg (Berry et al., 1988, Platero & Tejerina, 1976, McGuire & Kelman, 1984). Calcium ions improve the structure and integrity of

plant cell wall components, resulting in higher resistance to diseases involving tissue maceration.

Calcium fertilization is known to reduce soft rot caused by *Pectobacterium* spp. in Chinese cabbage (Park, 1969) and in bean (Platero & Tejerina, 1976). McGuire and Kelman (1984) showed, both in in vitro and field experiments, that bacterial soft rot caused by Pa was negatively correlated with calcium concentration in tubers. Resistance to blackleg and tuber soft rot appeared to be related to calcium concentration in seed tubers, but the results varied between the cultivars tested and over the three years of field experimentation (Pagel & Heitefuss, 1989).

Calcium is not equally distributed inside plant parts and in particular, potato tubers often have a low calcium level (Collier et al., 1980, Dunn & Rost, 1945). Soils naturally low in Ca can be amended by adding $CaSO_4$ (gypsum) to increase resistance not only to blackleg but also to soft rot of progeny tubers (Bain et al., 1996).

The level of nitrogen seems to be another factor that can affect susceptibility to soft rot pathogens. High levels of nitrogen, between 1050 and 1700 ppm (parts per million), significantly reduced bacterial leaf blight of *Philodendron selloum* caused by *Dickeya* spp., but resulted also in a significant deterioration of plant growth (Haygood et al., 1982). The effect of nitrogen levels on blackleg and soft rot in potato has not been explored apart from a study by Graham and Harper (1966) who showed that blackleg incidence caused by Pa was lower in field plots treated with high than with low level of N fertilizers (Graham & Harper, 1966).

A balanced fertilization of potato plants and an increase of the calcium content in soils alone will not provide sufficient control of blackleg and soft rot pathogens. It may however, be a part of an integrated control strategy.

Breeding for resistance

Traditional breeding

Commercial potato cultivars which are naturally immune to blackleg and soft rot caused by *Dickeya* and *Pectobacterium* species do not exist, but some cultivars show a partial resistance (Lyon, 1989). Attempts to breed potato cultivars with increased levels of resistance were only partially successful and never resulted in immune cultivars probably because of the narrow range of genetic diversity in parental breeding material used (Tzeng et al., 1990). In any event, breeding for

soft rot resistance has not been given a high priority in most breeding programmes relative to other diseases and desirable agronomic traits. At best, cross progeny was sometimes screened only at advanced selection stages for their resistance and the results then might be taken into consideration in the final selection.
Furthermore, screening for resistance is not straight forward. Cultivar resistance/susceptibility to blackleg and tuber soft rot are not always correlated, rather, different combination patterns are common (Perombelon & Salmond, 1995).

Several methods to screen for blackleg and soft rot resistance in breeding lines have been described. Ranking of cultivars for resistance to tuber soft rot caused by Pa varied according to inoculation method, presence or absence of oxygen and laboratory and field assessments (Łojkowska & Kelman, 1994, Bain & Perombelon, 1988). Moreover, resistance/susceptibility ranking of cultivars varied from one season to another (Perombelon & Salmond, 1995). Ultimately, field experimentations is the only reliable way to evaluate resistance of potato lines against blackleg and soft rot diseases (Allefs et al., 1995).

The relatively low resistance to blackleg and soft rot in cultivars can be strengthened by utilizing the high levels observed in wild potato species (Dobias, 1977, van Soest, 1983, French & de Lindo, 1985, Corsini & Pavek, 1986). More than 200 wild *Solanum* species present in North and South American and European potato collections contain a large reservoir of useful genetic material, including resistance to soft rot bacteria (Hijmans & Spooner, 2001). The majority of these species are diploids which facilitate hybridization with *S. tuberosum* compared to *Solanum* spp. tetraploids (Watanabe et al., 1994). These include *S. canasense* and *S. tarijense* (Carputo et al., 1997) and *S. tuberosum* subsp. *andigena*, believed to be the direct ancestor of the common potato, which showed relatively high levels of tuber and stem resistance to both *Pectobacterium* and *Dickeya* species (Hidalgo & Echandi, 1982). Sexual hybrids between *S. tuberosum* and *S. phureja* commonly used in breeding programs also displayed relatively high resistance but tuber yields were reduced (Rousselle-Bourgeois & Priou, 1995). When the wild *Solanum* species, *S. commersonnii*, known for its resistance to frost, nematodes and fungi, was crossed with *S. tuberosum*, the hybrids showed high resistance to both *Pectobacterium* and *Dickeya* species when screened on potato slices and in greenhouse experiments and field trials (Laferriere et al., 1999). Similarly, hybrids of commercial potato and *S. stenotomum* resulted in lines with a higher resistance to blackleg and soft rot bacteria (Fock et al., 2001). Sexual hybrids of *S. tuberosum* and *S. chacoense*, *S. sparsipillum* and *S. multidissectum* also showed higher resistance to blackleg than commercial S. tuberosum cultivars but at the same time

had a higher glycoalcaloid content, hence a higher toxicity to man and animals (Carputo et al., 1997).

S. brevidans is a diploid wild *Solanum* species that naturally does not produce tubers but bears resistance to several potato viruses and frost. Somatic hybrids obtained by fusion of protoplasts of *S. tuberosum* and *S. brevidans* showed a high level of resistance to blackleg and soft rot. Their resistance was attributed to the higher degree of esterification of cell wall binding pectin (McMillan et al., 1994). Resistance to *Dickeya* and *Pectobacterium* bacteria was stable and could be sexually transferred to the progeny in the F1 and F2 generations as well as in backcrosses with *S. tuberosum* cultivars (Zimnoch-Guzowska et al., 1999, Zimnoch-Guzowska & Łojkowska, 1993). This indicates that even relatively far-related Solanum species can be used to create hybrids with resistance to blackleg and soft rot (Austin et al., 1988).

So far traditional breeding has not succeeded in producing potato cultivars immune to *Pectobacterium* and *Dickeya* spp. Although none of the above results have been pursued further in breeding programmes, they indicate that classical (traditional) breeding for resistance against blackleg and soft rot can result in potato lines more resistant to *Pectobacterium* and *Dickeya* (Glendinning, 1983). However, breeding usually is a long process taking more than 10 years of screening and trialling to ensure that the selected material do not carry over undesirable traits. None of the hybrid lines have been commercialized at present.

Genetically modified potato plants (GMO-potato plants)

Genetic engineering is a promising alternative to traditional plant breeding, which is limited to closely related species and is time consuming. An already established cultivar could be modified to increase resistance without the need to undergo time consuming field trials for other traits. In Europe, however, introduction of genes into crops from non-crossable species to improve their quality is not readily accepted by society. For a long time, there was a moratorium on the cultivation of genetically modified (transgenic) crops and only recently, in March 2010, the first genetically modified potato cultivar with increased starch content for industrial use was allowed in Europe. (http://www.nytimes.com /2010/06/11/ world/europe/11sweden.html).

Only limited amount of work has been done so far using genetic modifications to increase resistance against bacterial pathogens. In the case of blackleg and soft rot bacteria, work has involved only in vitro plants and none of the transgenic potato lines have been exploited commercially. At present, single

genes used to improve resistance are those coding for proteins that are bactericidal, impede pathogen multiplication or prevent pathogen-host or pathogen-pathogen interactions. Few if any of the transgenic resistance in tubers was verified under anaerobic conditions essential for rotting initiation.

Lysozymes are enzymes that lyse bacteria cells by degradation of their cell wall. T4 and chicken lysozymes are well described proteins showing broad bactericidal activity. Potato plants modified to produce T4 or chicken lysozyme, showed an increased resistance to Pa in greenhouse assays (Düring, 1996, Serrano et al., 2000). However, introduction of these genes in potato plants may be harmful to naturally present beneficial bacterial populations of the potato rhizoplane and rhizosphere. It was observed that in transgenic potato plants, T4 lysozyme is released from roots into the soil and is able to kill *Bacillus subtilis* (Ahrenholtz et al., 2000) and probably also other bacteria present on the root surface.

Peptides and proteins, such as attacin and cecropin, that were found in the humoral immune response in insects, also showed antibacterial activity (Arce et al., 1999). Cecropins and its synthetic analogues showed antibacterial activity in in vitro tests, and potato plants transformed with genes coding for attracin and cecropin analog SB-37 were generally more resistant to blackleg than the untransformed control strains, but the results were variable.

When invading plant tissue, blackleg and soft rot pathogens produce large quantities of pectolytic enzymes that degrade plant cell wall components (Hugouvieux-Cotte-Pattat et al., 1996). Degradation of plant tissue cell wall generates polygalacturonate products that are used by the bacteria as a substrate for energy and biosynthesis but may also act as positive signals for the further production of pectolytic enzymes depending on the length of the galacturonate chain (Lyon, 1989). Production of pectolytic enzymes induced mainly by the presence of unsaturated digalacturonates released from the polygalacturonic polymers. Weber et al. (1996) proposed that expression of enzymes such as pectate lyases that can degrade these signals in transgenic potato plant tissue should inhibit the synthesis of large quantities of enzymes responsible for tissue maceration (Weber et al., 1996). Transgenic potato plants modified to produce pectate lyase were found to be resistant to infection caused by Pc in in vitro experiments and in planta tests (Wegener et al., 1996, Wegener, 2001).

Many secondary plant products including flavonoids (e. g. anthocyanin), steroidal alkaloids, and saponins show antibacterial activity against plant pathogenic bacteria (Hirotani et al., 2000). Their synthesis and modifications in plant tissues are controlled by different enzymes of which glucosyltransferases seem to play an important role. Lorenc-Kukula and co-workers (2005) produced transgenic potato

plants with increased 5-O-glucosyltransferase level. The ectopic over-expression of 5-O-glucosyltransferase improved resistance of potato tubers against P. carotovorum in in vitro experiments. The resistance to soft rot was at least 2-fold higher in transgenic lines than in non-transformed control tubers (Lorenc-Kukula et al., 2005).

Bacteria sense their population density by a cell to cell communication mechanism in which particular genes are expressed only when the threshold bacterial density (quorum) is reached (von Bodman et al., 2003). This mechanism, known as quorum sensing, controls diverse biological processes in human, animal and plant pathogenic bacteria including virulence. Communication between cells occurs via small, diffusible signal molecules and in Gram negative bacteria it is mediated mainly by acyl homoserine lactones (AHLs) (Fuqua et al., 2001). AHLs regulate virulence gene expression in *Pectobacterium* and *Dickeya* species possibly to ensure that infection will start only if the bacterial density is large enough to overwhelm plant response (Andersson et al., 2000, Reverchon et al., 1998). Bacteria of the genus *Bacillus* possess an AHL lactonase gene which blocks the quorum sensing mechanism by enzymatic degradation of signal molecules. When that gene was cloned and is expressed in commercial potato cultivars, the transgenic plants showed a high level of resistance against P. carotovorum. Either symptom expression was entirely blocked or symptoms development was significantly reduced (Dong et al., 2001).

Objection to GM plants could be overturned by producing cisgenic rather than transgenic potato plants. In a cisgenic approach, recipient plants are modified with resistance genes from cultivars or lines of the same or sexually compatible (crossable) species. The advantage of cisgenic over transgenic plants is that use of a gene of interest already present in the species for centuries does not alter the gene pool and/or provides no additional traits (Schouten et al., 2006). Current research is looking at the potato-soft rot bacteria interaction at a molecular level. Bacterial proteins active during infection provide clues on resistance responses in potato clones with known different resistance levels and markers suitable for marker-assisted breeding could be developed. It is hoped that this would allow rapid introgression of resistance into cultivars (König et al., 2004, Phipps & Park, 2002). To date, no resistance genes have yet been identified to control blackleg and soft rot of potato for use in a cisgenic approach. It is likely that more than one gene would be involved which would complicate the task.

Although promising results have been obtained, it is a long way before genetically modified potato plants resistant to soft rot bacteria become commercially available. Research has been restricted to laboratory or greenhouse

studies and none were conducted under field conditions to test the performance of resistant lines in the field or to assess potential environmental risks of release of the genetically modified plants. At present it is likely that future research will focus on identification of resistance genes which could be used in a cisgenic approach.

Physical seed tuber treatment

The traditional method to conserve seed tuber in good health has been storage in ventilated stores at low temperatures (ca. 5 °C). These conditions are easily met in temperate countries when temperatures in stores can be regulated using cold outside air over the winter until planting time in the spring. However, in tropical and subtropical regions, unless expensive refrigerated stores are available, storage is a real problem. At high ambient temperatures, not only dormancy is restricted but also infection by pests, fungi and particularly soft rot bacteria can cause serious losses. This is an important factor limiting potato production in these regions.

Disease can develop before cold storage or in transit to the market and specific treatments become useful. Physical control mainly by heat is recognized as competitive to biological and chemical methods as it does not require registration, and it may be effective against a broad range of pathogens. However, physical procedures affect not only superficially located pathogens but also beneficial micro-organisms and may negatively influence tuber emergence and health. Most of the information that is available on physical control of plant pathogens in potato comes from the control of *Pectobacterium* spp. under postharvest storage conditions. There is only limited information concerning *Dickeya* spp. The physical factors applied in controlling tuber soft rot infection are those involving hot water, steam (Robinson & Foster, 1987, Shirsat et al., 1991), dry hot air (Bartz & Kelman, 1985) and UV and solar radiation (Ranganna et al., 1997, Bdliya & Haruna, 2007).

Hot water treatment of potato tubers to control soft rot bacteria contamination was first applied in 1983 (Mackay & Shipton, 1983). Pcc and Pa could not be detected in tuber peel after dipping naturally infected potato tubers for 10 min in water at 55 °C. In field experiments, no blackleg was observed in plants grown from treated tubers. Similar results were obtained by Wale and Robinson (1986) and Shirsat et al. (1991), who showed that incubation in water at 44.5 °C for 30 min or at 56 °C for 5 min significantly reduced the periderm and lenticel contamination of seed potatoes and consequently blackleg incidence in the field (Shirsat et al., 1991, Wale & Robinson, 1986). However, failure to dry large

quantities of the tubers rapidly could result in multiplication of any surviving bacteria and even rotting. This difficulty was overcome by a continuous hot water treatment in which 50 kg batches were continuously treated for 5 min at 55 °C followed by drying under forced ventilation with air knives. Cooling of the water when large number of tubers are immersed is avoided and any residual moisture evaporated by the latent heat still in the tubers (Perombelon et al., 1989a). Effective blackleg control was obtained in field experiments with both vacuum infiltrated and naturally contaminated tubers. Moreover, the treatment led to the control of several fungal pathogens causing gangrene, skin spot, silver scurf and black scurf (Dashwood et al., 1991). The temperature/time combination used is critical, more so in bulk tuber dipping than in the continuous treatment. However, several side effects can adversely affect growth and have to be taken into consideration: depending on the cultivar used, tuber physiology can be altered resulting in delayed sprouting or may even be lethal to the tubers, and as a result, yield can be affected (Robinson & Foster, 1987, Perombelon et al., 1989a).

Steam was also tried as an alternative for hot water treatment to remove fungi and bacteria, especially Pc and Pa present superficially in the tuber periderm. The use of steam treatment reduced infestation of tuber periderm from 26 - 59% to 1-3% (Afek & Orenstein, 2002).

Bartz and Kelman (1985) reported that external but not internal populations of *Pectobacterium* spp. can be eliminated from washed tubers by application of hot dry air at 50 °C . Hot dry air also dries the tubers and stimulates wound healing without interfering with tuber sprouting as much as hot water treatment. However, generally heat transmission by air is less effective than by water necessitating a longer incubation time which could adversely affect tuber physiology.

Ranganna and co-workers (1997) tested the influence of UV radiation to control Pcc in potato tubers. When tubers were inoculated by vacuum infiltration 6 h before radiation, bacteria were totally eliminated by a relatively low UV dose of 15 kJ m^{-2}. Vacuum infiltrated tubers with Pcc and exposed to direct sun light for at least 180 min did not develop soft rot symptoms, probably more because of an increase in the temperature of tuber superficial tissues than the action of UV energy, which is unable to penetrate tuber tissue to reach the pathogens. However, the practical value of the last three methods when applied on a large scale involving several tonnes of tubers is doubtful.

In conclusion, physical control methods, especially hot water treatment, are environmental friendly, allows some control of blackleg caused *Pectobacterium*

spp. as well as of several superficial fungal pathogens simultaneously. Their limitations, however, are the inability to kill plant pathogenic bacteria located deep inside the tubers (vascular level) without a negative effect on plant growth, they are not cheap to run and can be difficult to expand for large scale use.

Chemical seed treatment

Chemical control strategies used against bacterial diseases are based on the eradication of the pathogen and/or the creation of unfavorable environmental conditions (e. g. low or high pH etc.) for disease development. Once disease has been initiated, disease control is limited because of rapid bacterial multiplication, and spread and inability of the chemicals to penetrate within the inner tissues (Bartz & Kelman, 1985). Therefore, disease control has focused on latently infected tubers rather than blackleg affected plants. A wide range of chemical compounds have been tested to reduce infection on or inside tubers by *Pectobacterium* spp. and *Dickeya* spp.

Most compounds used contain antibiotics (mainly streptomycin and its derivatives), inorganic and organic salts or combinations of the compounds. For a long time, streptomycin was considered as a promising control agent against blackleg and soft rot diseases in potato. Seed tubers immersed in a mixture of streptomycin and oxytetracycline hypochloride or streptomycin and mercury compounds before planting reduced the incidence of blackleg in the field and tuber decay in storage (Bonde & de Souza, 1954). Similar results were obtained when kasugamycin or virginiamycin was substituted for streptomycin (Bartz, 1999, Wyatt & Lund, 1981). However, although treatments with antibiotics showed promise, larger scale field studies are no longer allowed because of the risks of introducing resistance to bacterial human or animal pathogens.

As an alternative to antibiotics, a wide range of potential bactericides have been tested, more often in small laboratory scale experiments than in the field. Thus, organic compounds like hydroxyquinoline and 5-nitro 8-hydroxyquinoline were effective to control soft rot in wounded potato tubers (Harris, 1979). Similar results were obtained with chlorine based compounds, bronopol (2-bromo-2-nitropropane-1,3-diol) and the synthetic bacteriocide, 7-chloro-1-methyl-6-fluoro-1,4-dihydro-4-oxo-3-quinolinic carboxylic (Bartz & Kelman, 1986). Immersion of potato tubers in citric, acetic, ascorbic or malonic acids also reduced rotting by Pcc in freshly vacuum-infiltrated potato tubers without affecting sprouting in in vitro conditions. (Bartz & Kelman, 1986). Mills et al. (2006) showed that certain inorganic and organic salts like aluminium acetate, sodium metabisulphate, propyl

paraben, sodium benzoate, alum (hydrated potassium aluminium sulphate), potassium sorbate, calcium propionate, sodium hypochloride, sodium bicarbonate, aluminium chloride and copper sulphate could inhibit the growth of Pcc and Pa in vitro (Mills et al., 2006).

Some of these salts have already been approved as food preservatives and consequently their use to control soft rot bacteria would need limited additional registration testing. The activity of organic and inorganic salts may be attributed to the presence of cationic ions released from the salts that inhibit bacterial cell membrane protein functions or by modulation of the environmental pH by the anion moiety (Mills et al., 2006).

Synthetic antimicrobial peptides were evaluated as a group of antibacterial agents that by interacting with the bacterial cell membrane increase its permeability (Gabay, 1994). Kamysz et al. (2005) reported that synthetic peptides CAMEL was active against Pa and *Dickeya* spp., thereby protecting potato tuber tissue from rotting. CAMEL (an antimicrobial peptide: KWKLFKKIGAVLKVL) is a hybrid peptide derived from two naturally present antibacterial peptides cecropin A and melittin (Oh et al., 2000). CAMEL showed greater protection to potato tubers against Pa and *Dickeya* spp. than streptomycin (Kamysz et al., 2005).

These chemical treatments of tubers to control blackleg and tuber soft rot are far from being straight forward as the above results would suggest. First there is the problem of reaching the bacteria usually well protected in lenticels, suberised wounds and the vascular system. Even systemic bactericides, if available, would fail if applied post-harvest because there is no vascular activity in harvested tubers.

A gaseous bactericide might be more successful but penetration in tubers is likely to be poor and can be phytotoxic, as found by Eckert and co-workers (1988) in the case of chlorine gas.

The apparent success mentioned above can be explained by the fact that freshly harvested tubers with unsuberised lenticels and wounds were used. It may be also that testing was done on cut seed tubers; the use of cut seed is common practice in some countries (Eckert & Ogawa, 1988). In addition, treating large quantities of tubers after harvest with a liquid bactericide unavoidably would require efficient drying of the tuber surface to prevent multiplication of the bacteria and rotting depending on how they are stored. For example, one treatment option would be treating freshly harvested washed new tubers at the last rinse with hypochlorite solution to reduce superficial inoculum load, followed by drying by forced ventilation using air knives to minimize the risks of rotting when stored in plastic bags in supermarkets.

Biological control

Biological control of plant pathogenic bacteria could be an alternative to chemical and physical control and breeding for resistance. Biocontrol strategies comprise the use of antagonists affecting pathogen populations directly, via antibiosis, competition for nutrients or by induction of plant systemic resistance (Howarth, 2003). Although several attempts have been made to control *Pectobacterium* spp. and *Dickeya* spp. on potato by using biological control agents, most were restricted to in vitro overlay studies, potato slice assays or using in vitro raised potato plants and few included field experiments to check for consistency of results. Only the more recent work will be discussed here.

It has long been shown that bacteria isolated from the potato rhizosphere or those isolated from potato tuber periderm can be used successfully to protect potato tubers from *Dickeya* and *Pectobacterium* infections in laboratory conditions (Kloepper, 1983, Jafra et al., 2006, Rhodes & Logan, 1986). Initial selection of the control agent was based on random occurrence of bacteria which inhibited growth of soft rot bacteria in in vitro overlay studies. Further selection was based on characters likely to be inimical to soft rot bacteria. The agent is usually applied to seed tubers to control blackleg and rarely to control soft rot in stores.

In general soil fluorescent and non-fluorescent *Pseudomonas* spp. obtained by in vitro screening have shown to be potential candidates for biological control of blackleg and soft rot diseases (Kastelein et al., 1999). They are able to survive in the potato rhizosphere and in soil (Azad et al., 1985, Loper & Henkels, 1999, Kloepper, 1983, Gross, 1988) and produce a variety of secondary antibacterial metabolites (Weller, 1988) including mainly siderophores, antibiotics and surfactants (Kloepper et al., 1980, Cronin et al., 1997, Compant et al., 2005).

Fluorescent *Pseudomonas* spp. applied to tubers were able to reduce populations of blackleg and soft rot bacteria on potato roots and inside progeny tubers (Kloepper, 1983). They also apparently could control soft rot on potato when applied as a bacterial suspension directly to the tuber periderm (Colyer & Mount, 1984). Cronin and co-workers (1997) used *Pseudomonas fluorescens* strain F113 producing 2, 4-diacetylphloroglucinol (DAPG) to control Pa in vitro and on potato tubers. The wild type strain F113 was able to inhibit the growth in vitro and colonization of tubers by Pa, whereas a F113 mutant unable to produce DAPG was not effective, indicating that biocontrol occurred via antibiosis.

Kastelein et al. (1999) used strains of *P. fluorescens* to protect wounds and cracks on tubers from colonization by Pa. Application of individual and combination of strains resulted in the decrease of contamination of potato tuber

peel by 85% and 60-70% respectively indicating the potential of Pseudomonas spp. for controlling the soft rot disease caused by Pa.

Lactic acid bacteria are commonly found on fresh fruits, vegetables and milk products and pose no risk to human or animal health. *Lactobacillus plantarum, Lb. acidophilus, Lb. buchneri, Leuconostoc* spp. and *Weissella cibaria* isolated from fresh fruits and vegetables showed in vitro antagonistic activity in overlay assay towards Pcc which was attributed to production of hydrogen peroxide and acidification of the medium (Trias et al., 2008). In general, lactic acid bacteria possess different modes of action, mainly the production of organic acids, hydrogen peroxidase and siderophores which can be effective for biocontrol. Lactic acid bacteria are able to inhibit more than one phytopathogen, thus *Lb. plantarum*, *W. cibaria* and *Lb. acidophilus* also inhibit the fungus *B. cineria*. They have a wide range of growth temperatures, ranging from 8 to 45 °C, which provide possibilities for broad applications (Trias et al., 2008).

Gram positive *Bacillus subtilis* BS 107 that was selected for its broad antibiotic activity towards different plant pathogenic bacteria and fungi was used as a biocontrol agent against soft rot and blackleg causing bacteria (Sharga & Lyon, 1998). The strain was active in overlay assays against not only human pathogenic or opportunistic *P. aeruginosa*, *Klebsiella pneumonie*, *Micrococcus luteus*, *Staphylococcus aureus* and *Escherichia coli*, but also plant pathogenic Pcc, Pa and *Dickeya* spp., *P. syringae* and *Xanthomonas campestris*, which indicates that it is a potentially powerful agent to control different plant diseases. Cladera-Olivera et al. (2006) reported bacteriocin-like substance produced by *Bacillus licheniformis* P40 that was bactericidal to Pcc. This substance interacts with cell membrane lipids and provoking lysis of Pcc cells. It was also effective in protecting potato tubers against soft rot under standard storage (Cladera-Olivera et al., 2006).

Jafra et al (2006) focused on bacteria able to degrade quorum sensing signal molecules produced by *Pectobacterium* spp. and *Dickeya* spp. which is a useful and effective strategy for the control of the bacteria by preventing the secretion of large quantities of pectolytic enzymes to macerate tuber tissue. The result of this work was a selection of several bacteria isolates (e. g. *Delftia* spp., *Ochrobactrum* spp., *Rhodococcus* spp.) able to control pectinolytic bacteria by the quorum quenching mechanism in which infection of potato plants by target bacteria *Dickeya* and *Pectobacterium* was attenuated.

Predatory bacteria are ubiquitous in nature, present in different environments and able to invade and consume other bacteria (Stolp & Starr, 1963). *Bdellovibrio bacteriovorans* is a motile, δ-proteobacterium that preys on Gram-

negative bacteria (Rendulic et al., 2004). Epton et al. (1990) tested different strains of *B. bacteriovorans* to control Pa on potato (Epton et al., 1990). Only limited control of soft rot however was obtained in potato slice assays when co-inoculated with Pa and *B. bacteriovorans*. The main difficulty in using *B. bacteriovorans* as a biocontrol agent is that interaction with host prey bacterial cells is ruled by specific predator-prey relationship which assumes that the populations of both micro-organisms may fluctuate without complete eradication of the target bacterium (Crowley et al., 1980). Thus, Varon and Zeigler (1978) estimated that *Bdellovibrio* spp. were efficient as predators only when large populations of target bacterium are present. The minimum population density required for biocontrol of the target bacterium is about 10^5-10^6 cfu ml-1 which in case of *Dickeya* and *Pectobacterium* spp. may already be high enough to establish infection in plants under conditions favourable to disease (Varon & Zeigler, 1978). Finally, as *B. bacteriovorans* feeds on *Pectobacterium* spp., it is impossible to use *Bdellovibrio* spp. to prevent contamination in soft rot bacteria-free tubers.

Another possibility to control bacterial diseases of plants is the use of bacteriophages. Bacteriophages are viruses that infect and lyse bacterial cells. They are specific to their hosts and do not infect other micro-organisms. They are self-replicating, persistent in the environment and safe to use, as they cannot infect humans or animals. It has already been shown that bacteriophages possess the potential to control plant pathogenic bacteria (e. g. *Erwinia amylovora*, *Agrobacterium tumefaciens*) (Jones et al., 2007). However, their use is limited as they are non-motile and the target bacteria tend to become rapidly resistant. Until now, little attention was paid to the use of bacteriophages to control soft rot and blackleg bacteria in potato, but since Ravensdale et al (2007) had success in controlling up to 50% soft rot incidence on calla lily tubers inoculated with Pcc in greenhouse trials, there has been greater interest in this approach (Ravensdale et al., 2007).

Up to now, no commercial biocontrol agents active against blackleg and soft rot bacteria have been produced. In fact there are few instances of this approach being successful in other crop-pathogen systems. The main difficulty is the requirement for the antagonist to satisfy several criteria. It has to reach its target which in the case of potato would be located in lenticels, suberised wounds and vascular system, sites not readily available at all times. Then, to be active, the agent needs to survive and multiply, preferably becoming established in the tuber and in the rhizosphere microflora. Another requirement is the preparation of a stable formulation. Too often previous attempts have failed because some of the above criteria were not met. Moreover, transfer of small to large field scale testing can be

tricky because of annual variation in the weather, resulting in lack of consistency in the results. Finally, there is the costly and time-consuming registration of biological control agents which would require expensive large-scale field experiments and eco-toxicological studies (Weller, 1988).

A possible approach which takes into account most of the above requirements is the application of the selected antagonist bacteria, preferably spore forming, at the initial stage in seed stock multiplication. Inoculation of micro plants producing minitubers or of the microtubers before planting could allow establishment of the agent which could persist in later generations in the field. Protection of the first generations of seed crops is crucial, as control at that stage would reduce the risks of multiplication and spread of the pathogens at a later stage, at least in the high grade seed lots.

CONCLUSIONS AND PERSPECTIVES

Although many different control strategies against *Pectobacterium* spp. and *Dickeya* spp. have been employed, effective control of blackleg and soft rot diseases has not yet been achieved. Until highly resistant cultivars become available, disease control measures will continue to rely primarily on avoidance of contamination in the production of healthy certified seed. This is primarily based on seed derived from bacteria-free minitubers, the use of rigorous seed certification schemes and strict hygienic practices. Knowledge of the pathogen sources and contamination pathways should justify the application of hygienic measures, especially at harvest and post-harvest. Control strategies can be supported also by tuber treatments as discussed above. Only an integrated disease control strategy is likely to succeed in reducing blackleg and soft rot incidences effectively in seed and thence ware crops. The presence recently of the apparently more virulent forms of *Dickeya* spp. relative to *Pectobacterium* spp. should give a new urgency to research, notably on diagnostics, initial crop contamination and breeding for resistance.

Chapter 3

Distribution of *Dickeya* spp. and *Pectobacterium carotovorum* subsp. *carotovorum* in naturally infected seed potatoes

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European Journal of Plant Pathology (2009), **125**: 263-275

ABSTRACT

Detailed studies were conducted on the distribution of *Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya* species in two potato seed lots of different cultivars harvested from blackleg-diseased crops. Composite samples of six different tuber sections (peel, stolon end, and peeled potato tissue 0.5, 1.0, 2.0 and 4.0 cm from the stolon end) were analyzed by enrichment PCR, and CVP plating followed by colony PCR on the resulting cavity-forming bacteria. Seed lots were contaminated with *Dickeya* spp. and *P. carotovorum* subsp. *carotovorum* (Pcc). *Dickeya* spp. were found at high concentrations in the stolon ends, whereas relatively low densities were found in the peel and in deeper located potato tissue. Pcc was predominantly found in the peel and stolon ends and at a low incidence in deeper located tissue. Both seed lots have not contained *P. atrosepticum*. Rep-PCR, 16S rDNA sequence analysis and biochemical assays, grouped all the *Dickeya* spp. isolates from the two potato seed lots as biovar 3. The implications of the results for the control of *Pectobacterium* and *Dickeya* species, and sampling strategies in relation to seed testing, are discussed.

INTRODUCTION

Blackleg, a major bacterial disease of potato, is caused by bacteria belonging to *Dickeya* spp. (syn. *Erwinia chrysanthemi* or *Pectobacterium chrysanthemi* (Samson et al., 2005)), by *P. atrosepticum* (syn. *E. carotovora* subsp. *atroseptica*) (Gardan et al., 2003) (Pba), by *P. carotovorum* subsp. *carotovorum* (syn. *E. carotovora* subsp. *carotovora*) (Pcc) (De Haan et al., 2008) or by *P. c.* subsp. *brasiliensis* (Pcb) (Duarte et al., 2004). In temperate climates, *P. atrosepticum* was considered as the main causative agent of blackleg. *Dickeya* spp. was believed to be a major blackleg pathogen in tropical and subtropical regions, although 'atypical' strains of *Dickeya* spp. with a relative low growth temperature maximum were also isolated from blackleg-diseased plants in temperate regions (Janse & Ruissen, 1988). Pcc is considered to play a minor role in potato blackleg in temperate zones, although it has already been proven that tuber infections with virulent Pcc strains can result in true blackleg (De Haan et al., 2008). To date, Pcb has only been found in subtropical regions (Duarte et al., 2004)

Blackleg symptoms vary depending on the initial bacterial concentrations in seed tubers, the susceptibility of the potato cultivar and environmental conditions; particularly temperature and soil moisture content (Perombelon, 2002).

Trials using seed potatoes vacuum-infiltrated with Pba showed that even a low concentration of 10^3 colony forming units per ml was sufficient to cause blackleg disease in potato (Bain et al., 1990). As well as typical blackleg stem symptoms, *Dickeya* spp., Pba and Pcc can cause rotting of potato tubers (soft rot) during storage (Salmond, 1992, Van der Wolf & De Boer, 2007).

Control of potato blackleg is hampered by the absence of effective tools and strategies. In general, knowledge on the ecology of the blackleg-causing organisms is incomplete. For example, it is unknown how *Dickeya* and *Pectobacterium* species are introduced in seed potatoes grown from initially pathogen-free clonal selections or from *in vitro* material. It is reported that Pcc is able to spread via surface and rain water, by aerosols and also can be transmitted by insects (Perombelon & Kelman, 1980). However, for *Dickeya* spp. knowledge of the ecology and epidemiology in the potato production ecosystem is largely missing.

Selection for blackleg resistant potato cultivars was only partially successful and never resulted in cultivars completely resistant to *Dickeya* and *Pectobacterium* species (Lapwood & Read, 1984, Lapwood & Harris, 1982). The use of physical, chemical or biological control of blackleg also resulted in a reduction, but never in an elimination of the blackleg-causing pathogens (Perombelon & Salmond, 1995). Finally, there is still a need for effective seed testing protocols to eliminate contaminated seed lots from the production system. Most detection methods still lack specificity, sensitivity or are too costly for routine application. The lack of a cost-effective sampling strategy is another constraint in seed testing programs.

The major source of infection and the most important route of long distance dispersal of *Dickeya* or *Pectobacterium* species are contaminated seed tubers. Production of pathogen-free seed lots is therefore considered as the most important strategy in controlling spread of the blackleg pathogen. Tuber contamination can occur during plant growth, but harvesting and grading are considered the most important phases (Perombelon & Van der Wolf, 2002). Reduction of tuber contamination can be achieved by restricting the number of generations in the field, the application of disinfection procedures for mechanical equipment used during harvesting and grading, and disinfection of tubers (Perombelon, 2002). Several methods for the reduction of pathogen populations in infected tubers were tested: hot water treatment (Robinson and Foster, 1987), the use of bactericides such as streptomycin (Graham & Volcani, 1961) or copper-based compounds (Aysan et al., 2003), but none resulted in an eradication of the pathogen.

Knowledge of the distribution of *Dickeya* and *Pectobacterium* species in and on seed tubers is required for sampling in seed testing programs and also for the development of effective procedures for sanitation of tubers. This knowledge could also be used to understand how tuber infections occur. Helias and co-workers (2000) showed that Pba was mainly present in the stolon ends of infected potato tubers although bacteria were also found in the peel (Helias et al., 2000). The incidence of the presence of Pba in stolon ends was always higher than in tuber peel, in which Pcc was predominantly found (De Boer, 2002, Robinson & Foster, 1987, Samson et al., 2005). No information is known on the distribution of *Pectobacterium* species inside tubers, and information on the distribution of *Dickeya* species in and on seed tubers is entirely absent.

The aim of this work was to investigate in detail the distribution and the population structure of blackleg-causing bacteria in naturally infected seed potato lots.

MATERIALS AND METHODS

Bacterial strains and cultivation media

Bacterial isolates of *Pectobacterium* and *Dickeya* spp. were grown at 27 °C for 24-28 h on tryptic soya agar (TSA) (Oxoid) or nutrient agar (NA) (Oxoid) prior to use, unless otherwise stated. *Dickeya dianthicola* IPO1741, *Pectobacterium carotovorum* subsp. *carotovorum* IPO1990 and IPO1949 and *Pectobacterium atrosepticum* IPO1601 were used as reference strains in PCR amplification procedures. *Dickeya dianthicola* IPO2114, *Dickeya dadantii* IPO2120, *Dickeya* sp. IPO2222 and *Dickeya zeae* IPO2131 were used as reference strains for Rep-PCR. For long term maintenance, strains were kept on growth factor agar (0.4 g l^{-1} KH_2PO_4, 0.05 g l^{-1} $MgSO_4 \times 7\ H_2O$, 0.1 g l^{-1} NaCl, 0.5 g l^{-1} $NH_4H_2PO_4$, 0.01 g l^{-1} $FeCl_3$, 3 g l^{-1} yeast extract (Difco), 1 g l^{-1} glucose (Merck) 15 g l^{-1} agar (Oxoid)) (GF) at 17 °C .

Bacteria isolation from potato tubers

Two potato seed lots of cultivar Arcade and Konsul, grown in different regions in the North of the Netherlands and rejected because of a high blackleg

incidence in the field, were obtained from the Dutch Plant Inspection Service for agriculture seeds and seed potatoes (NAK).

For each seed lot, 10 composite samples of 10 tubers were analyzed. Seed lots were washed with running tap water and dried with tissue paper. After drying, the potatoes were peeled using a hand-held kitchen vegetable peeler, excluding the stolon end part (ca. 5 mm diameter). Peel (ca. 2 mm thick from the potato tuber periderm) from each lot of ten tubers (one composite sample) was collected. The peeled potato tubers were subsequently sterilized with 1% sodium hypochlorite (commercial bleach) for 5 min, washed once with tap water and subsequently sterilized with 70% ethanol for 5 min. After sterilization, potatoes were washed twice with tap water and dried with tissue paper. A 0.5 cm deep sample from the stolon end of each tuber was removed using a sterile cork-bore (0.5 cm diameter), and the 10 stolon ends were pooled. In a similar way, composite samples were made of transversely sliced potato disks taken at 0.5, 1.0, 2.0 and 4.0 cm from the stolon end of each tuber. The knife was sterilized with 70% ethanol between each cut to minimize the possibility of cross-contamination. Ten slices taken at a specific distance from the stolon end were combined as one composite sample. All composite samples were weighed and crushed for 1-2 min in a food processor (Combi Max 700, Braun) after adding twice the weight of quarter strength (1/4) Ringer's buffer (Merck) containing 0.02% diethyltdihiocarbamic acid (Acros Organics) as an antioxidant (Perombelon & Van der Wolf, 2002). For colony counts, duplicate 100 µl of 1:1, 1:10 and 1:100 dilution of the extracts in 1/4 Ringer's buffer were spread-plated on crystal violet pectate agar (CVP) and incubated for 72 h at 28 °C (Hyman et al., 2001). The colony forming units per gram of tuber sample (cfu g^{-1}) and cfu in tuber samples were calculated for cavity-forming bacteria. Cavity-forming bacteria were grown to pure culture by subsequent culture on CVP and TSA (Oxoid) for further analyses.

Incidence of Dickeya spp. *and P. atrosepticum presence in potato tubers*

To determine the incidence of tuber infection with *Dickeya* spp. and *P. atrosepticum*, 10 composite samples of 10 tubers each were tested using enrichment PCR. The probability of detecting *Dickeya* spp. and *P. atrosepticum* in the composite samples of the peel, stolon end and peeled tuber disk slices at 0.5, 1.0 cm, 2.0 cm, 4.0 cm distance from the stolon end was calculated. The incidence (*I*) was estimated using the statistical equation: $I = ([1-(N-p)/N]^{1/n})\times 100$ (De Boer,

2002), where *p* is the number of composite samples that tested positive for the presence of pectinolytic bacteria, *N* the total number of composite samples tested and *n* the number of potato tubers combined together in one composite sample.

Enrichment of Dickeya spp. *and P. atrosepticum in potato extracts*

For enrichment, 200 µl of the potato tuber disk extracts obtained from the different distances within the tubers were added to 1800 µl of polypectate enrichment broth (PEB) (0.3 g l^{-1} $MgSO_4 \times 7H_2O$, 1 g l^{-1} $(NH_4)_2SO_4$, 1.31 g l^{-1} $K_2HPO_4 \times 3H_2O$, 1.5 g l^{-1} polygalacturinic acid (Sigma), pH 7.2) (Perombelon & Van der Wolf, 2002) in 2 ml eppendorf tubes. The tubes were tightly closed to provide low oxygen conditions and incubated at 28 °C for 72 h. The enriched samples were used for purification of bacterial genomic DNA prior to PCR amplification.

Detection of Dickeya spp., *P. carotovorum sub*sp. *carotovorum, virulent P. c.* subsp. *carotovorum and P. atrosepticum by colony and enrichment PCR*

For characterization of pectinolytic bacterial isolates, a colony PCR procedure was used. Cells from a suspected colony were collected from CVP or NA plates using a sterile toothpick and resuspended in 50 µl of 5 mM NaOH. Suspensions were boiled for 5 min at 95 °C and immediately put on ice for 1-2 min. One or 2 µl of the cell lysate was used as a template in PCR. PCR detection of *Dickeya* spp. was performed according to Nassar et al. (1996), using ADE1/ADE2 primers (ADE1: 5' GATCAGAAAGCCCGCAGCCAGAT 3', ADE2: 5'CTGTGGCCGATCAGGATGGTTTTGTCGTGC 3') (Nassar et al., 1996). The expected fragment length of the amplicons was 420 bp. PCR detection of *Pectobacterium* spp. was performed according to Darasse et al. (1994), using Y1/Y2 primers (Y1: 5'TTACCGGACGCCGAGCTGTGGCGT 3', Y2: 5'CAGGAAGATGTCGTTATCGCGAGT '3) (Darrasse et al., 1994). The expected fragment length of the amplicons was 434 bp. PCR detection of virulent *P. c.* subsp. *carotovorum* was performed according to De Haan et al. (2008), using contig1R/contig1F (contig1F: 5' CCTGCTGGCGTGGGGTATCG 3', contig1R: 5'TTGCGGAAGATGTCGTGAGTGCG3') primers (De Haan et al., 2008). The expected fragment length of the amplicons was 500 bp. PCR detection of *P. atrosepticum* was performed according to Frechon et al. (1998), using Y45/Y46

(Y45: 5'TCACCGGACGCCGAACTGTGGCGT 3', Y46: 5'TCGCCAACGTTCAGCAGAACAAGT 3') primers (Frechon et al., 1998). The expected fragment length of the amplicons was 439 bp. In all cases, amplified DNA was detected by electrophoresis in a 1.5 % agarose gel in 0.5 × TBE buffer and stained with 5 mg ml^{-1} of ethidium bromide.

For enrichment samples, bacterial DNA was extracted from 500 µl of the enrichment broth using a Genomic DNA purification Kit (Qiagen) according to manufacturer's protocol for genomic DNA purification from Gram negative bacteria. After extraction, approximately 100-200 ng of DNA was used in the PCR assays which were conducted as described for the colony PCR.

Detection of Dickeya spp. *by microsphere immunoassay (MIA)*

A microsphere immunoassay for characterization of *Dickeya* spp. strains was performed as described by Peters et al. (2007) with slight modifications (Peters et al., 2007). Bacterial suspensions were prepared in 1/4 strength Ringer's buffer to a final concentration of approximately 10^8 cfu ml^{-1}. Subsequently, 50 µl of the prepared suspensions were added to a well of a 96-well V-shape microtitre plate (Greiner Labor Technik) with 50 µl of magnetic beads (1000 beads μl^{-1}) coated with IgG purified polyclonal antibodies against *Dickeya dianthicola* (8276-01) (Prime Diagnostics, Wageningen), prepared in 2 × concentrated PBS (pH 7.4) (16 g l^{-1} NaCl, 2 g l^{-1} KH_2PO4, 29 g l^{-1} $Na_2HPO_4 \times 12\ H_2O$) containing 0.1% Tween 20 (Acros Organics) and 1.0 % of skimmed milk (Difco). Samples were incubated for 30 min in the dark, at approx. 20-24 °C, with shaking (300 rpm). Plates were washed once with PBS containing 0.1 % Tween 20. Per well, 50 µl of the secondary Alexa Fluor 532 (Molecular Probes) conjugated antibody solution (final concentration 40 µg ml^{-1}) in PBS was added and plates were incubated for 30 min at 20 – 24 °C in the dark. Samples were analyzed with the Luminex 100 analyzer (Luminex Corporation). Analyses were finished after measuring 100 beads.

Identification of P. carotovorum spp. by biochemical assays

Biochemical assays, performed aseptically in test tubes, were used to differentiate *P. atrosepticum* from *P. carotovorum* subsp. *carotovorum* (Perombelon & Van der Wolf, 2002). Growth at 37 °C was evaluated by adding 100 µl of 10^8 cfu ml^{-1} to 3 ml of nutrient broth (NB) (Oxoid) and incubating for 72h

at 37 °C. The change in bacterial culture turbidity was observed visually. Acid production from maltose (Arcos Organics) and α-methyl glucosidase (Sigma) was performed as described by Perombelon and van der Wolf (2002) using minimal test medium (MTM) (10 g l^{-1} bactopeptone (Oxoid), 0.7 ml l^{-1} 1.5% bromocresol purple solution in water, 50 ml l^{-1} 20% maltose or α-methyl glucosidase solution in water). In each case, 100 µl of 10^8 cfu ml^{-1} was added to 2.5 ml of test medium. A change in medium color, due to the bromocresol purple serving as a pH indicator, was visually observed after 96 h. Production of reducing substances from sucrose was completed using MTM medium supplemented with 4% sucrose (Arcos Organics). 100 µl of 10^8 cfu ml^{-1} was added to 3 ml of MTM and incubated for 96 h. After adding an equal volume of Benedict's reagent (173 g l^{-1} $Na_3C_3H_5O(COO)_3$, 100 g l^{-1} $Na_2CO_3 \times H_2O$, 17.3 g l^{-1} $CuSO_4 \times 5H_2O$) to each tube, they were boiled in a water bath for 10 min before visually observing a change in medium color. As a positive control, in each test *P. atrosepticum* IPO 1601 and *P. carotovorum* subsp. *carotovorum* IPO 1990 were used.

Identification of Dickeya spp. *biovars by biochemical assays*

Biochemical tests in 96-well microtitre plates (Greiner Labor Technik) were used for biovar determination of *Dickeya* spp. (Palacio-Bielsa et al., 2006, Samson et al., 2005). Strains growth at 39 °C were evaluated by adding 15 µl of 10^8 cfu ml^{-1} to 150 µl nutrient broth (NB) followed by incubation for 72 h. The change in turbidity was observed visually and by determining the optical density at 600 nm (OD_{600}) using a spectrophotometer. Bacterial growth at 39 °C was compared with growth at 25 °C under the same conditions. Utilization of organic compounds, viz. D-tartrate, D-arabinose, D-raffinose, D-melibiose, D-mannitol, 5-keto gluconate (all from Arcos Organics) and β-gentobiose (Sigma) was performed as described by Palacio-Bielsa et al. (2006) using Ayers medium (1 g l^{-1} $NH_4H_2PO_4$, 0.2 g l^{-1} KCl, 0.2 g l^{-1} $MgSO_4 \times 7H_2O$) with bromothymol blue (0.08 g l^{-1}) as a pH change indicator. Anaerobic hydrolysis of arginine (Arcos Organics) (arginine dihydrolase) was evaluated as described by Palacio-Bielsa et al. (2006), and inulin (Sigma) assimilation in phenol red water according to Gallois et al. (1992) (Gallois et al., 1992). Per well, 15 µl of 10^8 cfu ml^{-1} in water was added to 150 µl of Ayers medium supplemented with 0.3% of test compound (Palacio-Bielsa et al., 2006). Plates were incubated at 27 °C for 72 h. A change in medium color was visually observed after 24, 48, 72 and 96 h, and compared with control wells without bacteria. Four replicate tests were performed per isolate.

Identification of Dickeya spp. *isolates by repetitive element PCR fingerprinting (Rep-PCR)*

As all *Dickeya* sp. isolates showed the same biochemical profile in the biovar determination assay, a selection of 20 isolates randomly chosen from different tuber samples and from both seed lots were used in repetitive element PCR fingerprinting (Rep-PCR).
Rep- PCR was executed according to Versalovic et al. (1991) using primers REP1R (5' IIIICGICGICATCIGGC 3') and REP2I (5' ICGICTTATCIGGCCTAC 3') (Versalovic et al., 1994) with the following modifications. Genomic DNA was purified using the Qiagen Genomic DNA purification kit (Qiagen) according to the protocol provided by the manufacturer. The DNA concentration was adjusted with Millipore water (MQ) to a final concentration of approximately 100 ng μl^{-1}. Rep-PCR was performed in a total volume of 28 µl using 6U of Taq polymerase (Roche) per reaction and 40 PCR cycles. Amplified DNA was analyzed by electrophoresis in a 1.5% agarose gel in 0.5 × TBE buffer stained with 5 mg ml^{-1} of ethidium bromide. Gels were developed for 6-7 h at 100 V and at room temperature (approx. 20-24 °C). A 1 kb ladder (Promega) was used as a size marker. Amplified fingerprints were compared using the Quantity One program (BioRad) according to instructions provided by the manufacturer. Cluster analyses were done with the UPGMA algorithm in order to calculate the percentage of similarity between isolates.

Identification of Dickeya spp. *biovars by 16S rDNA sequence analysis*

For purification of genomic DNA the Qiagen Genomic DNA purification Kit (Qiagen) was used. Purification was performed according to manufacturer's protocol for genomic DNA purification from Gram negative bacteria. Amplification of a 16S rDNA fragment between 968 and 1401 bp (numbering based on the *Escherichia coli* genome) was performed according to Heuer *et al.* (1999) using primers F968 (5'AACGCGAAGAACCTTAC 3') and R1401 (5'CGGTGTGTACAAGGCCCGGGAACG3') (Heuer et al., 1999). PCR products were purified with the PCR purification kit (Qiagen) according to manufacturer's protocol. For each strain, sequencing reactions were performed with both F968 and R1401 primers using the Big Dye Terminator Cycle Sequencing Kit (Applied

Biosystems). The DNA sequences obtained were compared with available sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) using nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) for nucleotides (blastn) alignments (http://www.ncbi.nlm.nih.gov/BLAST/). Acquired 16S rDNA sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) under accession numbers from EU515225 to EU515231. As a reference, GenBank sequences of *P. atrosepticum* (AY914794), *Dickeya paradisiaca* (AAF520710), *D. dianthicola* (AF520708), *D. chrysanthemi* subsp. *chrysanthemi* (Z96093), *D. chrysanthemi* subsp. *partheni* (AF520709), *D. dieffenbachia* (AF520712), and *D. dadantii* (AF520707) were used. The BioEdit Sequence Alignment Editor (Ibis Biosciences) was used for creating consensus 16S rDNA sequences from forward and reverse primers using pairwise alignment. The relationships between *Dickeya* spp. isolates were established by multiple alignment using the ClustalW2 program accessed via the Internet (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Phylogeny studies were performed with the use of the Phylip program (PHYLogeny Inference Package) (Felsenstein, 1980). For creation of dendrograms, the Neighbour-Joining method was applied followed by calculating the *p*-distance matrix for 16S rDNA sequences with the bootstrap support fixed to 1000 re-samplings. To root the tree, a 16S rDNA sequence from *Pectobacterium atrosepticum* (GenBank AY914794) was used.

RESULTS

Distribution of cavity-forming bacteria in potato tubers

To investigate the internal colonization and distribution of cavity-forming bacteria in two naturally infected seed potato lots harvested from blackleg-diseased crops, potato tuber extracts from six different sample types: peel, stolon end and (peeled) tuber slices from various distances from the stolon end were analyzed.

For all seed lots tested, the highest densities of cavity-forming bacteria were found in the tuber stolon ends (Fig. 1a). Relatively highly densities were also found in the tuber peel and at a 0.5 cm distance from the stolon end. The total number of bacteria producing cavities on CVP (calculated as total cfu per tuber and cfu g^{-1} of tuber sample) were highest in the stolon end and decreased with increasing distance

from the stolon end (Fig.1a and Fig. 1b). In most samples no cavity-forming bacteria were found at a distance of more than 2 cm from the stolon end.

Distribution of Dickeya spp. *and Pectobacterium carotovorum sub*sp. *carotovorum in potato tubers*

Two hundred and ninety six cavity-forming bacteria from CVP plates, selected from different tuber parts, were grown to pure culture on TSA or GF agar. 193 strains were taken from cv. Arcade and 103 from cv. Konsul. In a PCR assay specific for *Dickeya* spp., 157 isolates (81.3 %) from cv. Arcade and 73 isolates (70.8 %) from cv. Konsul were positive. 14 isolates (7.25%) from cv. Arcade and 23 isolates (22.3%) from cv. Konsul were positive in a PCR assay specific for *Pectobacterium* sp. 29 isolates were negative in both PCRs indicating that they did not belong to *Dickeya* spp. and/or *Pectobacterium* spp.

On the basis of tuber sample weight, the densities of cavity forming bacteria (in cfu g^{-1} of tuber sample) and the percentages of the cavity forming bacteria positive in colony PCR for *Dickeya* spp. and *Pectobacterium* spp., the numbers of *Dickeya* spp. and Pcc bacteria per gram of tuber sample and in tuber sample, were estimated (Fig. 2).

The densities of Pcc and *Dickeya* spp. in the stolon ends were ca. 100 times higher than in the peel (Fig. 2b, Fig. 2d). However, the total numbers were almost equal due to the higher weight of the peel (Fig. 2a, Fig. 2c). The total numbers of *Dickeya* spp. and Pcc decreased with increasing distance from the stolon end.

Table 1. Results of enrichment PCR for *Dickeya* spp. on 10 composite samples of different tuber samples of two seed lots cv. Arcade and cv. Konsul.

		composite sample [a]										
seed lot	**tuber sample** [b]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**estimated incidence (%)** [c]
Arcade	peel	-[d]	+[e]	+	+	+	+	+	+	+	+	**21**
	stolon end	+	+	+	+	+	-	+	+	+	+	**21**
	0.5 cm	+	+	+	+	+	-	+	-	-	-	**9**
	1.0 cm	+	-	+	-	+	-	-	-	-	-	**4**
	2.0 cm	+	-	+	-	+	-	-	-	-	-	**4**
	4.0 cm	-	-	-	-	-	-	-	-	-	+	**1**
Konsul												
	peel	-	+	-	-	-	-	-	-	-	-	**1**
	stolon end	-	-	+	-	+	-	+	+	-	+	**7**
	0.5 cm	+	-	-	-	+	-	-	-	-	-	**2**
	1.0 cm	-	-	-	+	+	-	-	-	-	-	**2**
	2.0 cm	-	-	-	-	-	-	-	-	-	-	**<1**
	4.0 cm	+	-	+	-	-	-	-	-	-	-	**2**

[a] Each composite sample represents 10 potato tubers combined together

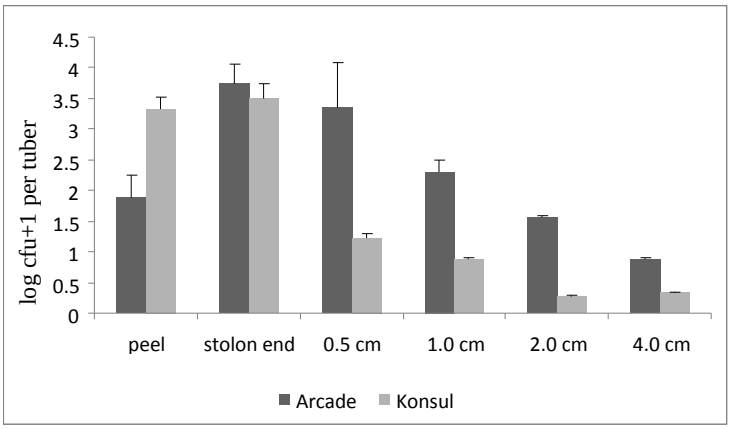
[b] Six different tuber samples were sampled: the peel, the stolon end and peeled tuber slices taken at a distance of 0.5, 1, 2 and 4 cm from the stolon end

[c] Calculated using the equation $I = ([1-(N-p)/N]^{1/n})\times 100$ (explanation provided in the text)

[d] Indicates a negative result in enrichment PCR for *Dickeya* spp.

[e] Indicates a positive result in enrichment PCR for *Dickeya* spp.

A



B

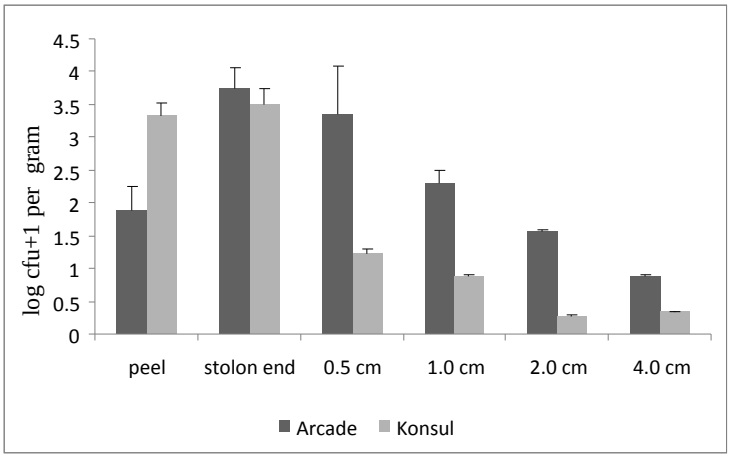


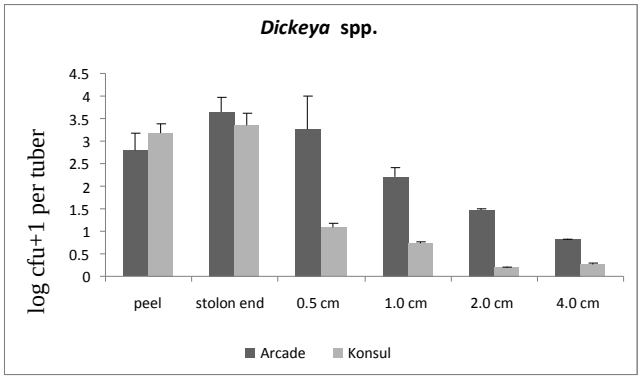
Figure 1. Distribution of pectinolytic bacteria in tubers of two seed potato lots, shown as (a) log cfu+1 of tuber sample and(b) as the average number of log cfu+1 g^{-1} of tuber sample, estimated from colony counts from CVP plating. Six different tuber samples were sampled: the peel, the stolon end and peeled tuber slices taken at a distance of 0.5, 1, 2 and 4 cm from the stolon end.

Incidence of Dickeya spp. *and P. atrosepticum in potato tubers*

Composite samples were analyzed for *Dickeya* spp. and *P. atrosepticum* with enrichment PCR, and the incidence of bacterial presence was estimated (Tab. 1). None of the tested samples was positive for *P. atrosepticum.* Overall, the highest incidence of *Dickeya* spp. was found in composite extracts of stolon ends. The

incidence was relatively high for peel extracts and in the peeled tuber sample extracts at 0.5 and 1 cm distance from the stolon end and relatively low for tuber sample extracts at larger distances from the stolon end.

A



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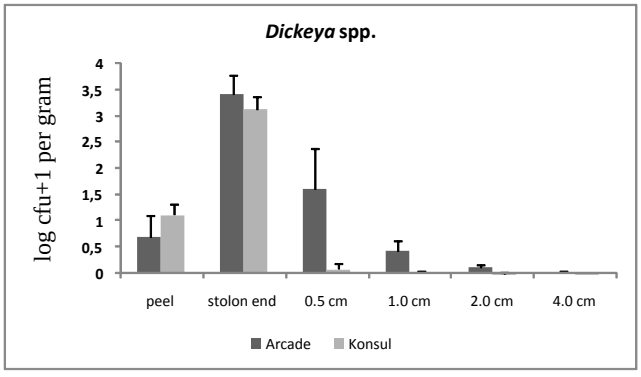
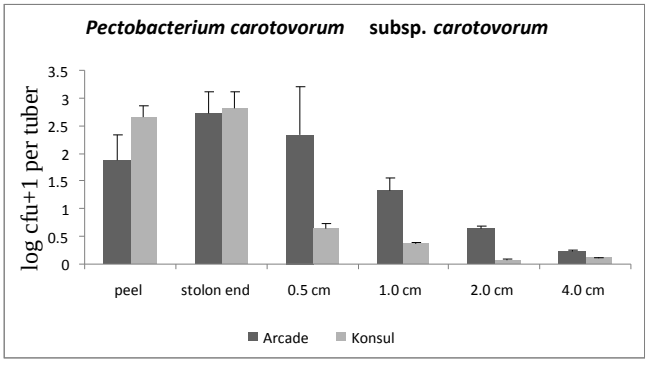


C



D

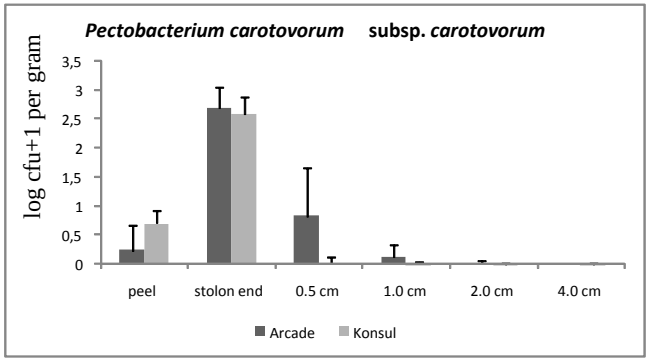


Figure 2. Distribution of *Dickeya* spp. (a, b) and *Pectobacterium carotovorum* subsp. *carotovorum* (c, d) shown as log cfu+1 of tuber sample (a,c) and as log cfu + 1 per g of tuber sample (b, d) for cv. Arcade and cv. Konsul. Densities were estimated on the basis of results from colony PCRs on cavity forming bacteria. Six different tuber samples were sampled: the peel, the stolon end and peeled tuber slices at a distance of 0.5, 1, 2 and 4 cm from the stolon end.

Characterization of Dickeya spp. *isolates by a microsphere immunoassay (MIA).*

To validate the colony PCR results for *Dickeya* spp., a selection of 38 isolates derived from different composite samples of cvs. Arcade and Konsul seed lots, and selected on the basis of different colony morphology, were characterized using a microsphere immunoassay. From cv. Arcade, 27 isolates were taken: 2 from peel extracts, 5 from stolon ends, 5 from 0.5 cm, 8 from 1.0 cm, 7 from 2.0 cm and 1 from 4.0 cm distance from the stolon end. From cv. Konsul, 11 isolates were taken; 2 from peel extracts, 7 from stolon end extracts, 1 from tuber slices taken 0.5 cm and 1 from 1.0 cm distance from the stolon end. All strains tested were positive in the microsphere immunoassay (data not shown).

Characterization of Dickeya spp. *isolates by biochemical tests.*

The same 38 isolates analyzed in MIA were characterized with different biochemical assays for *Dickeya* spp. biovar determination. Results of the biochemical assays were identical for all tested isolates. They utilized D-raffinose, D-melibiose, and D-mannitol but were not able to use D-tartrate, L-tartrate, D-arabinose or 5 ketogluconate as a carbon source and were not able to grow at 39 °C in NB. Strains neither assimilated inulin in peptone red water nor hydrolyzed arginine under anaerobic conditions. Results showed that all 38 isolates were

closest related to biovar 3 despite the fact that they did not grow at 39 °C and did not utilize arabinose (Samson et al. 2005, Palacio-Bielsa et al. 2006).

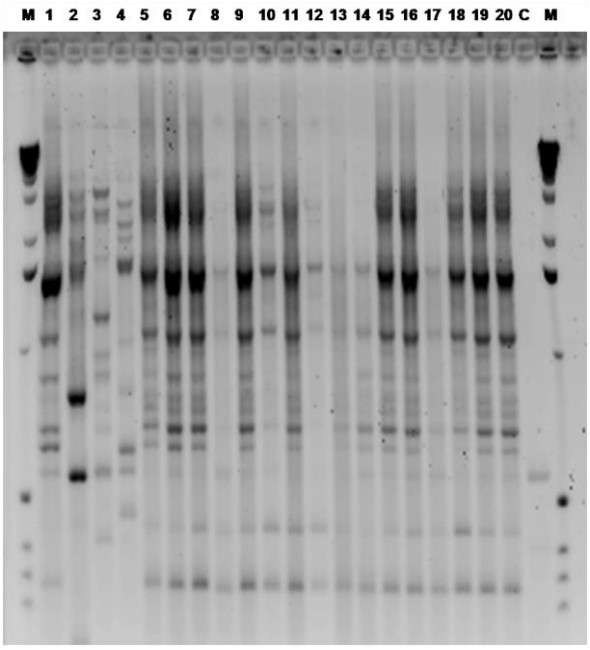
Characterization of Dickeya spp. *isolates by rep-PCR*

Twenty isolates, selected from the previous 38 that were characterized with the microsphere immunoassay and the biochemical tests, were analyzed using rep-PCR. Two isolates were selected from each tuber sample of cv. Arcade and cv. Konsul. Fingerprints from all isolates were identical to *Dickeya* spp. IPO2222, a strain isolated from Dutch seed potatoes in 2006 and closely related to *D. zeae* IPO2131 (Fig. 3).

Characterization of Dickeya spp. *isolates by 16S rDNA*

A selection of 4 isolates (2 derived from cv. Arcade and 2 derived from cv. Konsul seed lots) were characterized by 16S rDNA sequencing. The sequences of the four strains were identical and according to a cluster analysis with type strains of *Dickeya* spp. deposited in the Genbank, the strains were highly similar (> 99%) to *D. dadantii* and *D. dianthicola* (data not shown).

A



B

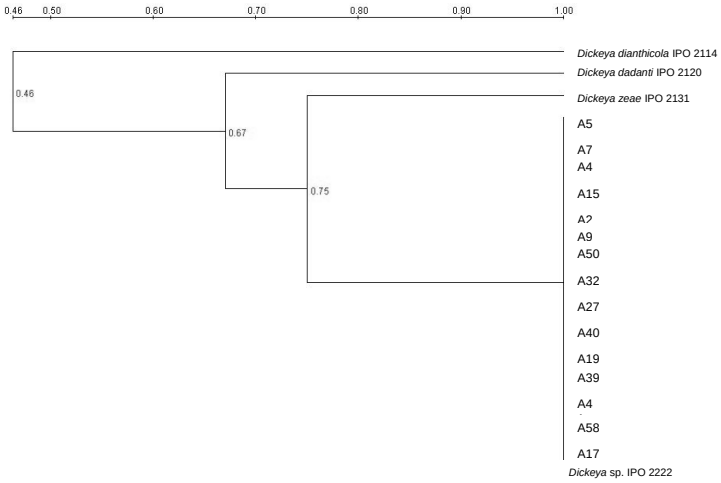


Figure 3. Rep-PCR analysis for selected isolates of *Dickeya* spp
A. Stained gel. M – 1 kb marker, C – negative control, 1 – *Dickeya* spp. IPO2222; 2 – *Dickeya dianthicola* IPO2114; 3 – *Dickeya dadantii* IPO2120, 4 – *Dickeya zeae* IPO2131, 5 – isolate A17; 6 – isolate A50, 7 – isolate A9; 8 – isolate A21; 9 – isolate A15, 10 – isolate A4, 11 – isolate A7, 12 – isolate A5, 13 – isolate A22, 14 – isolate A19, 15 – isolate A40, 16 – isolate A27, 17 – isolate A32, 18 – isolate A41, 19 – isolate A39, 20 – isolate A58.
B. Dendrograms showing the phylogenetic relationships between isolates. The numbers adjacent to the nodes are the similarities calculated using the Neighbour-Joining method. The A-numbers are test isolates. *Dickeya dadantii* IPO2120, *Dickeya dianthicola* IPO2114, *Dickeya* sp. IPO2222 and *Dickyea zeae* IPO2131 were used as reference strains.

Characterization of P. carotovorum subsp. *carotovorum and P. atrosepticum*

Fourteen isolates from cv. Arcade and 23 isolates from cv. Konsul positive in PCR for *Pectobacterium* spp. were all negative in the PCR specific for *P. atrosepticum* (data not shown). As there is no reliable PCR assay to characterize *Pectobacterium* spp., 10 isolates per seed lot, selected from different tuber samples were tested biochemically. Isolates were able to grow at 37 °C. They were not able to produce reducing substances from sucrose and were negative in acid production from maltose and α-methyl glucosidase. These characteristics are typical for *P. c.* subsp. *carotovorum*.

Characterization of P. c. subsp. *carotovorum by a PCR assay for virulent strains*

Twenty isolates previously classified as *P. c.* subsp. *carotovorum* by biochemical assays were additionally checked by PCR if they belonged to a virulent blackleg-causing subgroup of Pcc. All 20 isolates were negative in a PCR assay for virulent Pcc (data not shown).

DISCUSSION

In this publication, the distribution of pectinolytic, blackleg-causing *Dickeya* spp. in naturally infected potato tubers is described for the first time. New information is also provided on the distribution of *P. carotovorum* subsp. *carotovorum* in seed potatoes, of which a subgroup is able to cause blackleg disease symptoms in potato in temperate climate zones (De Haan et al., 2008). The information on the distribution is important for the sampling of potatoes in seed testing programs, to develop strategies to eliminate the pathogens in and on tubers and in general to acquire information on colonization routes in order to develop effective management strategies.

Composite samples of tubers of two naturally-infected seed lots belonging to different cultivars and harvested from crops rejected because of the high blackleg incidences were analyzed. We found that in these seed lots the highest numbers of pectinolytic bacteria were located in the stolon end and in tuber samples up to 0.5 cm distance from the stolon end, whereas only low numbers were found at larger distances from the stolon end. Frequently, the low densities of *Dickeya* spp. in deeper located tissue could only be detected after enrichment of the tuber extracts in a semi-selective broth, and not after direct plating on CVP. The estimated incidences of infection were relatively high for the peel extracts, stolon end extracts and the tissue at 0.5 cm from the stolon end, and low in deeper located tissues. It is likely that the tubers became infected via transport of bacteria through the vascular tissue from the stolon into the tuber. Also in field experiments with tubers vacuum-infiltrated with *Dickeya* spp., stolon ends became infected immediately at the formation of progeny tubers, indicating that *Dickeya* spp. readily move through vascular tissue in stems and stolons into tubers (Velvis et al., 2007). *Dickeya* spp. and *Pectobacterium* spp. seems to be less able to colonize tissues located deeper in the tuber. Relatively high numbers were also found in the peel due to lenticel infections during plant growth or contamination of tubers during harvesting and/or grading (Scott et al., 1996).

Although relatively high total numbers of pectinolytic bacteria were found in the peel, the densities (in cfu g^{-1}) were low compared to the stolon end. Due to its size and weight, the peel was found to harbor relatively large numbers of bacteria compared with the stolon end (Fig. 2). For pectinolytic bacteria, the onset of the infection process is density dependent and regulated, among other factors, by a quorum sensing (QS) mechanism (Pirhonen et al., 1993). Synthesis of pathogenicity determinants occurs only when the bacterial population is large enough to overwhelm the plant response. Population size is sensed by the production and secretion of signal molecules called autoinducers that in high concentration can stimulate expression of genes connected with pathogenicity. From this point of view it is more likely that tuber decay is initiated from the densely populated stolon ends than from the infected peel or deeper located tissues.
So far**,** knowledge on the distribution and the relative incidence of pectinolytic bacteria in potato tubers is limited. Studies have been done on *P. atrosepticum* only (De Boer, 2002, Helias et al., 2000), but never on *Dickeya* species or on *P. c.* subsp. *carotovorum*. Studies on *P. atrosepticum* have only been conducted to determine the relative incidence in the peel and stolon end; but never established to what depth bacteria were present in the vascular tuber sample.
For *P. atrosepticum*, de Boer (2002) found a higher incidence of infected stolon tissue than peel tissue, similar to *Dickeya* spp. Of 108 seed lots tested with enrichment ELISA for *P. atrosepticum*, 57 stolon end tissue samples were positive compared to 44 peel tissue samples (De Boer, 2002). Helias et al (2000) also found that the stolons of *P. atrosepticum* infected plants were more frequently infected than stems and daughter tubers, indicating the importance of transport via the vascular system in the stolons and adjacent tissues. At least these parts of the tuber should be sampled in seed testing programs, as was already advised by De Boer (2002) and is practiced in inspection services in The Netherlands. By using seed treatments, which only include superficial disinfection of tubers, a large part of the population will not be affected. Thirdly, because at harvest a large part of the contamination is already present, hygienic measures at this time will only partly avoid infection of seed.
Enrichment PCR and dilution plating on CVP combined with characterization of the isolates showed that seed lots were contaminated with *Dickeya* spp. and *P. c.* subsp. *carotovorum,* but not with *P. atrosepticum*. Until recently, *P. atrosepticum* was recognized as the major blackleg-causing pathogen of potato in cool and temperate climate regions. Although *D. dianthicola* had been reported to cause blackleg in Northern and Western Europe (Laurila et al., 2008), *Dickeya* spp. was more frequently found in regions with a higher temperature such as in Israel (Lumb

et al., 1986). *Dickeya* species have a higher growth temperature than *P. atrosepticum* (Perombelon & Kelman, 1980). The climatic change, resulting in higher temperatures during the potato growing season, may have contributed to the change in populations.

Biochemical tests, rep-PCR and 16S rDNA sequence analysis for *Dickeya* spp. proved that the test isolates were highly similar, if not identical, although they were isolated from two different potato cultivars grown at different locations in the Netherlands. They all belonged to serogroup O_1, and were similar to biovar 3, although they did not utilize arabinose and were not able to grow at 39 °C. 16S rDNA sequences were identical as was the fingerprinting pattern in rep-PCR. The 16S rDNA results and biochemical data did not allow them to be designated as known *Dickeya* species. According to 16S rDNA, the strains were closely related to *D. dianthicola* and *D. dadantii*, whereas the biochemical data suggested that they belonged to biovar 3, gathering *D. dadantii* and *D. zeae*. We were unable to identify the strains to species level, as straightforward methods for species determination are currently not available (Samson et al., 2005). Recently Tsror et al. (2008) described for the first time the presence of *Dickeya* spp. biovar 3 strains isolated from Dutch potatoes (Tsror et al., 2008). The strains isolated in this study from two potato cultivars Arcade and Konsul showed the same biochemical characteristics and rep-PCR fingerprints as presented in the work of Tsror et al. 2008. This indicates that the strains may have the same origin. It seems that this *Dickeya* sp. variant is more widely distributed in the Netherlands, suggesting that it possesses features which make it highly suitable to maintain itself as a pathogen in the potato production ecosystem. These features may include a high virulence, the production of antibacterial/antimicrobial compounds to compete with other bacteria including other *Dickeya* species, or the ability to survive conditions unfavorable for the other blackleg causing pathogens.

The negative results for all Pcc strains tested using PCR specific for a virulent, blackleg-causing subgroup of *P. c.* subsp. *carotovorum*, and the lack of *P. atrosepticum* present in the tested potato seed lots, indicated that the high blackleg incidence in the field was mainly due to the presence of *Dickeya* spp.

In conclusion, we have proven that tubers from blackleg diseased crops harbor relatively high densities and high numbers of *Dickeya* spp. and *P. c.* subsp. *carotovorum* in stolon ends, whereas the peel and deeper located tuber samples are less contaminated. We have also shown that although the sampled potato tubers were taken from different cultivars and obtained from different locations, all *Dickeya* spp. isolates were identical. Characterization of *Dickeya* spp. isolates both with biochemical assays and genetic techniques pointed to strains possessing

features of biovar 3 isolates and that they were closely related to strains isolated in Israel (Tsror et al., 2008). The lack of *P. atrosepticum* and virulent Pcc strains indicated that *Dickeya* spp. was the main blackleg-causing factor in these seed lots in the field.

ACKNOWLEDGEMENTS

The authors thank P. S. van der Zouwen (PRI, The Netherlands) for her technical assistance, L.J. Hyman (ex SCRI, UK) for her editorial work and S. Jafra (University of Gdansk, Poland) for critically reviewing the manuscript. The work was supported by the Dutch Ministry of Agriculture, Nature and Food Quality (program BO-04-006).

Chapter 4

Systemic colonization of potato plants by soilborne, green fluorescent protein-tagged strain of *Dickeya* sp. biovar 3

Robert Czajkowski, Waldo J. de Boer, Henk Velvis, Jan M. van der Wolf

Phytopathology (2010), **100**: 134-142

ABSTRACT

Colonization of potato plants by soil-borne GFP-tagged *Dickeya* sp. IPO2254 was investigated by selective plating, epifluorescence stereo microscopy (ESM) and confocal laser scanning microscopy (CLSM). Experiments were carried out in a greenhouse using plants with an intact root system and plants from which ca. 30% of the lateral roots was removed. One day after soil inoculation, adherence of the pathogen on the roots and the internal colonization of the plants was detected using ESM and CLSM of plant parts embedded in an agar medium. Fifteen days post soil inoculation, *Dickeya* sp. was found inside 42% of the roots, 13% of the stems and 13% of the stolons in plants with undamaged roots. At the same time-point, in plants with damaged roots, *Dickeya* sp. was found inside 50% of the roots, 25% of the stems and 25% of the stolons. Thirty days post inoculation, some plants showed 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. Microscopical observations were confirmed by dilution spread-plating the plant extracts onto agar medium directly after harvest. The implications of infection from soil-borne inoculum are discussed.

INTRODUCTION

Dickeya spp. (syn. *Erwinia chrysanthemi* and/or *Pectobacterium chrysanthemi*) (Samson et al. 2005), together with *Pectobacterium atrosepticum* (formerly *Erwinia carotovora* subsp. *atroseptica*) and P. *carotovorum* subsp. *carotovorum* (formerly *Erwinia carotovora* subsp. *carotovora*) are the causative agents of potato blackleg worldwide (Perombelon and Salmond 1995; Perombelon 2002; De Haan et al. 2008). Until recently, the majority of *Dickeya* spp. strains found in association with potato blackleg belonged to biovars 1 or 7 (*Dickeya dianthicola*). These strains have a relatively low maximum growth temperature compared to other *Dickeya* species and seem to be more adapted to European climate conditions (Janse and Ruissen 1988). In the last three years however, *Dickeya* spp. strains belonging to a new biovar 3 clade, probably constituting a new species, have been frequently isolated from potato tubers in Western Europe and Israel (Laurila et al. 2008; Tsror et al. 2008; Slawiak et al. 2009). The finding of this biovar 3 *Dickeya* spp. in seed potato tubers is associated with high incidences of potato blackleg in The Netherlands (NAK, personal communication).

In Western and Northern Europe in particular, *Dickeya* species are causing increasingly severe economic losses in potato crops. The costs in seed potato production resulting from *Dickeya* spp. caused blackleg infections are high due to rejection and declassification of seed lots (tubers) (Laurila et al. 2008; Prins and Breukers 2008).

Potato blackleg caused by *Dickeya* and *Pectobacterium* spp. is primarily a seed-borne disease. Typical blackening of the stem base (blackleg) and wilting of the potato plants are always associated with rotting of the seed (mother) tuber and spreading of the inoculum (Perombelon 1974). Symptom expression occurs when temperature and soil moisture are high, favoring multiplication of the blackleg bacteria and rotting of the seed potato (Stead 1999).

For P. *atrosepticum*, it is well-established that spreading of infections within a seed lot frequently occurs during harvest and grading, particularly if rotten tubers are present harboring high densities of inoculum (Elphinstone and Perombelon 1986). Dispersal of P. *atrosepticum* within a seed lot can also occur during plant cultivation. During tuber decay, massive amounts of bacteria are released into the soil from where they can move via free soil water up to a distance of 10 m (Graham and Harper 1967). The bacteria can be introduced into the lenticels, which are open during heavy rainfall under anaerobic condition (Adams 1975; Nielsen 1978). During harvest, a high percentage of tubers are bruised and are readily infected by contaminated equipment (Graham and Hardie 1971; Perombelon and Salmond 1995). Bacteria present on intact tuber peel will die off rapidly, but those in lenticels, suberized wounds and cracks may persist until the new growing season (Van Vuurde and De Vries 1994). It is assumed that contamination of potato tubers with *Dickeya* species occurs similarly.

Both lenticel contamination and infection of wounds during (post)harvest activities will primarily result in infections of the potato tuber periderm (peel). It is likely that tuber infection by *Dickeya* spp. can occur in a similar way.

For both *Dickeya* spp. and *P. atrosepticum*, however, a relatively high infection incidence is found in the stolon ends (De Boer et al. 1979; De Boer 2002; Czajkowski et al. 2009). This implies that during plant growth, internal colonization of progeny tubers via the stolons occurs more frequently than assumed. Internal infections of progeny tubers may take place in two different ways. Firstly, bacteria can be transported directly from the mother tuber via the plant vascular system into the stems, stolons and progeny tubers (Helias et al. 2000). Secondly, the soil-borne *Dickeya* sp. may also infect potato roots, from where the bacteria may further colonize the plant, including the progeny tubers. *Dickeya* spp., if present in soil, may enter not only the roots of the infected plant

but also the roots of adjacent plants by wounds caused by soil-borne pathogens and pest organisms or by natural openings which arise during lateral root formation.

The aim of this work was to investigate the ability of a representative potato strain of the new biovar 3 clade of *Dickeya* sp. to infect roots from inoculated soil and to colonize potato plants, including progeny tubers. To enable this, a GFP-tagged strain was generated and evaluated for growth and virulence. In greenhouse experiments population dynamics of the pathogen in plants with an intact (undamaged) and damaged (cut) root system was studied using epifluorescence microscopy, confocal laser scanning microcopy and dilution plating techniques.

MATERIALS AND METHODS

Bacterial strains and media used for cultivation

In all experiments a GFP-tagged strain of *Dickeya* sp. IPO2222, biovar 3 was used. Wild type strain IPO 2222 was isolated from seed potato tubers cv. Melody in The Netherlands in 2007 (Tsror et al. 2008). Strain was grown at 28 °C for 24-48 h on tryptic soya agar (TSA) (Oxoid), in nutrient broth (NB) (Difco), on crystal violet pectate (CVP) (Hyman et al. 2001) or pectate enrichment broth (PEB) (Perombelon and Van der Wolf 2002) prior to use. If required, growth media were supplemented with cycloheximide (Sigma) to a final concentration of 200 µg ml^{-1} and with ampicillin (Sigma) to a final concentration of 150 µg ml^{-1}.

Generation of GFP-tagged Dickeya sp. *strains*

Plasmid pPROBE-AT-*gfp* (Miller et al. 2000) was used for generation of GFP-tagged *Dickeya* sp. IPO2254 (parental strain *Dickeya* sp. IPO2222). The plasmid carrying *gfp* gene was introduced to bacterial cells by electroporation (Calvin and Hanawalt 1988). Briefly, suspensions of approximately 50 µl *Dickeya* sp. competent cells were mixed with 0.5 µl of plasmid DNA (approximately 100 ng $µl^{-1}$) and electroshocked at 2.5 kV for 1-2 sec at 4 °C using a BioRad Gene Pulser 200/2.0 (BioRad). After electroporation, cells were resuscitated for 1 h in 500 µl of NB broth at 28 °C with shaking. 100 µl of the transformed cells were plated on TSA containing 150 µg ml^{-1} ampicillin and incubated for 48 h at 28 °C for selection of positive transformants.

Maintenance of the pPROBE-AT-gfp plasmid in Dickeya sp. *cells in vitro plant model*

Maintenance of the plasmid carrying *gfp* gene in *Dickeya* sp. IPO2254 cells in planta was evaluated in a potato slice assay. Bacterial strains were grown overnight in NB supplemented with 150 µg ml^{-1} ampicillin (NBa) at 28 °C with a shaking rate of 200 rpm. Bacterial suspensions were diluted in Ringer's buffer (Merck) to a concentration of approximately 10^8 cfu ml^{-1} (OD600 = 0.1).
Dickeya-free minitubers of cultivar Kondor (Vitrocom, Westland, The Netherlands) were rinsed with running tap water, subsequently washed twice with 70 % ethanol for 5 min and washed twice for 1 min with demineralized water. Tubers were dried with tissue paper and cut into 0.7 cm transverse disk slices. One 5 mm deep well per slice was made using a sterile cork borer with a diameter of 5 mm. Wells were filled with 50 µl of the bacterial suspension. For disease development slices were incubated at 28 °C for 72 h in a humid box. After incubation, 200 mg of rotten potato tissue was collected and resuspended in 2 ml of Ringer's buffer supplemented with an antioxidant - 0.02% diethylthiocarbamic acid (Acros Organics). Fifty µl of the suspension was transferred to the well in a freshly prepared potato slice. Bacteria were transferred from one slice to another ten times in total. Serial dilutions of homogenized rotten potato tissue were plated on CVP at each transfer. The total number of GFP-positive and GFP-negative bacterial colonies, producing cavities on CVP were counted and the percentage of GFP positive cavity-forming colonies was calculated. Three replicate potato slices were used and the experiment was repeated twice. If present, cavity-forming bacteria that were negative in GFP fluorescence were randomly chosen at each time point and checked by a *Dickeya* spp. specific PCR to verify the identity of bacteria. For this, cells were collected from a suspected colony using a sterile toothpick and resuspended in 50 µl of 5 mM NaOH. Suspensions were boiled for 5 min at 95 °C and put on ice for 1-2 min. One µl of the cell lysate was used as a template in PCR specific for *Dickeya* spp. with ADE1/ADE2 primers (ADE1: 5' GATCAGAAAGCCCGCAGCCAGAT 3', ADE2: 5' CTGTGGCCGATCAGGATGGTTTTGTCGTGC 3') (Nassar et al. 1996). Amplified DNA was detected by electrophoresis in a 1.5 % agarose gel in 0.5 × TBE buffer stained with 5 mg ml^{-1} ethidium bromide. The expected fragment length amplified by the ADE1/ADE2 primers for *Dickeya* sp. was 420 bp.

Growth of the GFP-tagged strain

To assess bacterial growth under aerobic conditions, an overnight bacterial culture with a density of ca. 10^9 cells ml^{-1} in NBa was diluted 50 times in NBa. Bacteria were grown at 28 °C with a shaking rate of 200 rpm. Growth rate was determined by measuring the optical density (OD600) for a period of up to 25 hours.

To evaluate growth under anaerobic conditions, 5 ml of liquid paraffin was added over 30 ml of the bacterial suspensions in PEB, prepared as described above for growth in aerobic conditions. Samples were incubated at 28 °C without shaking. Growth was determined by measuring the OD600 at the same time intervals as for growth in aerobic conditions.

Ability of the GFP-tagged strain to macerate tuber tissue

The ability of GFP-tagged *Dickeya* sp. IPO2254 to macerate potato tuber tissue was evaluated in a potato slice assay, as described for estimation of the GFP plasmid stability with some modifications. Instead of one, three wells per tuber slice were used. The diameter of rotting tissue was measured after 72 h incubation at 28 °C. The result was compared with that of the wild type strain and with a water control. The experiment was repeated twice.

Growth of potato plants and soil inoculation with GFP-tagged Dickeya **sp.**

In a replicated experiment in 2008, minitubers of cultivar Kondor (Vitrocom, Westland, The Netherlands), highly susceptible to blackleg pathogens (Velvis, personal communication) were planted in potting compost in 5 liter plastic pots in the greenhouse and grown at a 16 h / 8 h (day / night) photoperiod, at 26 - 28 °C and 70% relative humidity (RH). Inoculation of soil was performed 3 weeks after planting, when plants were ca. 27-29 cm high and the stolons already formed. Plants were watered up to 1 h before soil inoculation. The lower part of the pots (ca. 40%) was immersed for 40 min in suspensions of *Dickeya* sp. IPO2254 containing 10^8 cfu ml^{-1} bacteria in water, or in bacteria-free sterile water. Half an hour before soil inoculation, while avoiding disturbing the plants, 30% of roots were cut off aseptically with a knife without removing the plants from pots, from half of the number of plants. Plants with damaged and undamaged root systems were inoculated in the same way. After inoculation, plants were left unwatered for

24 h. The greenhouse experiments were replicated in April and July 2008. In total 36 plants were used; per replication we used 6 plants inoculated with water (control), 6 plants with intact roots inoculated with GFP-tagged *Dickeya* sp. IPO2254 and 6 plants with damaged roots inoculated with the same strain. As well as sampling, plants were observed weekly for development of disease symptoms.

Sampling of potato plants for CVP plating

Plants were sampled 1, 15 and 30 days post inoculation (d.p.i.). At each time point, 4 plants per treatment were sampled. One g of soil was randomly collected from each pot and separately suspended in 2 ml of 1/4 strength Ringer's buffer (Merck) supplemented with 0.02% dieethydilthiocarbamic acid (Arcos Organics). The suspended soil was shaken for 10 min at 100 rpm and 100 µl of the undiluted, 10 times and 100 diluted samples were plated on CVP containing 100 µg ml^{-1} of cycloheximide (Sigma) and if appropriate with 150 µg ml^{-1} of ampicillin (Sigma).

Per plant, the total root system was collected and processed separately. Roots were washed with water to remove soil particles, sterilized in 70% ethanol for 1 min, washed 3 times with water for 1 min, incubated in 1% sodium hypochlorite (commercial bleach) for 4 min and finally washed three times with water for 4 min. Roots were weighed and Ringer's buffer (Merck) was added to twice the weight. Each sample was crushed in a Universal Extraction bag (BIOREBA) using a hammer. Extracts were plated as described for soil samples. To check the sterilization of root surface, the last washing water was collected, centrifuged (8000 rpm, 10 min), and the pellet was resuspended in 2 ml of Ringer's buffer. Three times 100 µl was plated on CVP and plates were incubated for bacterial growth and cavity formation at 28 °C for 16 h.

Six individual roots, three stolons and six progeny tubers with the diameter in range between 1 to 3 cm from each plant were randomly chosen. These were sterilized, crushed and plated in the same way as described for total roots. Each root, stolon and progeny tuber was processed separately.

From each plant, two or three 0.5 cm thick fragments from different stems cut 5 cm above ground level were jointly collected. The stem cuttings from each individual plant were sterilized, crushed and plated in the same way as described for the total root system.

Microscopic observations

Sample preparation

Three roots with a length of at least 30 cm, 2 stolons and 2 stems were cut randomly from every plant inoculated with the GFP-tagged strain, and 2 leaves was also collected from a plant expressing blackleg symptoms. The cut roots, stolons and stems were washed and sterilized before microscopic observation as described for CVP plating.

Epifluorescence stereo microscopy

Each root and stolon was cut into fragments of 1.5 – 2 cm long and each stem into fragments of 0.5 cm thick. Leaves were used without further cutting. Fragments were embedded in liquefied PT medium (Perombelon and van der Wolf, 2002) cooled down to 45-50 °C containing 200 µg ml^{-1} of cycloheximide and if required with 150 µg ml^{-1} of ampicillin in Petri dishes. After the medium had solidified, the plates were sealed with parafilm to prevent drying and incubated for 2 days at 28 °C. Samples were examined for the presence of *Dickeya* sp. IPO2254 under 495 nm blue light using an epifluorescence stereo microscope (Leica Wild M32 FL4) equipped with a mercury high pressure photo-optic lamp (Leica Hg 50W/AC) and GFP plus filter.

Confocal laser scanning microscopy (CLSM)

Samples for the confocal scanning laser microscope (Leica DM5500Q) were prepared in the same way as for the epifluorescence microscope. Most samples were counter-stained just before microscopic observations. For this, plant parts were washed from the agar and incubated for 0.5 -1 min in a 20 µg ml^{-1} propidium iodide (PI) (Invitrogen) solution. Samples were washed briefly in demineralized water and inspected under the microscope.
For excitation of GFP and PI, a 488 nm blue laser was used. For GFP, a 505 nm emission filter and for PI a 620 nm emission filter was used. Photographs were taken with a Leica Digital System (Leica) combined with a CSLM microscope using 10× and 63× water immersion objectives.

Isolation of the GFP fluorescent bacteria from the infected plant material

GFP-tagged bacteria were isolated from plant parts harboring GFP-tagged bacteria 30 d. p. i. Four roots, two cm long stem cuts taken 10 cm above the ground level and one leaf were cut into small pieces and incubated in 0.5 ml of Ringer's buffer for 20 min with shaking, and 100 µl of each suspension was plated onto TSA containing 150 µg ml^{-1} of ampicillin. GFP- positive colonies were collected from the plates.

Identification of GFP fluorescent bacteria by Dickeya **spp.** *specific PCR*

For characterization of the re-isolated bacteria, a colony-PCR procedure was used. Cells from a suspected colony were collected from tryptic soya agar (TSA) (Oxoid) using a sterile toothpick and processed in the same way as colonies sampled for the pPROBE-AT-*gfp* plasmid stability assay.

Identification of reisolated Dickeya **sp.** *by repetitive element PCR fingerprinting (Rep-PCR)*

For purification of genomic bacterial DNA the Qiagen Genomic DNA purification Kit (Qiagen) for Gram negative bacteria was used according to the manufacturer's instructions. Repetitive element PCR fingerprinting (rep-PCR) was done on 12 randomly chosen reisolates of GFP-tagged *Dickeya* sp. as described before (Versalovic et al. 1994) using REP1R/REP2I primers (REP1R: 5' IIIICGICGICATCIGGC 3', REP2I: 5' ICGICTTATCIGGCCTAC 3') with the following modifications. The DNA concentration was adjusted with demineralized, sterile water to a final concentration of approximately 100 ng $µl^{-1}$. Rep PCR was performed in a total volume of 28 µl using 6U of Taq polymerase (Roche) per reaction. Amplified DNA was analyzed by electrophoresis in a 1.5% agarose gel in 0.5 × TBE buffer and stained with 5 mg ml^{-1} of ethidium bromide. Gel was run for 6-7 h at 90-95 V and at room temperature. A 1 kb ladder (BioRad) was used as a size marker.

Statistical analysis

Bacterial count data were analyzed with ordinary linear regression using the statistical software package GenStat (Payne et al. 2009). To achieve approximate normality, the data were log transformed after adding a value 1 to avoid taking logs of zero. Effects were considered to be significant at P <= 0.05 and pair-wise differences were obtained using the t-test. Plates overgrown, due to high densities of cavity forming bacteria on CVP, were recorded as uncountable, taking the value 10^6 cfu g^{-1} as a likely cut-off level (censored observations). We used estimation of expected values for the censored observations based on normality assumptions as described in Schmee and Hahn (Schmee & Hahn 1979).
Data were analyzed according to the experimental design e.g. two replicated greenhouse experiments with treatments having 6 replications (plants) each. The linear model considered was a complete block design with replicates as complete blocks, main effects for time and treatment and the two-way-interaction between time and treatment.

RESULTS

Construction of GFP-tagged Dickeya sp.

Transformation of *Dickeya* sp. IPO2222 with pPROBE-AT-*gfp* resulted in 27 transformants, from which a highly fluorescent colony was selected (*Dickeya* sp. IPO2254). Repeated transfer of GFP-tagged bacteria onto TSA plates supplemented with 150 µg ml^{-1} of ampicillin or in liquid NBa showed that the transformant expressed GFP in a stable way. The presence of pPROBE-AT-*gfp* was proven by plasmid DNA purification and agarose gel electrophoresis (data not shown).

Maintenance of pPROBE-AT-gfp in Dickeya sp. *under nonselective conditions*

The stability of GFP expression in *Dickeya* sp. IPO2254 was evaluated in a potato slice assay. With a three day interval, *Dickeya* sp. IPO2254 was transferred from a potato slice derived from a *Dickeya* sp.-free minituber to a new slice, for a period of 1 month (10 times in total). During the first 9 days (3 transfers) no loss of plasmid was observed. After 21 days, 95% of cells were still GFP-positive. and after 30 days, 83% of the colonies still expressed GFP (data not shown). GFP

negative, cavity forming bacteria, collected at each time point, were all positive in a *Dickeya* sp. specific PCR (Fig. 1).

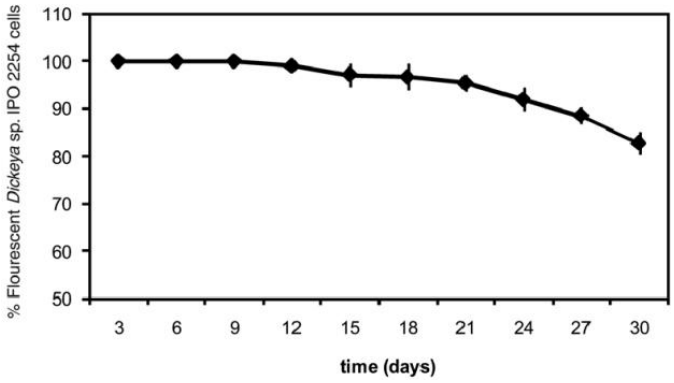


Figure 1. Maintenance of pPROBE-AT-*gfp* in *Dickeya* sp. IPO2254 during growth on potato slices. Potato slices of minitubers cv. Kondor, were inoculated with 10^8 cfu ml^{-1} *Dickeya* sp. IPO2254; slices were incubated for 3 days. Bacteria were harvest from rotten potato tissue and transferred to a fresh potato slice. Bacteria were harvested 10 times at 3 day intervals. At every transfer, serial dilutions of rotten potato tissue were plated on CVP, and the percentage of GFP-positive cavity forming *Dickeya* sp. was calculated.

Growth of GFP-tagged Dickeya sp.

GFP-tagged *Dickeya* sp. IPO2254 displayed similar growth characteristics as the parental wild type strain *Dickeya* sp. IPO2222 under aerobic (in NB broth) and anaerobic (in PEB medium) conditions, indicating that the growth rate was not affected either by the presence of the pPROBE-AT-*gfp* or by expression of the GFP protein (data not shown).

Tuber tissue maceration capacity of GFP-tagged Dickeya sp.

The ability of the *Dickeya* sp. IPO2254 to macerate potato tubers tissue was investigated using a potato slice assay. After incubation of slices for 3 days at 28 °C, the diameter of the rotting tissue was not significantly different from that of the parental wild type strain *Dickeya* sp. IPO2222 (data not shown).

Colonization of potato plants followed by CVP plating

Population dynamics of *Dickeya* sp. IPO2254 in soil and in plants were examined by CVP plating. One day after soil inoculation, the marker strain was found in all soil samples. Following a rapid decrease, populations stabilized at a low level of $10^2 – 10^3$ cfu g^{-1} soil during a 30 days experimental period (Fig. 2).
Densities of internal root populations of GFP-positive cavity forming bacteria in both damaged (cut) and intact (uncut) roots were high one day after soil inoculation ($10^4 – 10^6$ cfu g^{-1}) (Fig. 2). No statistically significant increase in densities of root populations was found during the course of the experiment, not even in plants showing visible blackleg symptoms. A significant decrease of density was found 15 d.p.i in roots derived from root-damaged plants, but the initial GFP-tagged bacterial densities were restored after 30 days. At 1 and 30 d.p.i. but not after 15 d.p.i., bacterial densities in plants with uncut roots were significantly lower than those in plants with cut roots. No visible symptoms were observed in roots during the experiment.
Low population densities of *Dickeya* sp. IPO2254 (approx. 1-160 cfu g^{-1} of stem) were detected in stems of potato plants with cut and uncut roots 15 and 30 d.p.i (Fig. 2). No statistical differences in population densities were found in stems derived from plants with damaged and intact roots.
GFP-tagged *Dickeya* sp. was detected in progeny tubers of plants with damaged and intact roots (Fig. 2). Population densities in stolons were variable 15 and 30 d.p.i but no statistical differences in densities were found in stolons derived from plants with intact and damaged roots. At 1 d.p.i. no stolons were present. Population densities in tubers were variable but no statistical differences were found in progeny tubers derived from plants with cut and uncut roots at time-points 15 and 30 days d.p.i.. No progeny tubers were present at time 1 d.p.i. No GFP-tagged *Dickeya* sp. was found in any sample of water-inoculated control plants.

Microscopic observations of plant colonization patterns

Epifluorescence stereomicroscopy (ESM)

Plant parts were analyzed with an epifluorescence stereo microscope (ESM) at a low magnification of 2.5 to 10 times. At 1 d.p.i., higher densities of *Dickeya* sp. IPO2254 were found on the surface of small roots (diameter: 0.25-0.5 mm) than on the surface of larger roots (diameter: 1-3 mm) (data not shown). At 1 d.p.i, bacteria were also observed inside approximately 20 % of the embedded roots after

disinfection. At 15 and 30 d.p.i., the GFP signal was frequently found inside roots, stolons and stem fragments taken 5 cm above the ground level (Fig. 3A and 3B). After 30 days, the GFP signal was detected in approximately 75% of the roots, 38% of the progeny tubers and 50% of the stolons from plants with intact roots, and in 92% of roots, 50% of progeny and 50% of stolons from plants with a cut root system (Fig. 4).

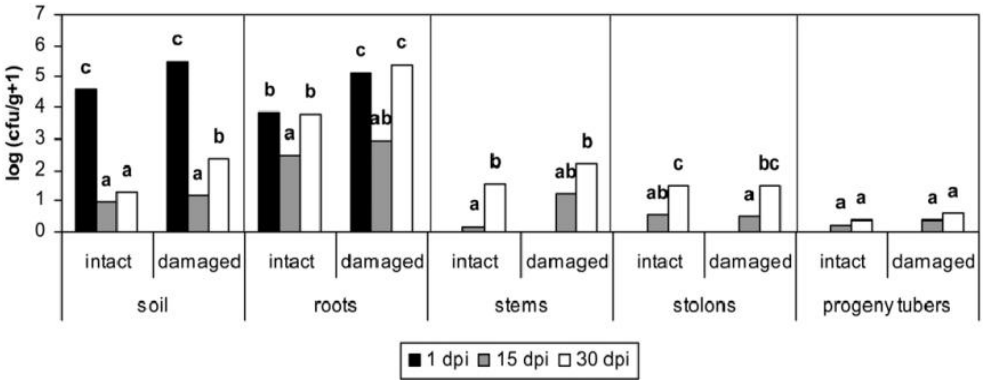
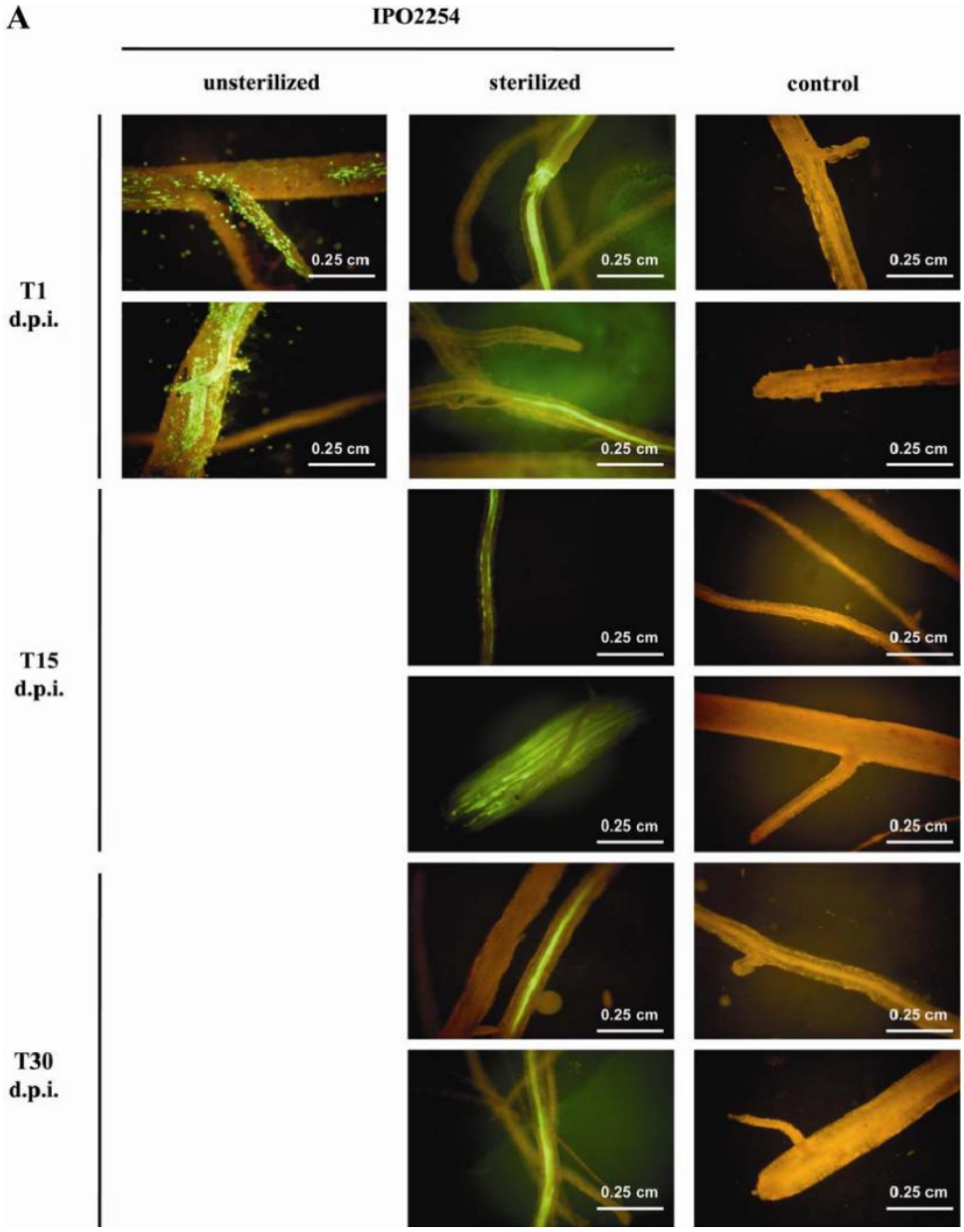


Figure 2. Population dynamics of *Dickeya* spp. IPO2254 in soil, roots, stems, stolons and progeny tubers taken from plants grown in inoculated soil. Samples were taken from plants with an undamaged (intact) and a damaged root system. Samples were surface sterilized before extracting the bacteria. Plant and soil extracts were plated on CVP 1, 15 and 30 days post soil inoculation (d.p.i.). Stem cuttings were taken 5 cm above the ground level. The average of the predicted values are shown from 4 plants per time point. Statistical analysis was done per subsample (soil n=4, roots n=4, stems n=4, stolons n=12, progeny tubers n=12). Values followed by identical characters are not significantly different (P=0.05)

Confocal laser scanning microscopy (CLSM)

Plant parts were analyzed with a confocal scanning laser microscope (CSLM) at a magnification of 640 – 1000 times. Detailed studies on the localization of *Dickeya* sp. IPO2254 in plant tissues using CLSM showed that bacteria were mainly present in the vascular tissue of roots and stems. In roots, *Dickeya* sp. IPO2254 was found in the pith (in medulla and cortex), both inter- and intracellularly. In stems, bacterial cells were found inside and between the xylem vessels and protoxylem cells (Fig. 5).


A
IPO2254
unsterilized
sterilized
control
T1
d.p.i.
T15
d.p.i.
T30
d.p.i.
0.25 cm

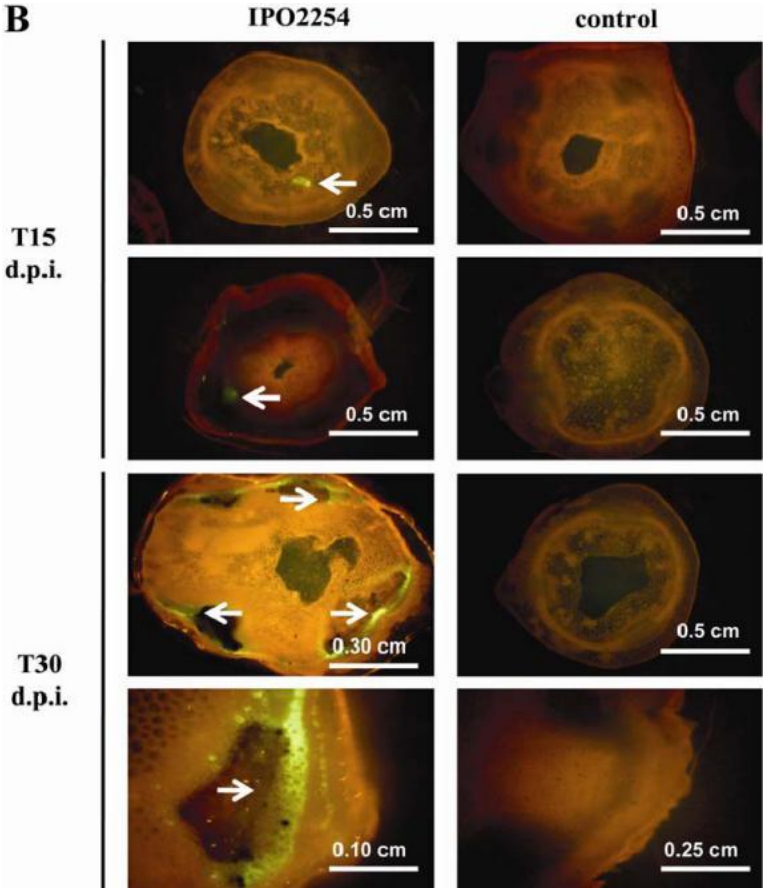


Figure 3. Colonization of potato roots and stems with GFP-tagged *Dickeya* sp. IPO2254 using epifluorescence stereo microscopy. Plant parts, embedded in PT agar and incubated for 1-2 days at 28 °C, were screened for a GFP-signal.
A – fragments of potato root. At 1 d.p.i , GFP-positive bacterial colonies on unsterilized roots were found on roots. After surface sterilization, a GFP signal was found in vascular and pith tissue of roots 1, 15, 30 d.p.i.
B – cross sections of surface sterilized potato stems embedded in PT agar. GFP signal was present in xylem and parenchyma tissue at 15 and 30 d.p.i, but not at 1 d.p.i. (results not shown)

Symptom development

No symptoms were observed in the first two weeks after soil inoculation. After two weeks symptoms started to develop and after 30 days all plants grown on *Dickeya* sp. infested soil showed wilting and chlorosis of the leaves, irrespective of root

treatment. One plant from a cut and another from uncut treatment showed typical blackening and soft rotting near the stem base. Control plants only expressed some chlorosis of lower leaves due to ageing (data not shown). Trans-sections of the stems from plants showing blackleg symptoms revealed a hollowing of the stems by degradation of pith tissue and a browning of the vascular tissue.

Characterization of GFP-expressing bacteria from infected plant tissue

Plant samples with a GFP fluorescence signal observed under the epifluorescence microscope were collected at 30 d.p.i. and were extracted and plated on TSA. Twelve green fluorescent isolates from various plant parts were selected; 8 from roots, 2 from stems and 2 from leaf material. All isolates produced typical cavities on CVP, were green fluorescent in ESM and were positive in a *Dickeya* sp. specific PCR, showing the expected 420 bp PCR product (data not shown). Rep-PCR analyses showed that all fingerprints were identical to strain *Dickeya* sp. IPO2254 used for soil inoculation and to the parental wild type strain *Dickeya* sp. IPO2222 used for GFP tagging (data not shown).

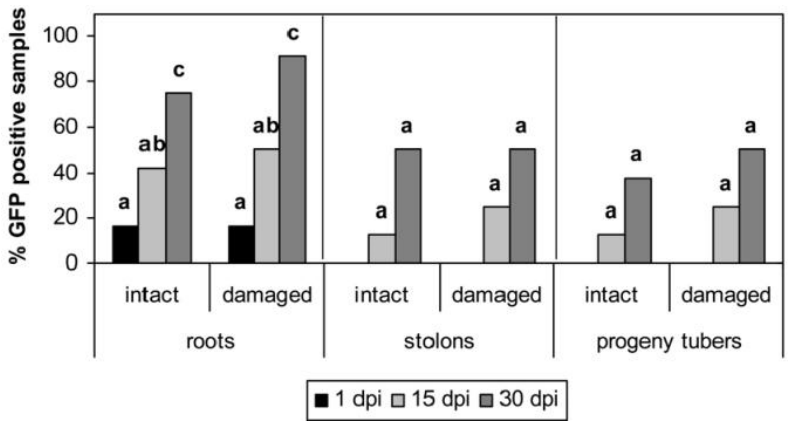


Figure 4. Percentage of samples embedded in PT agar found infected with GFP-tagged *Dickeya* sp. IPO2254, 1, 15 and 30 days post soil inoculation (d.p.i.). Plant parts were embedded in PT agar and incubated for 24-48 h at 28 °C before screening with an epifluorescence stereomicroscope. Samples were taken from plants with undamaged (intact) and damaged roots. From 4 plants in total 12 roots, 8 stolons and 8 progeny tubers, were screened per time point. Values followed by identical characters are not significantly different (P=0.05)

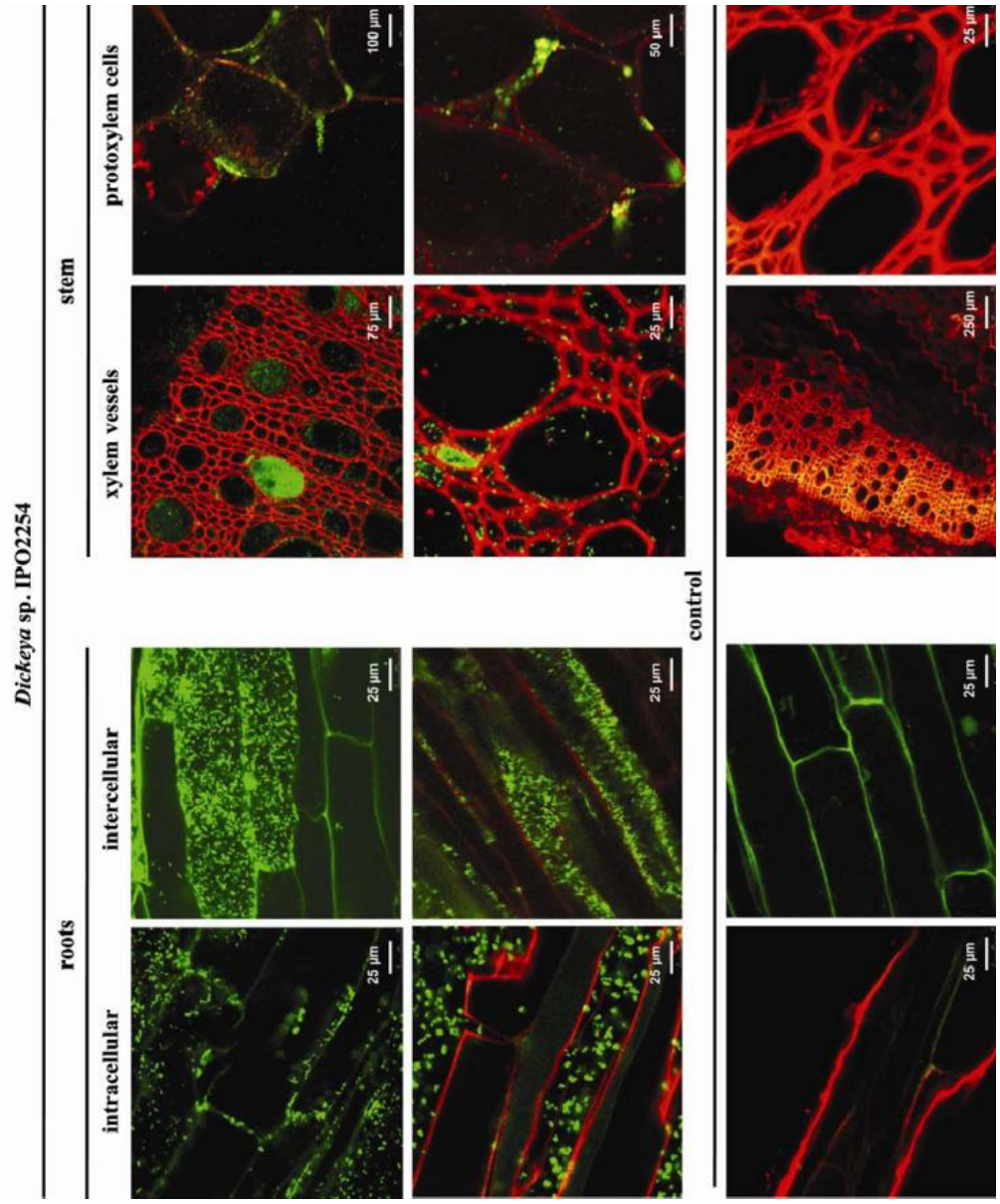


Figure 5. Colonization of potato stems and roots by GFP-tagged *Dickeya* sp. IPO2254 at 30 days post soil inoculation analyzed with confocal laser scanning microscopy (CLSM) after incubating embedded plant parts in PT agar for 2 days at 28 °C. (In most cases, plant cells were counter-stained with the red-fluorescing dye propidium iodide)

DISCUSSION

In this study we have shown for the first time that root infection with *Dickeya* spp. from soil-borne inoculum can result in the occurrence of typical blackleg symptoms and in a systemic colonization of potato plants. Systemic colonization of *Dickeya* sp. in stab-inoculated root cuttings of *Chrysanthemum morifolium* has been reported before (Pennypacker et al. 1981) as well as colonization of xylem vascular tissue with *Dickeya* spp. in naturally infected tomato plants (Alivizatos 1985). However, the colonization of intact potato roots from an inoculated soil has never been reported.

Using a GFP-tagged strain, roots were externally and internally colonized within one day of soil inoculation, and within one month, systemic spread of bacteria could be visualized in stolons and progeny tubers by dilution plating, epifluorescence stereo microscopy (ESM) and confocal laser scanning microscopy (CLSM).

The identity of the GFP-positive bacteria in roots, stems and leaves was confirmed by isolation, followed by colony PCR using *Dickeya* sp. specific primers and rep-PCR analysis, showing that the fluorescence was not due to conjugative transfer of the plasmid to other bacteria or caused by autofluorescent microorganisms, such as fluorescent Pseudomonas spp., present in the same niches.

GFP possesses excellent features as a reporter protein and is broadly used for studying bacterial populations in soil and the rhizosphere, colonization of plant tissue by pathogenic bacteria and for tracing particular proteins in the cytoplasm (Errampalli et al. 1999; Rosochacki & Matejczyk 2002). In our study, the GFP-tagged strain showed similar characteristics as the parental wild type strain with respect to growth under aerobic and anaerobic conditions and in the ability to macerate potato tuber tissue. The expression of GFP did not significantly affect the important biological features of *Dickeya* sp. IPO 2254. This confirms the observations of other research groups using GFP-tagged bacteria to monitor the destiny of bacterial cells in the environment (Errampalli et al. 1999).

GFP expression in *Dickeya* sp. during growth in plant tissue under non-selective conditions was stable. Only ca. 15% of the cells were negative in GFP expression after a total period of 30 days and after transferring the actively growing cells 10 times to fresh potato slices. Also in other studies, pPROBE- plasmids carrying *gfp* and various antibiotic resistance genes were stable up to 80 generations (80 doubling times) without any plasmid loss (Miller et al. 2000). The generation time of *Dickeya* spp. was determined at approximately 54 min in a rich

medium and 80 min in a poor medium, respectively (Hugouvieux-Cotte-Pattat & Baudouy 1994; Rincon-Enriquez et al. 2008). Assuming an average generation time of 60 min on potato slices, the pPROBE-AT-*gfp* carrying *gfp* gene in our study was steadily expressed in a large part of the *Dickeya* spp. IPO2254 population for more than 700 bacterial generations.

Low populations of GFP-tagged *Dickeya* sp. in potato tissue, embedded in PT medium, could be visualized with microscopical techniques after incubation. Microscopic observation of the GFP-tagged bacteria in plant tissue directly after harvest was often difficult due to the relatively low cell densities. Therefore, the embedded tissue was incubated for 24-48 h, enabling the bacterial cells to multiply. In this way we were able to monitor specifically culturable cells even if initial population densities were low.

Our microscopic observations may suggest that *Dickeya* sp. shares the same pattern as other root invading bacteria (Liu et al. 2006), comprising three stages. In the first stage, bacteria colonize the surface of lateral roots and junctions between the lateral and main roots. In the second stage bacteria penetrate the roots and establish infection of the cortex, and in stage three, they move into parenchyma cells of the pith and into xylem vessels of the stems, from where they can easily spread towards distantly located plant parts. Such colonization was observed both for *Ralstonia solanacearum*, a Gram-negative vascular plant pathogen (Vasse, Genin et al. 2000) and for a nitrogen-fixing Gram positive *Bacillus megaterium* (Liu et al. 2006).

Root colonization from soil borne inoculum was found irrespective if roots were damaged or not, indicating that *Dickeya* sp. enters via natural openings that occur during main and lateral root formation. In general, small but significant differences were found between incidences in infected tissues from plants with intact and damaged roots. It has been reported that both plant pathogenic bacteria, such as *Ralstonia solancearum*, and endophytic bacteria can enter plant roots via natural openings (Reinhold-Hurek and Hurek 1998; Huang and Allen 2000; James et al. 2002). In our study, higher numbers of GFP-tagged *Dickeya* sp. were found on the surface of lateral roots than on the surface of primary roots 1 day post soil inoculation (data not shown). Similar results have been reported in root cuttings grown under in vitro conditions, where *Dickeya* spp. was predominantly found near natural openings created during lateral root formation (Underberg and Vuurde 1989).

Infection of soil with *Dickeya* spp. can also occur via other routes. *Dickeya* spp. bacteria can be released from rotten tubers or from an infected root system. The motile bacteria can migrate via free water in soil up to a distance of 10 meters

(Graham and Harper 1967). Continuous infection of soils can occur during rainfall, if high numbers of bacteria are released into the soil from decaying tubers. Soil may also be infected from infected haulms after haulm destruction, as they can contain large numbers of cells which may contaminate the soil after rainfall (Perombelon 1982). Finally, soil-borne inoculum of *Dickeya* spp., a broad host range pathogen, may originate from other infected hosts, including crops grown in rotation with potato and/or weeds (Dickey 1980; Ma et al. 2007).

The risk of infection of a potato crop from soil borne inoculum will be dependent on the survival of the bacteria in the soil. Survival of *Dickeya* spp. in soil seems to be relatively short with a maximum period of six months (Rangarajan & Chakravarti 1970; Lim 1975). In our studies, a 1000-fold decrease in populations was found within 15 days. However, *Dickeya* sp. could survive at low densities of ca. $10^2 – 10^3$ cfu per gram of soil for 30 days. These densities may be sufficient to establish an infection when the conditions promote plant colonization . Moreover it cannot be excluded that *Dickeya* spp. may survive longer in plant debris of host plants as it was reported for closely related P. *carotovorum* (De Boer et al. 1979).

Systemic colonization of potato plants will be dependent on various factors such as potato cultivar, *Dickeya* species, initial inoculum present, soil moisture, soil type, temperature and pH. In our experiments, all plants showed symptoms, of which two plants had a typical blackening of the stem base, wilting, chlorosis and necrosis of plant tissue. In the glasshouse, conditions were highly favorable for disease development. The potato cultivar Kondor is highly susceptible to blackleg-causing pathogens (H. Velvis, unpublished results). The *Dickeya* sp. biovar 3 strain used is highly virulent, causes high blackleg incidences in the field and has been dominantly present in seed potatoes in several European countries in the past 5 years (Laurila et al. 2008; Tsror et al. 2008; Slawiak et al. 2009). The temperature in the glasshouse was high, favoring bacterial proliferation and symptom expression. Roots were submerged for 40 min in a bacterial suspension, probably long enough to create low oxygen conditions that can impair the host defense (Perombelon 1982). Field studies are required to further assess the risks of systemic colonization of plants via soil borne inoculum.

In general, these results suggest that systemic colonization of potato plants from contaminated soils can be highly significant in the epidemiology of potato blackleg caused by *Dickeya* spp. Progeny tubers can be infected systemically at the stolon end before harvest, limiting the possibility of controlling blackleg during harvest and post-harvest. To further estimate the role of stolon end infection the frequency of blackleg development from infected stolon ends relative to infected periderm needs to be investigated

ACKNOWLEDGEMENTS

The authors would like to thank I. Yedidia (Department of Ornamental Horticulture, ARO, Volcani Center, Israel) for providing the pPROBE-AT-*gfp* plasmid, P.S. van der Zouwen and M. Fiers (PRI, The Netherlands) for technical help, M. Perombelon (ex SCRI, UK) for the helpful discussion and Mrs L.J. Hyman (ex SCRI, UK) for her editorial work of the manuscript. The project was financed by the Dutch Ministry of Agriculture, Nature and Food Quality (programme BO-06-004)

Chapter 5

Downward vascular translocation of a green fluorescent protein-tagged strain of *Dickeya* sp. (biovar 3) from stem and leaf inoculation sites in potato

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Phytopathology (2010), **100**: 1128-1137

ABSTRACT

Translocation of a GFP-tagged *Dickeya* sp. from stems or from leaves to underground parts of potato plants was studied in greenhouse experiments. Thirty days after stem inoculation, 90 % of plants expressed symptoms at the stem base and 95 % of plants showed browning of internal stem tissue. GFP-tagged *Dickeya* sp. was detected by dilution-plating in extracts of 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, GFP-tagged *Dickeya* sp. was found in the xylem vessels and protoxylem cells. In progeny tubers, this strain was detected in the stolon end. Thirty days after leaf inoculation, GFP-tagged *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 and no translocation of the GFP-tagged *Dickeya* sp. to underground plant parts. The implications for contamination of progeny tubers are discussed.

INTRODUCTION

Dickeya spp. (syn. *Erwinia chrysanthemi* or *Pectobacterium chrysanthemi*) (Samson et al., 2005) , together with *Pectobacterium* spp. are the causal organisms of blackleg, stem wet rot, and tuber soft rot diseases of potato. In Europe, *Dickeya* spp. in particular are causing increasing economic losses in seed potato production, mainly due to downgrading and rejection of seed lots (Laurila et al., 2008, Prins & Breukers, 2008).

In 2005, the former *Erwinia chrysanthemi* species was reclassified into the genus *Dickeya*, which constitutes six different genomic species (genomo-species) inside 9 biovars (Samson et al., 2005). According to this classification, potato strains in Europe isolated before 2000 almost all belonged to *D. dianthicola* (previously *E. chrysanthemi* bv. *dianthicola*) (biovar 1, 7 and 9) (Janse & Ruissen, 1988, Slawiak et al., 2008). Since then, isolates belonging to a new genetic clade have been found frequently. This clade belongs to biovar 3 and possibly constitutes a new species (Slawiak et al., 2008). Strains belonging to this clade have been isolated from potatoes grown in Finland, Poland, the Netherlands and Israel (Czajkowski et al., 2009, Laurila et al., 2008, Slawiak et al., 2008, Tsror et al.,

2008), in the United Kingdom (J. Elphinstone, personal communication), France (Y. le Hingrat, personal communication) and Belgium (J. Van Varenbergh, personal communication). Increased losses in seed potato production due to *Dickeya* infection may be related to the occurrence of this new clade.

Potato blackleg is predominantly a seed borne disease and the use of pathogen-free seed lots is the best measure to control blackleg and stem rot diseases. Blackleg symptoms are nearly always associated with the presence of rotten contaminated seed potatoes indicating its seed-borne nature (Perombelon, 2000). An increase in tuber contamination and disease incidence within a seed lot occurs by spread of bacteria leaking from rotten tubers *via* soil water to daughter tubers in the field and, when soft rotting tubers are present, due to smearing during harvest and grading (Perombelon & Kelman, 1980).

Pathogen-free seed lots may become infected with blackleg and stem rot causing bacteria within a few generations growing in the field. In the Netherlands, minitubers became infected, as within only two field generations: 17 out of 50 seed lots were found contaminated with *Dickeya* spp. when tested by enrichment PCR (Velvis & van der Wolf, 2009). Possibly the use of contaminated machines during harvest and grading is responsible for introducing the bacteria but other sources of contamination cannot be excluded.

Initially clean seed may also become contaminated during cultivation. Infection of potato plants may originate from contaminated machines used for spraying crop protection agents, from contaminated insects, irrigation water, rain water, aerosols, human activity during field inspections or via animals entering potato fields (Charkowsky, 2006, Perombelon, 1992). In general, contamination via these sources more readily results in infection of haulms than underground plant parts. Aerial stem rot, which is frequently found in the field under wet conditions, may be the result of these introductions (Perombelon & Kelman, 1980).

Progeny tubers may become colonized from infected haulm indirectly via soil, or directly via translocation of bacteria inside plants. Bacteria washed off by rain from rotting stems and leaves into the soil may contaminate the progeny tubers. Despite the fact that *Dickeya* spp. cannot survive for a long time in soil, high numbers of bacteria washed from haulms and constantly reintroduced to the soil could result in tuber lenticel contamination (Scott et al., 1996). Bacteria in soil can also colonize roots and move *via* the vascular tissue of the roots into the stolons and finally in the progeny tubers (Czajkowski et al., 2010a).

It is unknown whether *Dickeya* spp. can be translocated from the haulm to underground plant parts, i.e. roots, stolons and progeny tubers. Translocation of bacteria internally from aerial to underground plant parts has been described for

only a few plant associated bacteria. For example, *Pseudomonas fluorescence* injected into stems of mature maize plants could move approximately 15 cm in stems above and below the inoculation point (Fisher et al., 1993), *Erwinia amylovora* was isolated from roots after stab-inoculation of apple seedlings (Bogs et al., 1998) and *Xanthomonas campestris* pv. *vitians* was recovered from stem sections 2 cm below the inoculation site in stems of lettuce (Barak et al., 2002).

This study examines the ability of a strain belonging to the new biovar 3 genetic clade of *Dickeya* spp. to infect roots, stolons and progeny tubers from inoculated potato haulm. In greenhouse experiments, the movement of the bacteria to underground parts was studied after stab-inoculation of stems and inoculation by abrasion of leaves. We used a GFP-tagged strain for monitoring the systemic movement of bacteria inside plants using epifluorescence stereomicroscopy, confocal laser scanning microscopy and dilution plating techniques.

MATERIALS AND METHODS

Bacterial strains and media used for cultivation

In all experiments a GFP-tagged *Dickeya* sp. strain IPO2254 was used. This strain is a derivative of the wild type biovar 3 *Dickeya* sp. IPO2222 (Tsror et al., 2008) and contains the pPROBE-AT-*gfp* plasmid (Miller et al., 2000) that confers stable production of GFP (Czajkowski et al., 2010a). Prior to use, *Dickeya* sp. IPO2254 was grown at 28 °C for 24-48 h on tryptone soya agar (TSA) (Oxoid) or in nutrient broth (NB) (Difco). For testing cavity formation, dilutions of isolates collected from infected plant material were plated on crystal violet pectate (CVP) (Hyman et al., 2001). For pour plating, bacteria were grown in PT medium at 28 °C for 24 h (Perombelon & Van der Wolf, 2002). Growth media were supplemented with 150 µg ml^{-1} of ampicillin and with 200 µg ml^{-1} of cycloheximide.

Growth of potato plants and inoculation with GFP-tagged Dickeya sp.

Minitubers of cultivar Kondor (Agrico, The Netherlands) were planted in potting soil in 5 liter plastic pots in a greenhouse and grown at a 16 / 8 h photoperiod at 26-28 °C and ca. 70% relative humidity. Replicated experiments were conducted in June and November 2008. Inoculation of potato stems was performed 3-4 weeks after planting, when plants were approximately 27-30 cm high and stolons were already formed. Twenty plants (10 per experiment) were

inoculated with the GFP-tagged *Dickeya* sp. strain and 10 plants (5 plants per experiment) for the water-inoculated control were used. Stems were inoculated 10 cm above the ground level with either 100 µl of 10^8 cfu ml^{-1} of GFP-tagged *Dickeya* sp. in water or 100 µl of sterile demineralized water (control). A 200 µl yellow pipette tip was stabbed halfway into the stem at an angle of 45° and subsequently wrapped around with parafilm to prevent drying and leakage of bacteria along the stem surface to the soil. Three stems per potato plant were inoculated. To minimize the risk of soil contamination with the GFP-tagged *Dickeya* sp. strain released from diseased (rotten) stems, the soil surface was covered with plastic film. Plants were watered from the bottom of the pots.
Inoculation of potato leaves was done at the same time and in the same greenhouse as being used for stem inoculation but with a different set of plants (cv. Kondor). For each plant, 7 randomly chosen leaves were inoculated by abrasion with 1 ml of water suspension containing 10^8 cfu ml^{-1} of *Dickeya* sp. strain IPO2254, 2.5% carborundum powder (Chemos GmbH) and 0.1% Tween 20 (Oxoid). Control plants were inoculated with sterile water containing 2.5% carborundum powder and 0.1% Tween 20. Both the axial and abaxial leaf surfaces were gently rubbed with the suspensions for 25 seconds. In total, sixteen plants (8 plants per experiment) were used for inoculation with the GFP-tagged *Dickeya* sp strain and a further 8 plants (4 plants per experiment) as a water-inoculated control. Direct soil contamination with the bacteria from inoculated leaves was prevented as with the stem-inoculated plants. Plants were also watered from the bottom of the pots.

Symptom development

Plants were visually inspected weekly for symptom development. Stem-inoculated plants were assessed for wilting, black rot on the stem base, aerial stem rot, haulm desiccation and plant death. Leaf-inoculated plants were assessed for wilting and chlorosis of leaves, wilting of primary stems, wilting of secondary stems, aerial stem rot, typical blackleg, haulm desiccation and plant death.

Sampling of stem and leaf-inoculated plants for PT pour plating

Per plant, 2 cm long stem fragments at the inoculation point were collected of three stems and processed as a composite sample. Similarly, 2 cm long stem fragments at the stem base were collected from the same 3 stems and processed as a composite sample. Per plant, the whole root system, 3 stolons and 6 progeny tubers were tested separately. The sampled material was washed with tap water to

remove soil particles, and subsequently surfacially sterilized with 70% ethanol for 1 min, washed 3 times with water for 1 min, held in 1% sodium hypochloride (commercial bleach) for 4 min and finally washed three times with water for 4 min each. Samples were weighted and a volume equivalent to twice the sample weight of 1/4 Ringer's buffer (Merck) added. Each sample was crushed in a Universal Extraction bag (BIOREBA) using a hammer. 100 µl of undiluted, 10 and 100 times diluted samples were added to wells of a 24 well plate (Greiner BioOne) with 300 µl of liquefied PT medium cooled down to 45-50 °C supplemented with 200 µg ml^{-1} of cycloheximide and with 150 µg ml^{-1} of ampicillin. After the medium had solidified, plates were sealed with parafilm to prevent drying and incubated for 1 day at 28 °C. Plates were examined for the presence of GFP-tagged *Dickeya* sp. IPO2254 colonies under 495 nm blue light using an epifluorescence microscope (Leica Wild M32 FL4) equipped with a mercury high pressure photo-optic lamp (Leica Hg 50W/AC) and GFP plus filter at a low magnification of 10 and 20 times. Leaf- inoculated plants were sampled 30 d.p.i. Seven inoculated leaves, 7 leaf petioles, 7 axils, seven 2 cm stems segments taken 15 cm above the ground level and seven 2 cm stem base segments, whole root system, 3 stolons and 6 progeny tubers were collected per plant and pooled per sample source. Plant parts were sterilized and processed for pour plating similarly as described for stem-inoculated plants.

Microscopic observations

Sample preparation

From every stem-inoculated plant, 4 roots at least 5 cm long, two 1 cm stem base segments, two 2 cm stem segments taken at the inoculation point, 3 stolons and 6 progeny tubers were randomly selected.

From every leaf-inoculated plant, 2 leaves, 4 leaf petioles, 4 leaf axils, 2 stem base segments of 1 cm, 2 stem samples of 2 cm were randomly selected. Stem segments, stolons and stolon ends of progeny tubers were transverse sectioned into 0.1 cm thick pieces. All plant parts were washed and sterilized before microscopic observations as described for pour plating.

Epifluorescence stereo microscopy

All plant samples were embedded in PT medium in Petri dishes. The PT medium was liquefied by heating, cooled down to 45-50 °C and supplemented with 200 µg ml^{-1} of cycloheximide and 150 µg ml^{-1} of ampicillin before embedding. After the medium had solidified, plates were sealed with parafilm to prevent drying and incubated for 1 day at 28 °C. Samples were examined for the presence of GFP-tagged *Dickeya* sp. under 495 nm blue light using an epifluorescence stereo microscope (Leica Wild M32 FL4) equipped with a mercury high pressure photo-optic lamp (Leica Hg 50W/AC) and GFP plus filter.

Confocal laser scanning microscopy

Samples for the confocal laser scanning microscope (Leica DM5500Q) were prepared in the same way as for the epifluorescence microscope. Samples were counter-stained just before microscopic observations. For this, plant parts were washed from the agar and incubated for 0.5 -1 min in a 20 µg ml^{-1} propidium iodide (PI) (Invitrogen) solution. Samples were washed briefly with demineralized water and inspected under the microscope. For excitation of GFP and PI, a 485 nm blue laser was used. For GFP, a 505 nm emission filter and for PI a 620 nm emission filter was used. Photographs were taken with a Leica Digital System (Leica) combined with a CSLM microscope using 10× and 63× water immersion objectives.

Isolation of the GFP-tagged bacteria from infected plant material

GFP-tagged bacteria were isolated from plant parts harboring GFP-tagged bacteria 30 days post inoculation. For this, 2 stem samples at the inoculation point, 2 stem samples 10 cm below the inoculation point, 2 randomly chosen roots, 2 stolons and 2 progeny tubers were collected from the stem-inoculated plants; 2 leaves, 2 axils, 2 petioles and 2 stem base cuts were collected from leaf-inoculated plants. Collected parts were cut into small pieces and incubated in 0.5 ml of Ringer's buffer for 20 min with shaking (approx. 200 rpm), and 100 µl of each suspension was plated onto TSA containing 150 µg ml^{-1} of ampicillin or on CVP plates for testing cavity formation. GFP- positive and cavity forming colonies were collected from the plates.

Identification of GFP-tagged bacteria by a Dickeya spp. *specific PCR*

For characterization of re-isolated bacteria, a colony-PCR procedure was used. Cells from GFP fluorescent or cavity forming colonies were collected from tryptone soya agar (TSA) (Oxoid) using a sterile toothpick, and resuspended in 50 µl of 5 mM NaOH. Suspensions were boiled for 5 min at 95 °C and put on ice for 1-2 min. One µl of the cell lysate was used as a template in PCR specific for *Dickeya* spp. with ADE1/ADE2 primers (ADE1: 5' GATCAGAAAGCCCGCAGCCAGAT 3', ADE2: 5'CTGTGGCCGATCAGGATGGTTTTGTCGTGC 3') (Nassar et al., 1996). Amplified DNA was detected by electrophoresis in a 1.5 % agarose gel in 0.5 × TBE buffer stained with 5 mg ml^{-1} ethidium bromide. The expected fragment length amplified by the ADE1/ADE2 primers for *Dickeya* spp. was 420 bp.

Identification of re-isolated Dickeya sp. *by repetitive element PCR fingerprinting (Rep-PCR)*

Repetitive element PCR fingerprinting (rep-PCR) was done on randomly chosen reisolates of GFP-tagged *Dickeya* sp. as described before (Versalovic et al., 1994) using REP1R/REP2I primers (REP1R: 5' IIIICGICGICATCIGGC 3', REP2I: 5' ICGICTTATCIGGCCTAC 3'). The Qiagen Genomic DNA purification Kit (Qiagen) for Gram negative bacteria was used according to the manufacturer's instructions for purification of genomic bacterial DNA. The DNA concentration was adjusted with demineralized, sterile water to a final concentration of approximately 100 ng $µl^{-1}$. Rep PCR was performed in a total volume of 28 µl using 6U of Taq polymerase (Roche) per reaction. Amplified DNA was analyzed by electrophoresis in a 1.5% agarose gel in 0.5 × TBE buffer and stained with 5 mg ml^{-1} of ethidium bromide. The gel was run for 6-7 h at 90-95 V and at room temperature. A 1 kb ladder (BioRad) was used as a size marker.

Statistical analysis

Bacterial count data were analyzed using a generalized linear model (McCullagh & Nelder, 1989) implemented within the statistical software package GenStat (Payne et al., 2009). Before applying the model, we estimated expected counts for samples that were recorded as uncountable due to high densities of cavity forming bacteria (Czajkowski et al., 2010a). Briefly, the value 10^6 cfu g^{-1} was taken as a likely cut-off level for the censored observations and bacterial

density estimation was based on normality assumptions (Schmee & Hahn, 1979). Count data were modelled using a standard Poisson regression method (Cameron & Trivedi, 1998). In real count data, the underlying assumption of equality of mean and variance was rarely met. Most count data had variance greater than the mean or were Poisson overdispersed. The negative binomial distribution was the natural choice to model that overdispersed count data (Hilbe, 2007). Type of tissue effects were considered to be significant at $P \leq 0.05$ and pair-wise differences were obtained using the t-test.

RESULTS

Symptom development

Fifteen days after stem inoculation, first symptoms started to develop in inoculated plants. At 30 d.p.i. all stem-inoculated plants showed wilting and chlorosis of leaves. In 90% of plants aerial stem rot and/or a typical blackening of the stem base were observed. Trans-sections of stems of symptomatic plants showed degradation of vascular tissue and pith above and below the inoculation point resulting in a hollowing of stems and browning of the internal stem tissue of 95% of screened plants (Fig. 1).

In leaf-inoculated plants, the first symptoms appeared at 7 d.p.i.; all inoculated leaves showed chlorosis and wilting. In the following two weeks these symptoms were also found frequently in adjacent leaves (Fig. 2). We did not observe symptoms on or in stems during the period of 30 days (data not shown).

Dilution plating of stem-inoculated plants

Thirty days after inoculation, plant material was disinfected, extracted and pour-plated in PT to determine the percentage of infected plant tissues and to quantify populations of *Dickeya* sp. IPO2254. GFP-tagged cells were found at the point of inoculation of all plants. GFP-tagged cells were found in 90% of the stem bases, inside 80% of the roots in 55% of the stolons and in 24% of progeny tubers (Tab. 1A). We did not detect GFP-tagged *Dickeya* spp. in any water-inoculated control plants.

At the inoculation point, the highest estimated densities of $10^6 – 10^7$ cfu g^{-1} of GFP-tagged *Dickeya* sp. were found (Fig. 3A). Densities at the stem base were still relatively high (10^4 cfu g^{-1}), but relatively low (<100 cfu g^{-1}) in roots, stolons

and progeny tubers. Bacterial densities in stolons and progeny tubers varied largely per sample.

In 10 percent of stem-inoculated plants no systemic colonization of GFP-tagged *Dickeya* sp. IPO2254 was found. In those plants, disease symptoms (rotting and browning of the tissues) and presence of bacteria were restricted to the inoculation site.

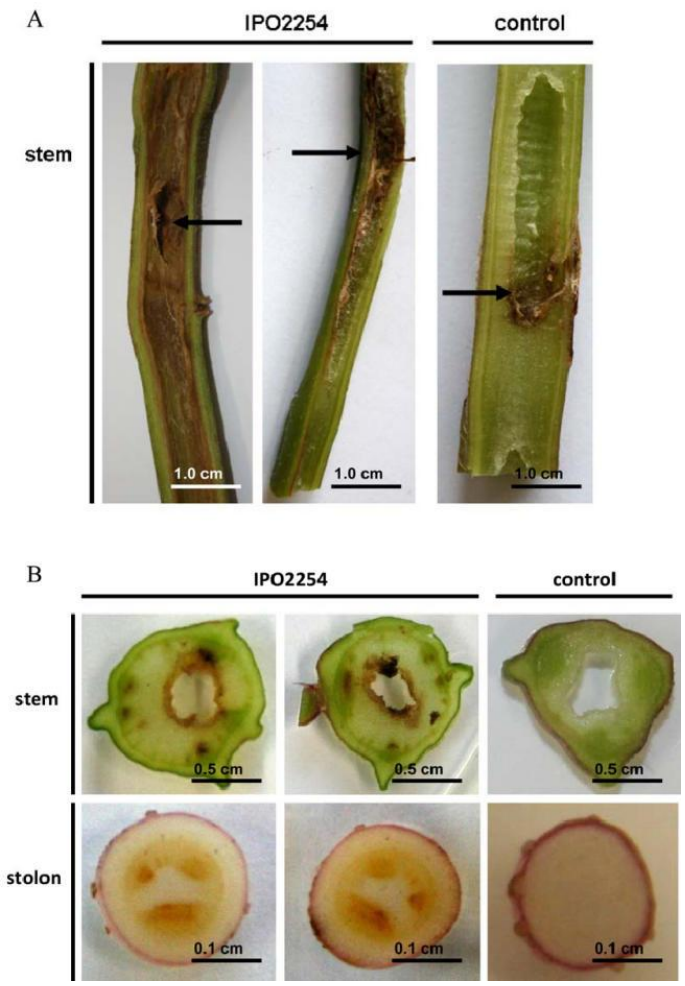

Figure 1. Symptoms inside stems and stolons 30 days after stem inoculation.
(A) Sections of stems taken around the inoculation point (inoculation point marked with an arrow). Browning and blackening of the vascular- and pith tissue of stems observed above and below the inoculation point.

(B) – Trans-sections of stems and stolons. Inoculated plants showing necroses (discoloration) of pith tissue. In both stems and stolons of inoculated plants, a necrosis of vascular tissue was found, resulting in light and dark brown lesions. Trans-sections of stems were taken 5 cm below the inoculation point. Control plants (water inoculated) were free of disease symptoms.
Water inoculated control plants showed only browning of pith tissue at the inoculation side.

Table 1. Plant samples analyzed by dilution plating for the presence of GFP-tagged *Dickeya* sp. IPO2254, 30 days after stem or leaf inoculation

A. stem inoculation

plant part	nr. tested	nr. positive	% positive
inoculation point [a]	20	20	100
stem base [a]	20	18	90
roots [b]	20	16	80
stolons [c]	60	33	55
progeny tubers [d]	120	32	24

B. leaf inoculation

plant part	nr. tested	nr. positive	% positive
leaves [e]	16	12	75
petioles [e]	16	14	88
axils [e]	16	10	63
stems [e]	16	4	25
stem bases [e]	16	0	0
roots [b]	16	0	0
stolons [c]	48	0	0
progeny tubers [d]	96	0	0

[a] composite sample of 3 stem parts analyzed per plant
[b] total root system analyzed per plant
[c] three stolons individually analyzed per plant
[d] six progeny tubers individually analyzed per plant
[e] composite sample of 7 plant parts analyzed per plant

Dilution plating of leaf-inoculation plants

Leaf-inoculated plant samples were also examined 30 d.p.i. by pour dilution plating (Fig. 3B). The densities of GFP-tagged *Dickeya* sp. found in inoculated leaves were generally low. The estimated density in the inoculated leaves was 1000 cfu g^{-1}, in leaf petioles and axils 100 cfu g^{-1} and in stems only 10 cfu g^{-1} (Fig. 3B). On average, GFP-tagged *Dickeya* sp. was found in 75% of the inoculated leaves, in 88% of the leaf petioles, 63% in the axils and 25% of stems directly attached to the axils of inoculated leaves (Tab. 1B). We did not detect GFP-tagged *Dickeya* sp. in stem segments collected at ground level (stem base), inside roots, stolons and progeny tubers. Seventy percent of the leaf-inoculated plants exhibited disease symptoms at the inoculation point. Only low populations of GFP-tagged *Dickeya* sp. were observed in axils of leaves (approx. 14 cfu g^{-1}) and main stems (approx. 1.5 cfu g^{-1}) (Fig. 3B). However, in 25 % of the tested plants GFP-tagged bacteria were isolated from stem fragments taken near the inoculated compound leaves, but not from the stem bases or underground part of the plants (roots, stolons and progeny tubers). The densities of GFP-tagged *Dickeya* sp. in inoculated leaves, in leaf petioles and in axils were not statistically different (Fig. 3B).

Microscopic observations of infected plant tissues

To acquire information in which plant tissue *Dickeya* sp. IPO2254 is present, different plant parts were analyzed with an epifluorescence stereomicroscope at low magnifications (2.5 to 10 times) and with a confocal laser scanning microscope (CLSM) at a magnification ranging from 640 to 1000 times.

Epifluorescence stereomicroscopy (ESM)

In stem-inoculated plants, a GFP signal was found in the vascular tissue of stems, stem bases and stolons. In progeny tubers, the signal was observed in the vascular ring of the stolon end. In roots the signal was detected in pith tissue (Fig. 4).
In leaf-inoculated plants, a GFP signal was detected in the main vein of the leaves and inside petioles (Fig. 5), but not inside the stem basis (data not shown). We did not detect GFP-tagged *Dickeya* sp. in any water-inoculated control plant.

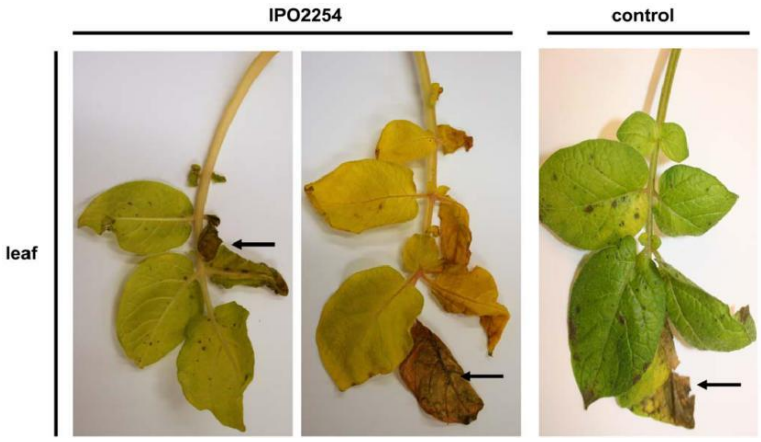

Figure 2. Symptom expression on leaves inoculated with *Dickeya* sp. IPO2254 by abrasion with carborundum powder. The inoculated leaves are indicated with black arrows. After 30 days, maceration, rotting and necrosis of leaf tissue was observed in plants inoculated with *Dickeya* sp. IPO2254, whereas leaves of water-inoculated control plants only showed chlorosis and slight necrosis due to mechanical damage.

Confocal laser scanning microscopy (CLSM)

Detailed studies with CLSM on the localization of GFP-tagged *Dickeya* sp. showed that 30 days post stem inoculation, bacteria were mainly present inside xylem vessels and between protoxylem cells of stems, stem bases and stolons. In roots of stem-inoculated plants, GFP-tagged bacteria were found inside parenchyma cells of pith tissue both inter- and intracellularly. In progeny tubers, GFP-tagged bacteria were found inside xylem and between xylem vessels of the stolon ends (Fig. 6). We did not do the CLSM studies on material from leaf-inoculated plants.

Characterization of GFP expressing bacteria from infected plant tissue

Plant samples with a typical GFP signal observed under the epifluorescence microscope 30 d.p.i. were collected, extracted and plated on TSA or CVP. From stem-inoculated plants, 10 isolates were selected (2 from inoculation points, 2 from stems 10 cm below the inoculation point, 2 from roots, 2 from stolons and 2 from progeny tubers). From leaf-inoculated plants 8 isolates were selected (2 from the inoculated leaves, 2 from petioles, 2 from the axils and 2 from stems). All isolates produced typical cavities on CVP, were green fluorescent in ESM and were positive in a *Dickeya* sp. specific PCR, showing the expected 420 bp PCR product

(data not shown). Rep-PCR analyses showed that all fingerprints of isolates from stem- and leaf-inoculated plants were identical to strain *Dickeya* sp. IPO2254 and the parental wild-type strain *Dickeya* sp. IPO2222 used for GFP-tagging (data not shown).

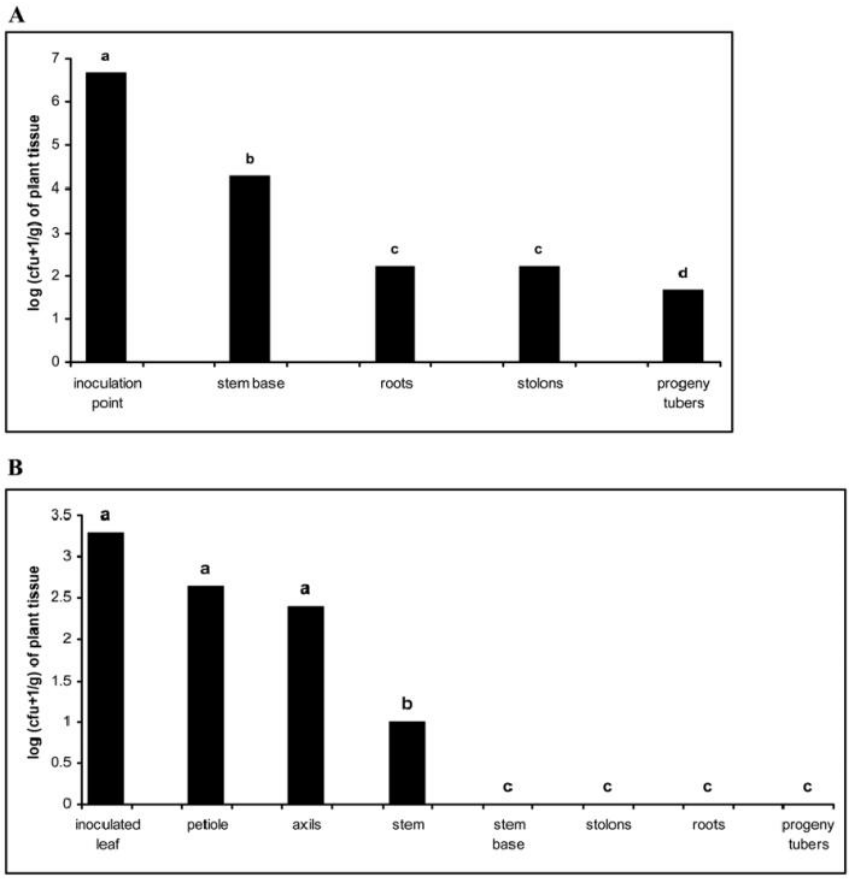

Figure 3. Densities of *Dickeya* sp. IPO2254 (in log cfu+1 g^{-1} of plant tissue) in stem- inoculated plants (A) and in leaf-inoculated plants (B) 30 days post inoculation as determined by pour plating. Predicted values are averages from in total 20 (A) and 16 individual plants (B) from two independent experiments. Values followed by identical characters are not significantly different (P=0.05)

DISCUSSION

This study showed that inoculation of *Dickeya* sp. biovar 3 into the vascular system of stems can result in downward translocation and colonization of

underground plant parts including roots, stolons and progeny tubers. Stem inoculation resulted in typical blackleg symptoms in the majority of infected plants. It was already known that *Dickeya* spp. could move upward in the vascular system inside plant tissue and cause systemic colonization of plants (Perombelon & Hyman, 1988) as shown for stab-inoculated root cuttings of *Chrysanthemum morifolium* (Pennypacker et al., 1981, Perombelon, 2002) and naturally infected tomato plants (Alivizatos, 1985). The downward movement of this bacterium, however, has not been described before.

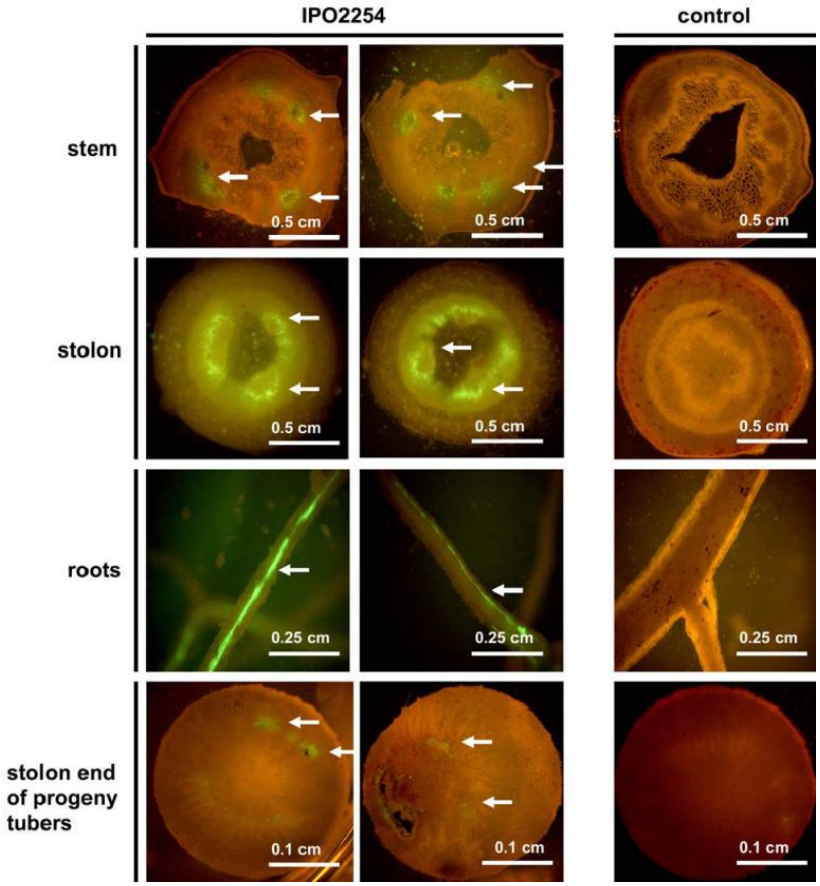

Figure 4. Colonization of potato stems, stolons, roots and progeny tubers with GFP-tagged *Dickeya* sp. IPO 2254 at 30 days post stem inoculation analyzed with epifluorescence stereo microscopy. Plant parts, embedded in PT agar for 24 h at 28 °C were screened for the presence of a GFP signal. The GFP signal was found in vascular tissue of stems, stolons and stolon end of progeny tubers, and in pith tissue of roots (white arrows). Control – water inoculated plants.

The downward translocation of *Dickeya* sp. inside potato plants via xylem vessels is in agreement with our previous observations in which, after soil inoculation, GFP-tagged *Dickeya* sp. was recovered from the xylem of symptomless stems (Czajkowski et al., 2010a). There are also reports of other plant pathogenic bacteria that use xylem vessels to systemically colonize plant tissue. *Xanthomonas campestris* pv. *vitians* is able to move inside xylem vessels in stab-inoculated lettuce plants (Barak et al., 2002) and *Pseudomonas syringe* pv. *lachrymans* is able to colonize cucumber seedlings *via* the xylem vessels (Kritzman & Zutra, 1983).

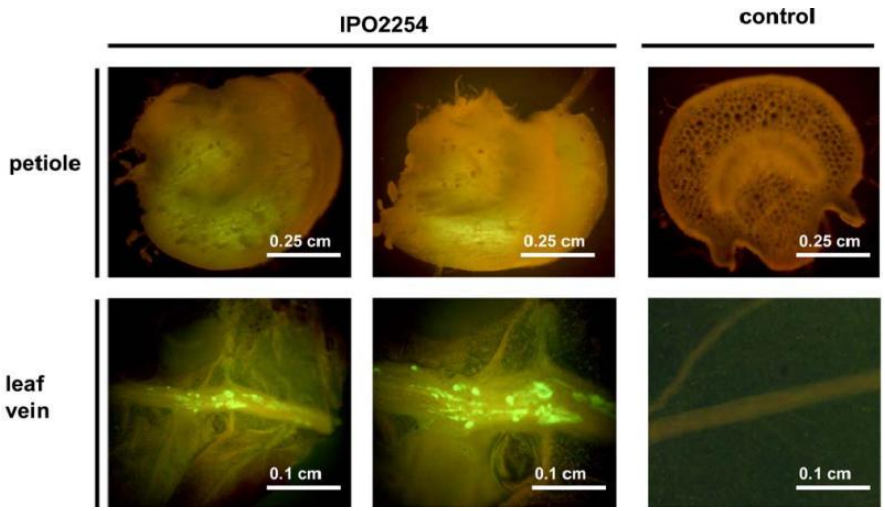

Figure 5. Colonization of potato leaves by GFP-tagged *Dickeya* sp. IPO 2254 at 30 days post leaf inoculation analyzed with epifluorescence stereo microscopy. Plant parts were sterilized and embedded in PT agar for 24 h. Control: water inoculated plants. GFP signal was found inside main veins and inside petioles.

In theory, there are three mechanisms by which bacteria are transported downward in xylem vessels: via degradation and embolism of xylem vessels followed by colonization of the xylem elements (Nelson & Dickey, 1970), via reverse water transport in xylem during dark periods (Tatter & Tatter, 1999) and upstream via pilus-driven twitching motility (Bove & Garnier, 2002, Meng et al., 2005). Degradation of xylem vessels leads to creation of large horizontal and vertical spaces filled with rotten tissue and bacterial slime that can be easily colonized. In general, degraded plant tissue near the infection point allows bacteria to move only short distances in infected tissue. The movement of *Xanthomonas*

campestris pv. *vitians* in lettuce stems, for example, was a result of mechanical damage of stems and embolism of the xylem (Barak et al., 2002) and the bacteria were only detected up to 2 cm below the inoculation point.

Water movement in xylem vessels drags the bacteria rapidly to distant plant parts. Movement of sap in xylem vessels is dependent upon evaporation of water from the surface of mesophyll cells (transpiration) in leaves thereby pulling water up from the soil via the root system. Therefore, the directional movement of water is usually from roots to leaves (Rand, 1983). A reverse (downward) water movement in xylem from leaves to roots only occurs when xylem sap is subjected to negative hydraulic pressure as a result of low water uptake from roots and reduced leaf evaporation. This is consistent with the cohesion-tension theory (Tatter & Tatter, 1999, Tyree, 1997). *Erwinia amylovora*, for example, was recovered from the roots of apple seedlings after stem inoculation due to negative hydraulic pressure of the plant sap in xylem vessels (Bogs et al., 1998).

Upstream migration in xylem vessels via twitching motility has been described for the non-motile plant pathogen *Xylella fastidiosa* in grapevine (Meng et al., 2005). There is strong evidence that *Ralstonia solanacearum* can also move in xylem vessels of tomato plants via twitching motility (Liu et al., 2006). With *D. dadanti*, however, twitching motility could not be detected (El Hassounti et al., 1999). Therefore we consider it unlikely that the *Dickeya* sp. biovar 3 strain can move via this form of migration.

Leaf-inoculated plants only harbored low *Dickeya* sp. populations in stems 30 days after inoculation, indicating that the risk for translocation from infected leaves to progeny tubers is small. Downward vascular translocation from infected leaves may be limited to the low densities of *Dickeya* sp. present in leaf tissue, but also by the stem-leaf junction physiology. Many vessels end in these junctions and those nearby, contain inter-vessel pit membranes (Fisher et al., 1993). These membranes are part of the defense mechanism of the plant to protect vascular tissue from microorganisms that can infect leaves (Chatelet et al., 2006).

The risks of systemic infections after contamination of leaves will not only depend on the efficiency of translocation of bacteria but also in the ability of *Dickeya* sp. to survive the phyllosphere. The phyllosphere is recognized as a harsh environment in which bacteria most of the time are subjected to nutrient limitation, desiccation, direct UV and visible radiation stress (Burrage, 1976, Gouesbet et al., 1995, Sundin, 2002). It has been reported that *Dickeya* spp. are sensitive to drought and direct UV radiation (Sundin, 2002), therefore they are not expected to survive for long periods on the leaf surface.

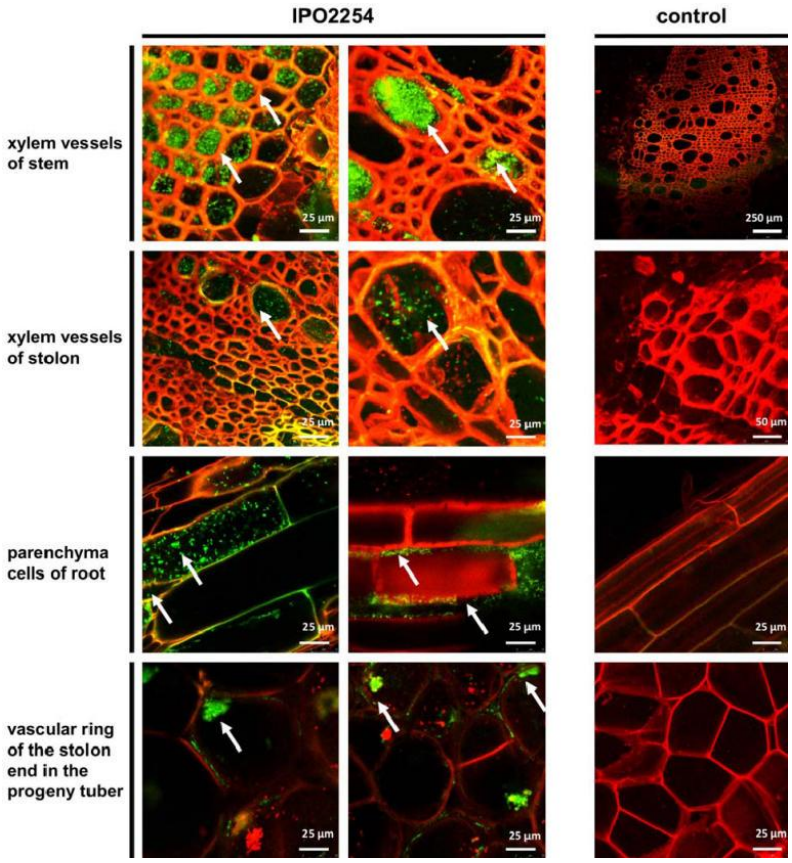

Figure 6. Colonization of xylem vessels of potato stems, stolons, parenchyma cells of roots and stolon end containing xylem elements of progeny tubers by GFP-tagged *Dickeya* sp. IPO2254 at 30 days post stem inoculation analyzed with confocal laser scanning microscopy. Plant parts were embedded in PT agar for 24 h prior to analyses. Plant cells were counter-stained with the red-fluorescent dye propidium iodide. Bacteria were found inside xylem vessels in stems and stolons, inside and between parenchyma cells in roots and inside xylem cells of the vascular ring of the stolon end in progeny tubers (white arrows).

Our results suggest that stem infection during cultivation practices can result in infected progeny due to the internal movement of *Dickeya* spp. to underground plant parts. Infection of stems resulting in aerial stem rot under wet conditions occurs via the use of contaminated machines, pest insects or via humans and animals (Charkowsky, 2006, Perombelon & Kelman, 1980).

The risks of systemic colonization of potato plants from aerial stem infections will depend on various factors such as potato cultivar, *Dickeya* species, initial inoculum, air temperature and humidity. The conditions in our experiments

were highly favorable for disease development. Tubers of cultivar Kondor were used, which are highly susceptible to *Dickeya* spp. (Henk Velvis, personal communication). High bacterial densities of a strain belonging to a new genetic clade of biovar 3 *Dickeya* sp. were used, which appears to be highly virulent (unpublished results). Also the high temperature and high relative humidity in the greenhouse favored disease development. Field studies are required to further determine in practice the risks of infection of progeny tubers via haulm infections.

ACKNOWLEDGEMENTS

The authors would like to thank I. Yedidia (Department of Ornamental Horticulture, ARO, Volcani Center, Israel) for providing the pPROBE-AT-*gfp* plasmid. Thanks are indebted to M.C.M. Pérombelon (SCRI, UK) and Mrs. L.J. Hyman (ex SCRI, UK) for their comments on the manuscript and their editorial work. The project was financed by the Dutch Ministry of Agriculture, Nature and Food Quality (Research programme BO-06) and by STW Foundation (Technologiestichting STW) (grant nr. 10306 "Curing seed potato from blackleg-causing bacteria")

Chapter 6

Characterization of bacterial isolates from rotting potato tuber tissue showing antagonism to *Dickeya* sp. biovar 3 in vitro and *in planta*

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Plant Pathology (2011) (DOI: 10.1111/j.1365-3059.2011.02486.x)

ABSTRACT

Possibilities for biocontrol of biovar 3 *Dickeya* sp. in potato were investigated, using bacteria from rotting potato tissue isolated by dilution plating on non-selective agar media. In a plate assay, 649 isolates were screened for antibiosis against *Dickeya* sp. IPO2222 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 control of the *Dickeya* sp. Strains able to reduce rotting of potato tuber tissue by at least 50% of the control were selected. Strains were characterized by 16S rDNA analysis as *Bacillus*, *Pseudomonas*, *Rhodococcus*, *Serratia*, *Obesumbacterium* and *Lysinibacillus* genera. 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, 4 selected antagonists on the base of the *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%. The potential use of *S. plymuthica* strain A30 for the bio-control of *Dickeya* sp. is discussed.

INTRODUCTION

Soft rot and blackleg diseases of potato caused by pectinolytic bacteria belonging to *Pectobacterium* and *Dickeya* spp. are a continuous threat to (seed) potato production worldwide. Potato plants and tubers are affected by the presence of pectinolytic bacterial pathogens in virtually all phases of tuber production including storage. In Western and Northern Europe, in particular *Dickeya* spp. cause increasing economic losses in seed potato production, generally due to declassification and rejection of seed lots (Netherlands General Inspection Service for Agricultural Seeds and Seed Potatoes, personal communication). *Dickeya* spp. cause pre-emergence seed piece decay, blackleg and aerial stem rot, and soft rot of progeny tubers (Perombelon, 2002). Increased losses in seed potato production in

Europe resulting from *Dickeya* spp. infection are related to the recent occurrence and spread of a new *Dickeya* sp. genetic clade belonging to biovar 3 which probably constitutes a new *Dickeya* species (Tsror et al., 2008).

Potato (seed) tubers are the primary source of soft rot and blackleg inoculum (Perombelon, 1974). Production of pathogen-free seed tuber lots or eradication of the existing bacteria in seed lots is consequently of major interest. Selection for blackleg and soft rot resistant potato cultivars has not resulted in seed lots completely free from *Pectobacterium* and *Dickeya* spp. bacteria. Soft rot and blackleg control therefore, is presently based on an integrated strategy which includes the use of pathogen-free initial propagation material and practices which avoid the wounding of tubers and plants, the occurrence of oxygen depletion by free water on tuber surface and smearing and dissemination of the pathogens within or between seed lots by mechanical equipment (Perombelon, 2002).

Simultaneously, the use of physical (e. g. hot water treatment) and chemical disinfection procedures for controlling blackleg and soft rot have resulted in a reduction, but not in an elimination of the bacterial inoculum (Perombelon & Salmond, 1995). During chemical and/or physical sterilization bacteria present on or in the periderm may be destroyed, but not those present inside the tubers. It has already been reported that the highest densities of pectinolytic *Pectobacterium* and *Dickeya* spp. are located in the vascular system at the stolon end of tubers (Czajkowski et al., 2009). Inoculum present inside tuber tissue will not be affected by superficial sterilization procedures.

Bacterial antagonists can be considered as an alternative to chemical and physical control strategies to reduce *Dickeya* spp. populations in plant vascular tissue and in tubers during storage. Successful, commercially available bio-control agents were, however only developed for a limited number of bacterial phytopathogens. *Agrobacterium radiobacter* K84 was developed to control of *A. tumefaciens* the causative agent of grown gall (Vicedo et al., 1993). A product base on *Erwinia herbicola* (*Pantoea agglomerans*) successfully controlled *E. amylovora*, responsible for fire blight in apple and pear (Stockwell et al., 1998).

Several publications describe promising results for biocontrol of bacterial pathogens in potato. For *Clavibacter michiganensis* subsp. *sepedonicus*, the causal agent of bacterial ring rot, in field experiments, reasonable levels of control have been obtained with *Arthrobacter histidinolovorans* and *Serratia* sp. in field experiments. Both strains reduced tuber infections by 35-70% and infection in plants by 60-80% (Gamard & De Boer, 1995). Wilt symptoms caused by *Ralstonia solanacearum* could be reduced by up to 83 and 73% using *Bacillus subtilis* and *Pseudomonas fluorescens* strains respectively (Aliye et al., 2008). *Streptomyces*

scabies, causing common scab, was antagonized by a *Bacillus* sp. strain in pot assay experiments and reduced infections in potato tubers by up to 35% (Han et al., 2005). Fluorescent *Pseudomonas* spp., applied to tubers, reduced populations of blackleg and soft rot bacteria on potato roots and inside progeny tubers. They were also effective in controlling postharvest soft rot on potato (Kloepper, 1983). Finally, *Bacillus subtilis* producing antibiotics active against a broad spectrum of bacteria, showed a high level of control towards *Pectobacterium* spp. in *vitro* and on potato tubers (Sharga & Lyon, 1998). None of the bacterial bio-control agents, however have been used in commercial application.

Only limited attempts have been made for the biocontrol of *Dickeya* spp. Various Gram-positive and Gram-negative bacteria isolated from different hosts and environments producing antibiotics against *Dickeya* spp. or siderophores competing with *Dickeya* spp. for iron have been characterized (Kastelein et al., 1999, Jafra et al., 2006b). Bacteria isolated from the potato tuber surface (Kastelein et al., 1999) or the potato rhizosphere (Jafra et al., 2006b) proved useful in the protection of tuber tissue from *P. atrosepticum* and *Dickeya* spp. However, to date, these bacteria have not been tested for the control of *Dickeya* spp. in potato plants.

The purpose of this study was to select and characterize bacterial biocontrol agents against the recently described genetic clade of *Dickeya* sp. biovar 3 in potato. Bacteria were isolated from rotten potato tuber tissue to obtain isolates able to compete with *Dickeya* spp. in this harsh environment. Selection of antagonists involved the antibiotic production, the ability to compete for iron with the pathogen by the production of siderophores, the production of (antibacterial) surfactants and the ability to interfere with quorum sensing and the ability to grow under different environmental conditions. The selection also included greenhouse experiments with treated tubers.

MATERIALS AND METHODS

Bacterial strains and media used for cultivation

Bacterial strains isolated and used in this study are listed in Table 1. Tryptic soya agar (TSA) (Oxoid, Badhovoevendorp, The Netherlands), King's B agar (Fluka, Zwijndrecht, The Netherlands), nutrient agar (NA) (Difco, Breda, The Netherlands) and R2A (Difco) agar media supplemented with 200 µg ml^{-1} of cycloheximide (Sigma) to prevent fungal growth were used to isolate bacteria from rotting tuber tissue. NA or TSA and/or nutrient broth (NB) (Difco) were used for

bacteria maintenance. Bacteria were grown on or in media for 24 - 48 h at 28 °C. Growth of bacteria in liquid media was done by shaking at 200 rpm during incubation. Biovar 3 type strain, *Dickeya* sp. IPO2222 (Tsror et al., 2008) was grown on TSA or in NB and *Dickeya* sp. IPO2254 pPROBE-AT-*gfp* (Czajkowski et al., 2010a) was grown on TSA or in NB supplemented with 150 µg ml^{-1} of ampicillin.

Rotting of potato tubers and isolation of potato endophytes

Dickeya spp.-free minitubers of cv. Kondor, and seed tubers of cv. Arcade, Agria, Konsul and Kondor were used to isolate bacteria from rotting potato tissue. To isolate bacterial endophytes indigenously present in potato tubers, tubers were sterilized by two times 10 min incubation in 70% ethanol followed by washing in running tap water. Surface-sterilized tubers were firstly mechanically wounded and subsequently enclosed in Saran Wrap (foil) and kept for 5 to 10 days at 28 °C. Isolation of bacteria able to colonize rotting tuber tissue from soil was done using tubers buried either in loamy sand, a rich soil collected in the region of Wageningen, NL (S1) (Garbeva et al., 2003), or in potting compost (S2) (basis potgrond nr. 4, Hortmea group, The Netherlands) and kept for 5 to 10 days under low-oxygen conditions obtained by immersion of pots in water to 80% its capacity and 90% relative humidity at 28 °C in closed humid boxes. Rotting tubers in soil were washed with tap water before processing. Approximately 20 g of the rotting potato tissue, collected if possible from the inside of each tuber, was resuspended in 50 ml of a quarter strength (1/4) Ringer's buffer (Merck) supplemented with 0.02% diethyldithiocarbamic acid (DIECA) (Acros Organics) as an antioxidant. Rotting tuber suspensions were incubated at room temperature for 1 h with shaking (200 rpm). Serial dilutions of the rotting tissue extracts were made in a Ringer's buffer and 100 µl of 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated on King's B (Fluka), R2A (Difco), TSA (Oxoid) and NA (Difco) plates supplemented with 200 µg ml^{-1} cycloheximide (Sigma, Zwijndrecht, The Netherlands). Plates were incubated at 28 °C for 24 – 48 h. Single bacterial colonies of different colony morphologies were collected from plates inoculated with material from different potato tubers and different treatments for further analyses. Bacteria were grown to pure cultures on TSA or NA and stored at -80 °C in NB/glycerol (60%/40%) stocks.

Pre-screening of candidate antagonists against Dickeya sp. *IPO2222*

Pure cultures of collected strains were tested for the ability to produce antibiotics against *Dickeya* sp. IPO2222. or siderophores.

The availability of iron ions is crucial during plant infection. In an iron poor environment the virulence of *Dickeya* spp. is seriously reduced (Expert, 1999). The presence of bacteria able to produce strong siderophores and sequester iron from the environment will theoretically reduce disease development by *Dickeya* sp..

In each case, two independent replications were made for each tested strain and the experiment was independently repeated one time. Results from both experiments were averaged.

Production of antibiotics

Production of antibiotics was tested in an overlay assay with *Dickeya* sp. IPO2222 as indicator strain. 200 µl of an overnight culture of *Dickeya* sp. (ca. 10^9 cfu ml^{-1}) in NB was mixed with 20 ml of soft top agar (NB supplemented with 0.7% agar) at 45-50 °C and poured onto square (15 x 15 cm) TSA plates. Once the agar had solidified, 2.5 µl of an overnight culture of the antagonist grown in NB (ca. 10^9 cfu ml^{-1}) was spotted on the surface of the agar plate. Plates were incubated for 24 – 48 h at 28 °C. Isolates inhibiting growth of *Dickeya* sp. IPO2222 and causing a clear 'halo' around their colonies were collected for further analyses.

Siderophore production assay

A CAS plate assay was used to evaluate siderophore production by test isolates (Schwyn & Neilands, 1997). 2.5 µl of overnight isolate culture in NB (approx. 10^9 cfu ml^{-1}) was spotted on the surface of the CAS agar plate. Plates were incubated for 24 – 48 h at 28 °C. Isolates producing an orange/pink halo around colonies, indicating siderophore production, were collected for further analyses.

16S rDNA sequence analyses

To identify the bacterial isolates, a 16S rDNA fragment between 968 and 1401 bp (numbering based on the *Escherichia coli* genome) was sequenced with primers

F968 (5'AACGCGAAGAACCTTAC 3') and R1401 (5'CGGTGTGTACAAGGCCCGGGAACG3') in both directions. A colony PCR procedure was used to amplify DNA. Individual colonies were collected from TSA plates using a sterile toothpick and suspended in 50 µl of 5 mM NaOH (Sigma). Suspensions were boiled at 95 °C for 5 min and kept at 4 °C. One µl of the cell lysate was used per PCR reaction. PCR products were purified with the PCR purification kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Ijssel, The Netherlands). DNA sequences were compared with available sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) using nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) for nucleotides (blastn) alignments accessed from the website (http://www.ncbi.nlm.nih.gov/BLAST/).

Determination of species in risk groups

The DSMZ (German Collection of Micro-organisms and Cell Cultures) (http://www.dsmz.de/) data base was used to classify bacterial isolates into risk categories on the basis of their ability to cause disease in humans (WHO – World Health Organization http://www.who.int/en/).

Screening for antagonism in a potato slice assay

A potato slice assay was used to evaluate the ability of selected isolates to reduce potato tissue maceration by *Dickeya* sp. IPO2222. Bacteria were centrifuged (5 min, 6000 x g) and washed twice with 1/4 Ringer's buffer. Densities of test isolates were adjusted to ca. 10^8 cfu ml^{-1} and *Dickeya* sp. to ca. 10^6 cfu ml^{-1} with sterile water. Potato tubers of cv. Agria (Agrico, The Netherlands) were rinsed with tap water, surface-sterilized with 70% ethanol for 10 min, rinsed with tap water and dried with tissue paper. Tubers were cut into 0.7 cm transverse slices using a sterile knife. Three wells (5 x 5 x 5 mm) per slice were made using a sterile cork borer and were filled with 50 µl suspension containing 10^8 cfu ml^{-1} of test isolate and 10^6 cfu ml^{-1} of *Dickeya* sp. For each test strain, 3 potato slices derived from three different tubers were used. Negative control was 50 µl of sterile water instead of bacterial suspension. The effect of test isolates on potato tissue was measured by comparing ratio of the average diameter of rotting potato tissue around wells subsequently-inoculated with *Dickeya* sp. and an antagonist with the average diameter of rotting potato tissue around wells of the positive control (*Dickeya* sp.

only). Two replications were made for each tested strain and the experiment was independently repeated one time. Results from both experiments were averaged.

Characterization of selected isolates for features potentially involved in antagonism

Isolates showing at least 50% decrease of rotting ability of *Dickeya* sp. in the potato slice assay were screened for motility, AHLs production and degradation, auxin production with and without supplementation of L-tryptophan, biosurfactant production, growth at pH 4.0 and pH 10.0 and at 10 °C in anaerobic and aerobic conditions and for pectinolytic enzyme production.

Motility is an important feature in active colonization of plant surfaces (e. g. roots, leaves), migration in soil and in establishment of high bacterial populations on plants (Andersen et al., 2003). Motile antagonistic bacteria will have an advantage in competition with motile *Dickeya* spp. in the plant and soil environment.

AHL based quorum sensing mechanism is involved in regulation of different physiological processes in bacteria including production of pathogenicity factors. The ability to interfere with this mechanism by degradation of signal molecules (so-called quorum quenching) proved useful in the biocontrol of plant pathogenic bacteria (Uroz et al., 2003). Due to the fact that the bioassays employed in this study for testing the AHLs degradation cannot be used if a strain produce own AHLs, firstly the AHLs production in the selected strains was tested. All the isolates able to produce own signal molecules were excluded from AHLs degradation assay.

Antagonists were tested for their ability to produce auxins. Microbial plant hormones many contribute to plant development and fitness (Leveau, 2010).

Biosurfactants are surface active substances decreasing the surface tension acting as dispersants or detergents. In the bio-control, the main mode of surfactant action is a destabilization of the cell membranes and lysis of the cells. Biosurfactants can also induced plant systemic resistance towards plant pathogens (Tran et al., 2007).

For the field application of a bio-control agent important is that the isolate can survive under different environmental conditions. Potatoes may be cultivated using most types of soil from sandy rich to clay poor that largely differ in pH and organic matter and salt content. The optimal therefore would be if the antagonist was able to persistent in different pH and temperature conditions.

Competition for the same carbon and nitrogen sources released during degradation of plant tissue may be advantageous in biological control. Although there is a risk for secondary infections and enhanced tuber decay.

For each assay, two independent replications were made for each tested strain and the experiment was independently repeated one time. The results from both experiments were averaged.

Motility assay

The motility of selected isolates was tested using a motility agar assay (NB supplemented with 0.3% agar) (S. Jafra, University of Gdansk, Gdansk, Poland, personal communication). Briefly, 2.5 µl of an overnight culture of the test isolate (10^9 cfu ml^{-1}) grown in NB and diluted 10 times in NB (ca. 10^8 cfu ml^{-1}) was spotted on the surface of the motility agar plates and incubated for 24 h at 28 °C. The colony diameter after incubation time was measured. Bacterial motility was assessed using the following indexation based on the diameter of the colony: "-"below 5 mm, "+" 6 – 15 mm, "+ +" 16 – 30 mm, "+ + +" 31 – 50 mm, "+ + + +" above 51 mm.

AHLs production

Production of AHLs was tested using two AHL reporter strains; *Chromobacterium violaceum* CVO26 and *Escherichia coli* JB534 (Andersen et al., 2001). These strains produce a purple pigment (CVO26) or GFP (JB534) in the presence of exogenous AHLs. Overnight culture of the reporter strains grown on agar plates were streaked on the surface of TSA plates in two lines at a distance of 3 cm using a sterile 1 µl inoculation loop. Thereafter, 2.5 µl of an overnight culture of the test isolate grown in NB was spotted at 0.5 cm distance from reporter strain lines. Prepared plates were incubated at 28 °C for 24-48 h and visually inspected for the presence of purple pigment (CVO26) and for GFP fluorescence (JB534). The following indexation was used: "+/+" for strains positive for both reporter strains, "+/-" for strains positive for one reporter strain, and "-/-" for strains negative for both reporter strains.

AHLs degradation

To test the ability of bacterial strains to degrade AHLs, a fast screening method was used with AHLs reporter strain *Escherichia coli* JB534. AHL degradation was studied for four synthetic signal molecules: hexonoyl-L-homoserine lactone (HHL) (Sigma, Zwijndrecht, The Netherlands), 3-oxo-hexonoyl-L-homoserine lactone

(OHHL) (Sigma), octanoyl-L-homoserine lactone (OHL) (Sigma) and 3-oxo-octanoyl-L-homoserine lactone (OOHL) (Sigma). 50 µl of 10 µg ml^{-1} of the individual AHL and 20 µl of the test isolate (10^9 cfu ml^{-1} in NB) was added to 100 µl of NB buffered to pH 6.4 with 1M MOPS (Sigma) in the well of a 96-wells microtitre plate. Plates were incubated for 4 h at 28 °C. Bacteria were killed under UV light for 30 min and wells were filled with 100 µl of a suspension of *E. coli* JB534 (10^8 cfu ml^{-1} in NB). Plates were then incubated at 37 °C for 12 h and screened for GFP fluorescence using the Fluoroscan Ascent FL (Thermo Fisher Scientific, Roskilde, Denmark) with the filter sets 495 nm (excitation) and 530 nm (emission)

Auxin production

To screen for L-tryptophan (L-Trp)-independent or L-Trp-dependent auxin production, test isolates were grown overnight in NB or in NB supplemented with 0.5% L-tryptophan (Sigma) in shaken cultures respectively. Cultures were centrifuged (8000 x g, 10 min) and 3 ml of the clear supernatant was collected per sample. Auxin production was measured as IAA (indole-3-acetic acid) equivalents: 3 ml of bacterial supernatant were mixed with 2 ml of freshly prepared Salkowski reagent (2ml of 0.5M $FeCl_3$ + 98 ml 35% $HClO_4$) A standard curve of IAA was made by preparing solutions of 1 µg ml^{-1} to 30 µg ml^{-1} IAA (Sigma) in NB. As a negative control, non- auxin producing strain *Escherichia coli* strain DH5α (Invitrogen, Breda, The Netherlands), which does not produce auxins, was used. For color development, solutions were left at room temperature for 30 min. The intensity of the color was measured spectrophotometrically at 535 nm wavelength. The following indexation was used: '-' for isolates not producing auxins, "±" auxin concentration 0.1 – 0.4 µg ml^{-1}, '+' 0.5 – 1.0 µg ml^{-1}, '++' 1.1 – 2.0 µg ml^{-1}, '+++' 2.1 – 5.0 µg ml^{-1}, '++++' 5.1 – 15.0 µg ml^{-1}, and '+++++' > 15.1 µg ml^{-1} of auxins.

Biosurfactant production

Biosurfactant production was investigated by using a modified oil spreading method (Youssef et al., 2004) (S. Jafra, University of Gdansk, Gdansk, Poland, personal communication). Test isolates were grown overnight in NB at 28 °C. Cultures were centrifuged for 20 min at 8000 x g to remove the bacteria. A 40 µl of mineral oil (BioRad, Veenendaal, The Netherlands) was spotted on the surface of the 30 ml demineralized water and 5 µl of the supernatant or sterilized water

(control) was placed on the top of the oil drop. The diameter of the circle created by oil is proportional to the biosurfactant concentration. The diameter of the circle was measured for each isolate and compared with the control. The following indexation was used: '-' no biosurfactant production, '+' diameter of the circle 1 – 6 mm, '++' – 7 – 15 mm, '+++' – 16 - 30 mm, '++++' – > 30 mm.

Growth under different conditions

- *Growth at pH 4.0 and pH 10.0*

To test the growth of isolates at low and high pH, 100 µl of a suspension of 10^8 cfu ml^{-1} of the test strain in NB was added to 20 ml of NB buffered to pH 4.0 or pH 10.0 with 1M MOPS (3-(N-morpholino) – propane-sulfonic acid) (Sigma) or to 20 ml of NB, pH 7.0 (control) (S. Atkinson, Institute of Infection, Immunity and Inflammation, Nottingham, United Kingdom, personal communication). Shaken cultures (200 rpm). were incubated for 48 h at 28 °C. The turbidity of the bacterial cultures was inspected by eye and measured spectrophotometrically at 600 nm wavelength at the start (T=0) and end of the experiment (T=48). The following indexation was used: '-' no growth (increase of OD_{600} < 0.050, '+' growth (increase of $OD_{600} \geq 2$).

- *Growth at 10 °C under anaerobic and aerobic conditions*

To test the growth of isolates at 10 °C under anaerobic and aerobic conditions, 100 µl of 10^8 cfu ml^{-1} of bacteria in NB was added to 10 ml of potato dextrose broth (PDB) (Difco) buffered to pH 7.0 with 1M MOPS (Sigma). For anaerobic incubation, 5 ml of liquid paraffin (Sigma) was added on the top of the inoculated medium. Tubes were kept at 10 °C for 3 to 5 days and inspected by eye daily for turbidity. On, day 5, turbidity of the cultures was measured spectrophotometrically at 600 nm wavelength. Two experiment was repeated one time and the results were averaged. The growth of the tested isolates was evaluated by comparing the optical density (OD_{600}) at T= 0 and T= 120 h. The indexation used was: '-' increase of OD_{600} < 0.050, '+' increase of OD_{600} between 2 and 5 times, '++' increase of OD_{600} between 5 and 10 times, '+++' increase of $OD_{600} \geq 10$ times.

Production of pectinolytic enzymes

Pectinolytic activity of the test isolates was determined on polygalacturonic minimal medium (PMM) (per L: 3 g KH_2PO_4, 7 g K_2HPO_4, 2 g $(NH_4)_2SO_4$, 4 g polygalacturonic acid (PGA), 15 g agar; pH 7.0. As a positive control *Dickeya* sp. IPO2222 was used as a positive control. 2.5 µl of the test isolate (10^9 cfu ml^{-1} in NB) was spotted on the surface of PMM. Plates were incubated for 24-48 h at 28 °C. After this time, plates were washed with 10% copper acetate solution in water and left for 15 min at room temperature for halo development. The diameter of the white halo around of the bacterial colony is proportional to the concentration of pectinolytic enzymes. The following indexation was used, based on the diameter of the halo: '-' no halo, '+' – 0.1 – 10 mm, '++' 11 – 15 mm, '+++' 16- 25 mm.

Inoculation of potato tubers and growth of potato plants

In the first greenhouse experiment, in May – June 2009, 12 selected antagonists were tested for their ability to protect potato plants from infection by *Dickeya* sp. IPO2254 and disease development. Suspensions of test isolates and *Dickeya* sp. IPO2254 were prepared in sterile demineralized water to achieve densities of 10^{11} cfu ml^{-1} (antagonist) and 10^6 cfu ml^{-1} (*Dickeya* sp. IPO2254). Certified *Dickeya* spp.-free minitubers of cv. Kondor (Dutch Plant Inspection Service for agricultural seeds and seed potatoes (NAK), Emmeloord, The Netherlands) were used. Half of the tubers were wounded by removing 0.5 cm of stolon end with a sterile knife and others left intact. Both types of minitubers were immersed in the antagonist suspension and vacuum infiltrated for 10 min at -800 mBar in a desciccator followed by 10 min incubation at atmospheric pressure to allow the bacteria to penetrate the tuber lenticels and wounds. For control, intact and wounded mini tubers were vacuum infiltrated with sterile demineralized water. Tubers were dried in a flow cabinet overnight and the next day they were vacuum infiltrated with *Dickeya* sp. IPO2254 suspension or, for the control, with sterile demineralized water and dried overnight in flow cabinet. Tubers were planted in potting compost in 5 l plastic pots and kept at a 16/8 h photoperiod, 80% relative humidity (RH) and 28 °C for 3 weeks in a greenhouse. To eliminate the bias effect of the environmental conditions, a random plot design of the pots was applied (8 blocks containing 5 pots from each individual strain). In each treatment, 20 intact and 20 wounded (tubers from which 0.5 cm of the stolon end was cut off with sterile knife) Kondor minitubers were used per test strain. In the subsequent second

greenhouse experiment in June and July 2010, the four most promising candidates from first greenhouse experiment, using the same design and under the same conditions as in the first experiment were tested.

Symptom development

Plants were visually inspected weekly for symptoms development and were evaluated for wilting, chlorosis of leaves, black rot at the stem base, aerial stem rot, haulm desiccation and whole plant death.

Sampling of potato plants derived from vacuum infiltrated tubers

Potato plants were sampled 25 days post tuber planting. Approx. 1.5 cm long stem segments taken 5 cm above ground level were collected per plant and pooled. Stems were then surfacially sterilized for 1 min. in 70% ethanol, washed once with tap water, disinfected in 1% sodium hypochloride (commercial bleach) for 3 min, washed 3 times with tap water and dried with tissue paper. Samples were weighted and 1/4 Ringer's buffer (Merck) containing 0.02% of diethyl-dithiocarbamic acid (Acros Organics) was added to twice the weight of the sample. Samples were crushed in Universal Bioreba Bags (BIOREBA AG, Reinach, Switzerland) using a hammer. 100 µl of undiluted and decimal dilutions (0, 10^{-1} and 10^{-2}), of plant extract were mixed with 300 µl of pre-warmed to 45-50 °C PT medium containing 200 µg ml^{-1} of cycloheximide and 150 µg ml^{-1} of ampicillin in the well of a 24 well plate (Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands). After the medium had solidified, plates were incubated for 24-48 h at 28 °C to allow the bacteria to grow. Wells were inspected for the presence of GFP-tagged *Dickeya* sp. IPO2254 under 495 nm blue light using an epifluorescence stereo microscope (Leica Wild M32 FL4) equipped with a mercury high pressure photo-optic lamp (Leica Hg 50W/AC) and GFP plus filter (Czajkowski et al., 2010a). GFP positive bacterial colonies were counted.

Statistical analyses

Greenhouse experiments were done according to a completely randomized block design. In the experiment with 12 selected strains 40 plants (20 with wounded and 20 with intact tubers) were used per treatment divided over 8 complete blocks containing 5 plants each. In the experiment with a sub-selection of 4 strains, 40

plants (20 with wounded and 20 with intact tubers) were used per treatment divided over 5 complete block containing 6 plants and one complete block containing 5 plants. Observations were as follows: (1) incidence of tuber sprouting or not, (2) level of emerged plants with blackleg symptoms and (3) measurements of plant height and weight. Sprouting and the incidence of blackleg symptoms were analyzed using a standard generalized linear mixed model (GLMM) with the binomial distribution and a logit link to map the discrete outcome onto the real line (Cramer, 2004). A normal distribution was assumed for plant height and weight. Effects were considered to be significant at $P \leq 0.05$ and pair-wise differences were obtained using the *t* test. Data were analyzed using the statistical software package GenStat (Payne et al., 2009).

RESULTS

Collection of candidate bacterial antagonists

In total, 649 isolates were collected from tubers belonging to different potato cultivars and from various agar media (NB, R2A, King's B and TSA): 289 isolates from tubers rotting in soil type I (S1), 143 isolates from tubers rotting in soil type II (S2) and 217 isolates from tubers rotting in plastic foil (foil). Bacteria with different colony morphologies were selected. In a first screening, all isolates were tested for antibiosis against *Dickeya* sp. IPO2222 in an overlay plate assay and for production of siderophores. Forty-one isolates (6.3 %) produced antibiotic factors against *Dickeya* sp. IPO2222 strain whereas 112 isolates (17.3 %) produced siderophores, from which 41 isolates (6.3 %) produced a large orange/pink halo on the CAS agar plates (data not shown). None of the strains produced both antibiotics and siderophores. Forty one strong siderophore producers together with the 41 strains producing antibiotics were selected for further studies.

Table. 1 Characterization of 23 bacterial isolates from rotting potato tubers able to reduce tuber decay caused by *Dickeya* on the basis of motility, AHLs production and degradation, production of auxin, siderophores and biosurfactants, antibiosis, pectinolysis and growth in a medium at pH 4.0 and 10.0 and at 10 °C, under aerobic and anaerobic conditions

Isolate[1]	origin: potato cultivar	origin: rotting type[2]	Motility[3]	AHLs production[4]: CVO26/JB534	AHLs degradation[5]: HHL	AHLs degradation[5]: OHHL	AHLs degradation[5]: OHL	AHLs degradation[5]: OOHL	Auxin[6]: -Trp	Auxin[6]: +Trp	Pectinolysis[7]	Siderophore[8]	Antibiosis[9]	Biosurfactant[10]	growth in NB[11]: at pH 4.0	growth in NB[11]: at pH 10.0	growth at 10 °C[12]: anaerobic	growth at 10 °C[12]: aerobic
A3 * (*Lysinibacillus sphaericus*)	Kondor	S1	++	-/+	nd.	nd.	nd.	nd.	+	+++	+++	-	+	+++	-	-	+++	+
	Kondor (minitubers)	S1																
A6 * (*Bacillus simplex*)	Kondor	S2	-	-/-	+	+	+	+	+	+++	-	-	+	-	-	-	+	+
A10 * (*Rhodococcus erythropolis*)	Agria	foil	++++	-/+	nd.	nd.	nd.	nd.	±	+++	+++	-	+	±	-	-	+	++
A12 * (*B. subtilis*)	Arcade	S1	-	-/-	+	+	+	+	±	+++	++	-	+	+	-	-	-	-
A13 * (*Pseudomonas brassicacearum*)	Arcade	foil	++++	-/-	+	+	+	+	±	+++	-	-	+	-	-	-	-	+
A17 (*B. subtilis*)	Konsul	S1	-	-/-	-	-	-	-	+	+++	+	-	+	+++	-	-	-	-
A19 ** (*B. simplex*)	Arcade	foil	-	-/-	+	+	+	+	+	+++	-	-	+	-	-	+	-	+
A20 (*B. simplex*)	Arcade	foil	-	-/-	-	-	-	-	+	+++	-	-	+	++	-	-	-	-
A21 (*B. subtilis*)	Arcade	foil	++	-/-	-	-	-	-	±	+++	-	-	+	++	-	-	-	-
A23 (*Serratia plymuthica*)	Arcade	foil	++++	-/+	nd.	nd.	nd.	nd.	±	+++++	-	-	+	+	-	-	++	+++
A30 ** (*S. plymuthica*)	Arcade	foil	++++	-/+	nd.	nd.	nd.	nd.	±	+++++	-	-	+	+++	-	+	++	+++
A34 (*S. plymuthica*)	Agria	foil	++++	-/+	nd.	nd.	nd.	nd.	±	+++++	-	-	+	++	-	+	++	+++
A36 (*B. subtilis*)	Agria	foil.	-	-/-	+	+	+	+	+	+++	++	-	+	+	-	-	-	+
S3 * (*P. putida*)	Arcade	S1	++++	-/+	nd.	nd.	nd.	nd.	++	+++	-	+	-	-	-	-	-	+
S9 * (*Obesumbacterium proteus*)	Konsul	S1	++++	-/+	nd.	nd.	nd.	nd.	++	+++	-	+	-	+	+	+	-	++
S10 (*P. putida*)	Konsul	S2	++++	-/+	nd.	nd.	nd.	nd.	++	+++	-	+	-	++++	-	-	-	+
S20 ** (*P. putida*)	Konsul	S1	++++	-/-	+	+	+	+	++	+++	-	+	-	+	-	-	-	+
S21 (*P. putida*)	Agria	foil	++++	-/+	nd.	nd.	nd.	nd.	++	+++	-	+	-	-	-	+	-	+++
S23 * (*P. fulva*)	Arcade	foil	++++	+/+	nd.	nd.	nd.	nd.	++	+++++	-	+	-	+++	-	+	++	+
S26 (*P. putida*)	Agria	S2	++++	-/-	+	+	+	+	+	+++++	-	+	-	+++	-	+	-	++
S31 ** (*P. fulva*)	Arcade	foil	++++	-/+	nd.	nd.	nd.	nd.	+	++	-	+	-	+++	-	+	-	+
S37 (*P. putida*)	Arcade	foil	++++	-/-	+	+	+	+	+	++	-	+	-	-	-	+	-	-
S38 (*P. putida*)			++++	-/-	+	+	+	+	++	+++	-	+	-	-	-	+	-	+

[1] Bacterial isolates from rotting potato tissue; 12 isolates were used in a first greenhouse experiment (*) and 4 isolates were used in a second (**) , only 23 isolates belonging to risk category 1(+) and protecting potato from maceration causing by *Dickeya* sp. IPO2222 in a potato slice assay were characterized in detail

[2] Potato tubers were rotting in loamy sand, a rich soil collected in the region of Wageningen, NL (S1) (Garbeva et al., 2003), or in potting compost (S2) or wrapped in plastic foil (foil). Tubers were kept for 5 to 10 days under low-oxygen conditions and 90% relative humidity at 28 °C in closed humid boxes

[3] Motility was tested in a motility agar assay: "-" diameter colony < 5 mm, "+" diameter colony 5 - 15 mm, "++" 16-30 mm, "+++" 31 - 50 mm, "++++" > 50 mm

[4] AHLs production tested in a bioassay with two indicator strains *C. violaceum* (CVO26) and *E. coli* (JB534); "-" strain does not produce AHLs, "+" strain produces AHLs

[5] AHLs degradation was determined using an *E. coli* reporter strain responding to the presence of AHLs by production of a green fluorescent protein. Degradation was determined for HHL – hexanoyl-L-homoserine lactone, OHHL – 3-oxo-hexanoyl-L-homoserine lactone, OHL – octanoyl-L-homoserine lactone and OHHL – 3-oxo-octanoyl-L-homoserine lactone. ""+" degradation of the tested signal molecule, "-" lack of the degradation, "nd." – activity not determined

[6] Auxin concentrations were spectrophotometrically determined in media supplemented with L-tryptophan (+Trp) (auxin precursor) or without (-Trp): ."-"no production of auxins, "±" concentration 0.1 – 0.4 µg ml-1, "+" 0.5 – 1.0 µg ml-1, "++" 1.1 – 2.0 µg ml-1, "+++" 2.1 – 5.0 µg ml-1, "++++" 5.1 to 15.0 µg ml-1 and "+++++" > 15.1 µg ml-1

[7] Pectinolysis was determined on a polygalacturonic minimal medium on which pectinolysis results in a white halo: '-' no halo, '+' – diameter 0.1 – 10 mm, '++' 11 – 15 mm, "+++" 16- 25 mm

[8] Siderophore production was tested in a CAS agar plate assay, in which siderophore production resulted in a pink halo "-" no halo "+" halo

[9] Antibiosis was tested *in vitro* in an overlay assay with *Dickeya* sp. IPO2222 as indicator strain; "-"no inhibition , "+" inhibition

Suppression of soft rot development on potato slices

Selected isolates (41 antibiotics and 41 siderophore producers) were tested in a potato slice assay for their ability to reduce tuber decay caused by *Dickeya* sp. IPO2222. Thirty two antibiotic producers (from 41 isolated - 78%) and 41 strong siderophore producers in CAS agar assay (from 112 isolated - 37%) were able to reduce the tuber rot to at least 50% of the control, i.e. potato slices inoculated only with *Dickeya* sp. IPO2222 (data not shown).

Identification of potential antagonists

Identification of the eighty two isolates (41 antibiotic producers and 41 siderophore producers) was achieved by partially sequencing their 16S rDNA. Alignment with

sequences deposited in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) resulted in a classification of isolates into the following genera: *Alcaliges*, *Lysinibacillus*, *Aeromonas*, *Bacillus*, *Proteus*, *Haemophilus*, *Rhodococcus*, *Pseudomonas*, *Serratia*, *Acinetobacter*, *Enterobacter*, *Obesumbacterium*, *Stenotrophomonas*, *Pantoea* and *Raoultella* (data not shown). Alignments showed similarities of the 16S rDNA sequences of test isolate and reference species in the GenBank database between 95 and 100% (data not shown). Fifty nine out of 82 tested isolates (72%) were classified as risk category 2 species (human, animal or plant pathogens) and were therefore excluded from further studies.

Characterization of features involved in antagonism

Twenty three isolates classified as risk category 1 or 1+ bacteria belonging to *Lysinibacillus sphaericus*, *Bacillus simplex*, *Rhodococcus erythropolis*, *B. subtilis*, *Pseudomonas brassicacearum*, *Serratia plymuthica*, *P. putida*, *Obesumbacterium proteus*, and *P. fulva* species, that did not pose a risk for human and animal health and were not plant pathogens, were characterized in detail (Tab. 1). Isolates were investigated for the ability to produce quorum sensing signal molecules (AHLs), biosurfactants, pectinolytic enzymes and auxins, for growth at low and high pH, for growth under anaerobic and aerobic conditions at 10 °C and for motility and AHL degradation. No situation was observed in which a particular strain reacted differently in repeated experiments. Average values of each test were calculated and distributed into categories (Tab. 1)

AHLs production. Eleven out of 23 strains (A3, A10, A23, A30, A34, S3, S9, S10, S21, S23 and S31) were able to produce AHLs in both bioassays. AHLs producers belonged to the genera *Pseudomonas* (S21, S23 and S31), *Serratia* (A23, A30 and A34) and *Obesumbacterium* species (S9). As expected, no AHLs production was observed in the Gram positive *Bacillus* spp. isolates (A17, A19, A20 andA36).

AHLs degradation. Twelve isolates that were not producing AHLs were tested for their ability to degrade 4 synthetic signal molecules (HHL, OHL, OHHL and OOHL). Nine (A6, A13, A19, A36, S20, S37 and S38) degraded all four synthetic AHLs. These strains were *Pseudomonas* and *Bacillus* species.

Biosurfactant production. Sixteen strains produced biosurfactant in the *in vitro* assay (all isolates except A6, A13, A19, S3, S21, S37 and S38). The concentration of the surfactant produced varied largely per isolate as indicated by the oil drop spread assay. The largest average diameter of the oil circle was produced by *P. fulva*, *L. sphaericus* and *B. subtilis* (A3, A17, S10, S23, S26 and S31) isolates and

the smallest by *Rhodococcus erythropolis* isolate A10. Most isolates of *P. putida* and *P. brassicacearum* were negative for biosurfactant production.

Motility. The majority of test isolates were highly motile (17 isolates out of 23), especially those identified as *Pseudomonas* and *Serratia* species. Non-motile isolates (A6, A12, A17, A19, A20 and A36) were all characterized as *Bacillus* species.

Production of pectinolytic enzymes. Five isolates (A3, A10, A12, A17 and A36) out of 23 produced recordable amounts of pectinolytic enzymes under *in vitro* conditions, characterized as *L. sphaericus*, *R. erythropolis*, *B. subtilis*, *B. subtilis* and *B. subtilis*, respectively. Isolates A3 and A10 showed the highest concentration of pectinolytic enzymes in the *in vitro* plate assay.

Auxin production. All 23 isolates produced auxins in the absence of L-tryptophan; the supplementation of L-tryptophan increased auxin production in all strains. The highest increase was observed for isolates A23, A30, A34, S23 and S26 characterized as *S. plymuthica*, *P. putida* or *P. fulva*.

Growth at low and high pH. None of the isolates, except *O. proteus* were able to grow in NB at pH 4.0. Ten out of 23 isolates showed growth at pH 10.0, namely *P. putida* and *P. fulva* species (S21, S23, S26, S31, S37 and S38), *S. plymuthica* (A30 and A34), *B. simplex* (A19) and *O. proteus* (S9).

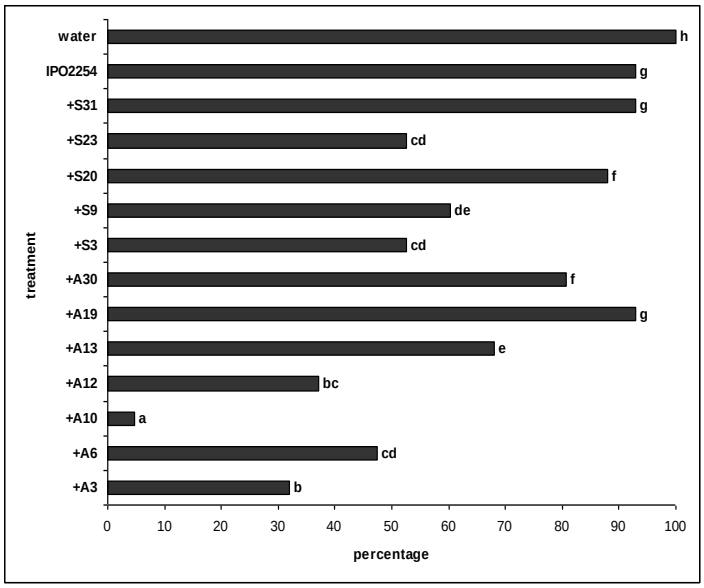
Growth under aerobic and anaerobic conditions. Under anaerobic conditions, seven strains (A3, A6, A10, A23, A30, A34 and S23) and under aerobic conditions 18 strains (all except A12, A17, A20, A21 and S37) grew at 10 $^{\circ}$C in PDB.

Greenhouse studies with vacuum-infiltrated minitubers

In the first greenhouse experiment 12 bacterial isolates (A3, A6, A10, A12, A13, A19, A30, S3, S9, S20, S23 and S31) belonging to 9 different species (*B. simplex, B. subtilis, R. erythropolis, P. brassicacearum, S. plymuthica, P. putida, P. fulva, O. proteus*, and *L. sphaericus*) were tested for protection of potato plants against biovar 3, *Dickeya* sp. IPO2254. They were selected on the basis of results in *in vitro* assays and features that may play a role in antagonism, in order to maximize variation of strains and modes of antagonistic actions. Antagonists were tested for their ability to reduce pre-emergence rot of tubers, blackleg symptoms and colonization of stems by *Dickeya* sp. IPO2254. Wounded (cut) or intact mini tubers of cv. Kondor were subsequently-inoculated subsequently with an antagonist and a GFP-tagged biovar 3 *Dickeya* sp. strain (*Dickeya* sp. IPO2254) by vacuum infiltration. Plants were screened for pre-emergence rot 15 days after planting, and weekly for symptom development. In a second greenhouse experiment, 4

antagonists (A19, A30, S20 and S31) which showed the best protection in the first greenhouse trial were used.

A



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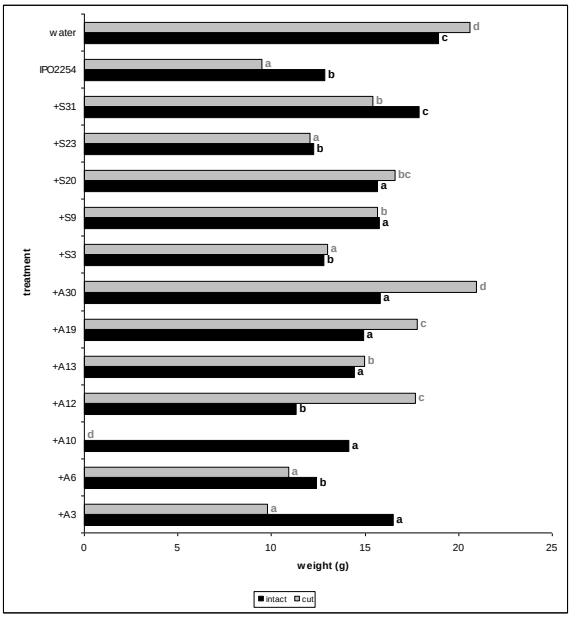


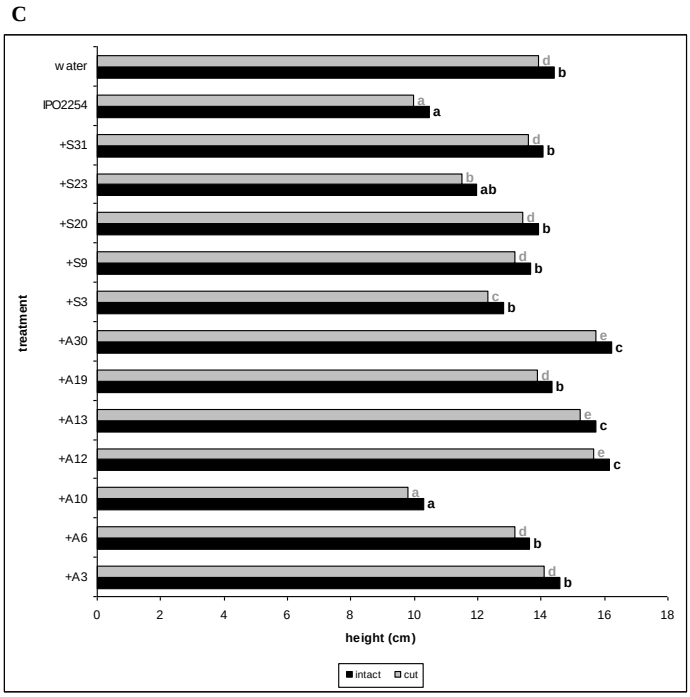


Figure 1. Effect of co-inoculation of seed tubers with *Dickeya* sp. IPO2254 and different antagonists on the percentage of sprouted tubers (a), weight (b) and maximum shoot length (c).
A) Average percentage of sprouted tubers 15 days post tuber co-inoculation. Control tubers were inoculated with water or only with *Dickeya* sp. IPO2254. No significant difference between treatments containing intact and cut tubers was observed therefore treatments were analyzed together. Values followed by identical characters are not significantly different (P=0.05)
B)Average fresh weight of shoots determined 25 days post tuber co-inoculation. Intact tubers (intact) and tubers from which stolon end part was removed (cut) were used. Control tubers were inoculated with water or only with *Dickeya* sp. IPO2254. Data from two independent experiments were analyzed together. Values followed by identical characters are not significantly different (P=0.05)
C) Average height of the highest shoot measured 25 days after tuber co-inoculation. Intact tubers (intact) and tubers from which stolon end part was removed (cut) were used. Control tubers were inoculated with water or only with *Dickeya* sp. IPO2254. Data from two independent experiments were analyzed together. Values followed by identical characters are not significantly different (P=0.05)

Non-emergence of potato plants

Non emergence of subsequently-inoculated potato tubers and controls was evaluated 15 days after planting. The percentage of tubers that generated shoots was calculated (Fig. 1A). No significant differences between treatments with intact

and cut tubers were found (data not shown), therefore treatments were analyzed together. Interestingly, inoculation with *Dickeya* IPO2254 resulted in a relative low percentage of non-emergence averaging at 7%. Equal percentages of non-emergence were found for treatments with *Pseudomonas fulva* S31 and *Bacillus simplex* A19. Co-inoculations of *Dickeya* sp. with other antagonists resulted in a higher percentage of non-emergence, from on average 19% for *S. plymuthica* A30 to a maximum of 95% for *R. erythropolis* A10.

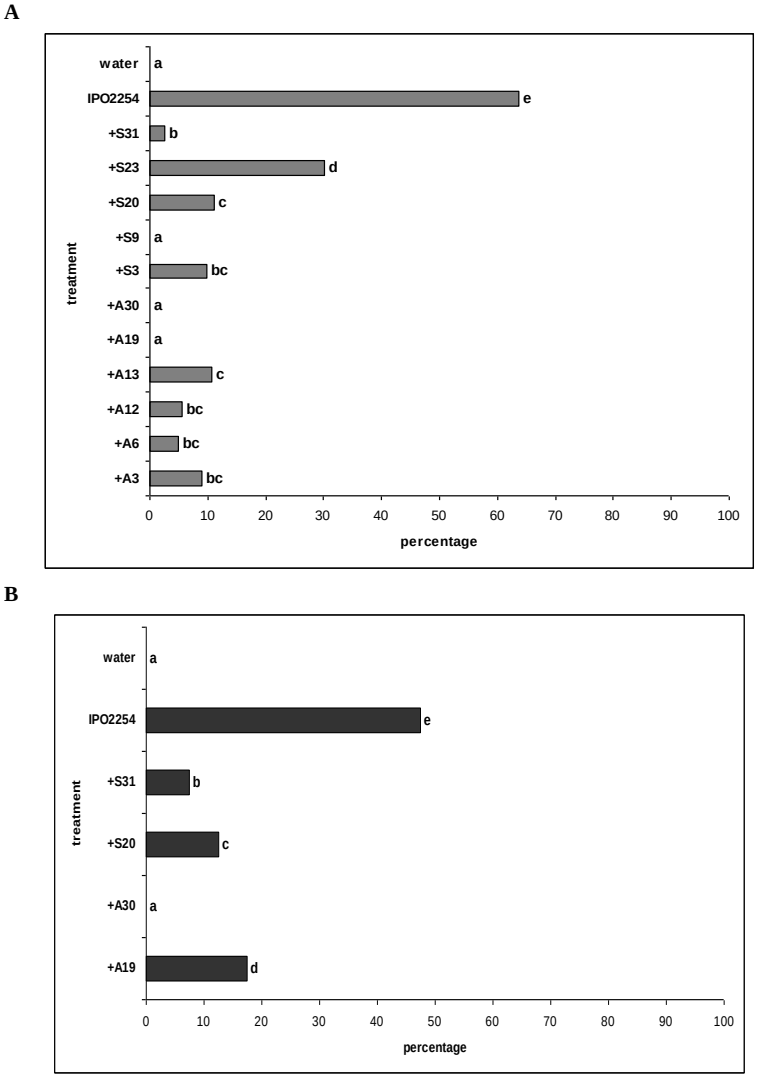
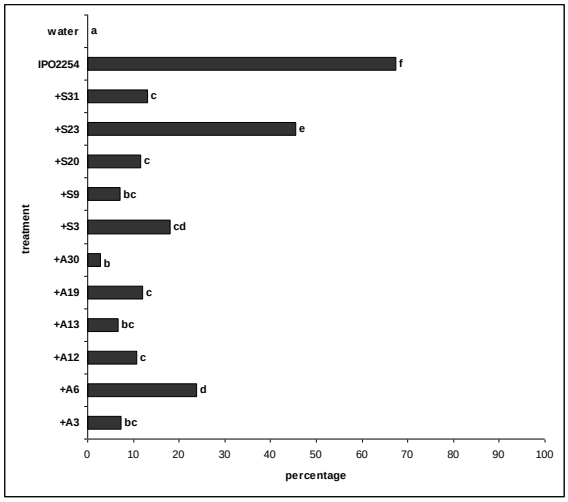


Figure 2. Effect of co-inoculation of seed tubers with *Dickeya* sp. IPO2254 and antagonists on the average percentage of symptomatic plants (showing blackleg or browning and rotting of internal stem

tissue) 25 days after incubation. In experiment 1, 12 antagonists were tested (A) and in experiment 2 a sub-selection of 4 antagonists were tested (B).
Control tubers were inoculated with water or only with *Dickeya* sp. IPO2254. No significant differences between treatments with intact and cut tubers were observed in both experiments therefore treatments were pooled before analyses. Values followed by identical characters are not significantly different (P=0.05).

A



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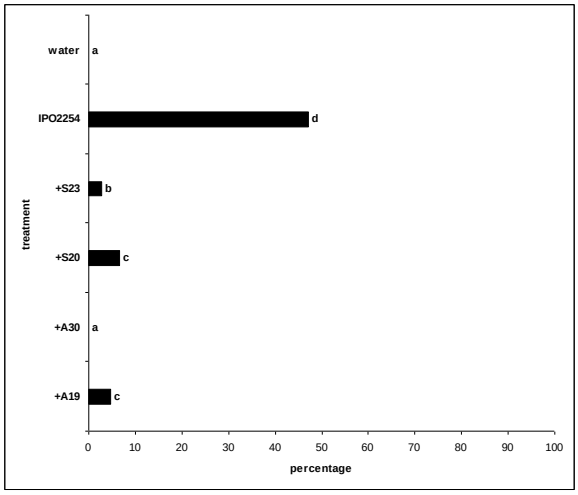

Figure 3. Effect of co-inoculation of seed tubers with *Dickeya* sp. IPO2254 and antagonists on the average percentage of plants harboring *Dickeya* sp. IPO2254, 25 days after co-inoculation. In

experiment 1, 12 antagonists were tested (A) and in experiment 2 a sub-selection of 4 antagonists were tested (B). Control tubers were inoculated with water or only with *Dickeya* sp. IPO2254.Values followed by identical characters are not significantly different (P=0.05).

Effect of antagonistic strain on the fresh weight of potato shoots

Twenty-five days after planting, the average fresh weight of potato shoots per treatment was determined (Fig. 1B). For intact tubers, fresh plant weight was significantly decreased by all treatments except for tubers subsequently-inoculated with both *Pseudomonas fulva* S31 and *Dickeya* sp. IPO2254 for which the weight of the shoots was similar to that of the water inoculated control plants. For plants raised from cut (wounded) tubers, only a treatment with *Serratia plymuthica* A30 resulted in an improvement on the adverse effect of *Dickeya* sp. on shoot weight.

Effect of antagonistic strains on the average length of the highest shoot

Twenty-five days after planting, the average length of the highest potato shoot per plant per treatment was determined for the sprouted tubers (Fig. 1C). For both intact and wounded tubers, all antagonists except *Rhodococcus erythropolis* A10, at least partially increased shoot length relative to *Dickeya* sp. Treatments with the antagonists *Bacillus subtilis* A12, *Pseudomonas brassicacearum* A13 and *Serratia plymuthica* A30 resulted in an increase in shoot height of plants derived from both cut and intact tubers relative to the control.

Effect of antagonistic strains on blackleg incidence

Twenty-five days after planting, the average percentage of symptomatic plants showing blackleg or internal discoloration and hollowing of stem tissue was determined (Fig. 2A). A significant difference between the results of intact and cut tubers was not found in any of the treatments (data not shown), therefore results of both treatments were analyzed together. Results of the treatment with joint inoculation with *R. erythropolis* A10 and *Dickeya* spp. were removed from the analysis, because of the high percentage of pre-emergence rot. All other antagonists reduced the percentage of symptomatic plants significantly. Treatment with *B. simplex* A19, *S. plymuthica* A30 and *O. proteus* S9 completely prevented symptom development. Co-inoculation of *P. fulva* S23 with *Dickeya* sp. IPO2254 resulted in 47% reduction of symptomatic plants, but for plants with the other

bacterial co-inoculations, blackleg incidence was reduced from at least 52 to 96% in all other treatments.

In a second greenhouse experiment, 4 antagonists were selected on the basis of results from the first experiment (i. e. average percentage of sprouted tubers, height and weight of shoots, reduction of blackleg symptoms and *Dickeya* sp. IPO2254 densities in stems), namely *S. plymuthica* A30, *B. simplex* A19 and *P. putida* S20 and *P. fulva* S31. A significant difference in the results between intact and cut tubers was not found in any of the treatments (data not shown), therefore data for both treatments were analyzed jointly. Approximately 47% of control plants inoculated with *Dickeya* sp. IPO2254 expressed blackleg symptoms. All four antagonists reduced the blackleg incidence significantly. Co-inoculation of potato tubers with *S. plymuthica* A30, *P. fulva* S31, *P. putida* S20 or *B. simplex* A19, resulted in 0, 7, 12 and 17% diseased plants, respectively.

Effect of antagonistic strains on Dickeya sp. *populations in stems*

Twenty-five days after planting, the percentage of plants harboring GFP-tagged *Dickeya* sp. inside stems was determined by pour-plating of stem extracts and screening for typical green fluorescent colonies (Fig. 3A). A significant difference in the results between intact and cut tubers was not found in any of the treatments (data not shown) therefore results of both treatments analyzed together. All antagonists reduced infection incidence significantly, relative to the *Dickeya* sp. control at 70 % , from 45% for *Pseudomonas fulva* S23 to 3% for *S. plymuthica* A30. *Dickeya* sp. IPO2254 was not present in any of the water inoculated plants.

In a second greenhouse experiment, as above, results of both cut and intact tubers treatments were analyzed jointly. Twenty-five days after inoculation, approximately 47% of control plants inoculated with *Dickeya* sp. IPO2254 harbored the pathogen. In contrast, the four antagonists reduced infection incidence significantly, 0, 2, 6 and 5% infected plants grown from potato tubers subsequently-inoculated with *S. plymuthica* A30, *P. fulva* S31, *P. putida* S20 or *B. simplex* A19, respectively.

Densities of *Dickeya* sp. IPO2254 in both experiments varied largely per treatment and plant screened. No statistically significant differences in results between intact and cut tubers were found (data not shown). On average in both experiments, in plants inoculated with IPO2254, 25 dpi $10^3 – 10^4$ cfu $g^{-1}$of stem tissue were present. Co-inoculation of tubers with A19, S20 and S31 antagonists resulted in a reduction of *Dickeya* sp. populations on average to 10, 100 and 100 cfu g^{-1} stem tissue, respectively (data not shown).

DISCUSSION

This study was conducted to assess the potential of antagonistic bacteria isolated from rotting potato tubers to control a distinct genetic clade of *Dickeya* sp. biovar 3, that is currently one of the dominant potato blackleg causing pathogens in Europe.

Although bacterial antagonists against *Dickeya* spp. have been isolated previously, this study is the first dealing with antagonists isolated from rotting potato tuber tissue. This approach was taken to acquire bacteria able to grow in an environment in which *Dickeya* spp. are highly active. It is generally accepted, that success of a biocontrol agent in controlling a pathogen depends on the occupation of the same niches, utilization of the same carbon and nitrogen sources or adaptation and multiplication in the same environmental conditions (Völksch & May, 2001). Therefore we speculated to find potential antagonists present in rotting potato tissue where they have to face high levels of antimicrobial metabolites and oxygen depletion rather than in healthy tuber tissue. It is known that rotting tubers contain high concentrations of different plant metabolites including ethanol, acetone, 2-butanone, acetalaldehyde, methyl acetate, ethyl acetate, propanol and butanol that show antimicrobial activity towards a variety of Gram-positive and Gram-negative bacteria (Maga, 1994). The high microbial activity in rotting tubers is also expected to result in high concentrations of antimicrobial metabolites produced by micro-organisms, including antibiotics and siderophores.

Perhaps because of the few sources from which bacteria were isolated, only a limited number of bacterial species antagonistic to *Dickeya* spp. were found in rotting tissue. The initial selection based on cultivation, and antibiotic and siderophore production resulted in bacteria belonging to only 18 genera. It was previously mentioned that potato tubers host a limited number of culturable bacterial species such as fluorescent and non-fluorescent *Pseudomonas* spp., *Bacillus* spp., *Serratia* spp. and *Xanthomonas* spp. (Sturz, 1995). To our knowledge, only very limited information is available on bacterial species present in potato rotting tissue, and no intensive study has been conducted till now.

The majority of the 82 selected bacterial strains were classified as (opportunistic) human pathogens (i.e. *Enterobacter cloace, Bacillus cereus, Alcaligens feacalis, Proteus vulgaris, Aeromonas salmonicida* and *Enterobacter cancerogenus*). Tuber decay can result in the occurrence of high densities (10^3-10^5 cfu g^{-1}) of these potential human pathogenic bacteria. They can spread over tubers

during harvesting, grading and packaging and may pose a health risk for people and animal exposed. Protection in rotting tuber tissue may result in enhanced survival periods of these pathogens. Growth of (opportunistic) human pathogens in rotting plant material has been reported for other crops. For example, it was found that vegetables (e. g. beet, broccoli, cabbage, cucumber, carrot, pepper, radish, squash and tomato) infected with pectinolytic bacteria often harbored higher densities of *Salmonella* spp. than healthy plant tissues (Wells & Butterfield, 1997). Carlin and co-workers (Carlin et al., 1995) reported an increase in the density of *Listeria monocytogenes* in decaying endive leaf tissue in comparison with healthy tissues.

The bacteria isolated from rotting potato tissue possessed different mechanisms that can interfere with *Dickeya* spp. *in planta* such as competition for iron ions by production of siderophores, antibiosis which may be partially based on the production of biosurfactants, degradation of quorum sensing signal molecules and competition for nutrients (e. g. pectinolysis). These features have been reported previously to play a role in bio-control (Uroz et al., 2003).

Selected isolates possessed various other features that potentially enhance their colonization, adaptation and survival in decaying potato tissue such as spore formation, motility and the ability to grow under anaerobic conditions. Oxygen depletion is one of the factors inducing rotting of potato tubers by pectinolytic bacteria, by attenuation of the plant defense mechanism (Perombelon & Lowe, 1975a). Antagonists that are able to grow under low oxygen conditions might have an advantage when competing with *Dickeya* spp. during tuber decay. Spore forming strains, such as *Bacillus* spp. are able to persist for long periods under harsh conditions as they are tolerant to heat and desiccation (Weller, 1988). Use of these strains may result in a longer protection of tubers compared to non spore forming strains. Motility enhances colonization of (plant) surfaces, which may be therefore an advantage in the competing with the motile *Dickeya* spp. both in plants and in soil.

The presence of high numbers of antagonistic bacterial species isolated from rotting potato tissue may explain partially the variation in symptom expression in plants homogeneously inoculated by vacuum infiltration with *Dickeya* sp. (unpublished results) and contributes to other factors affecting blackleg development (Perombelon, 2002). The presence of high numbers of antagonistic bacteria may also explain false negative results in isolations from diseased tubers or plants or detection procedures based on enrichment for *Dickeya* spp. (Degefu et al., 2009).

The strategy of selecting antagonists, based on *in vitro* assays and a tuber slice test appears to be successful in obtaining antagonists effective in the biocontrol of biovar 3 *Dickeya* sp. *in planta*. From a sub-selection of 12 strains, 10 were able to reduce the blackleg incidence by more than 50%. This is in contradiction with former studies in which *in vitro* assays had only a limited value in predicting the antagonist potential for *in planta* tests. It must, however, be noted that co-inoculation of tubers with 11 out of 12 strains and *Dickeya* sp. resulted in an increased incidence of non-emergence. In particular, co-inoculation with A3, A10 and A12, characterized as *Lysinibacillus sphaericus, R. erythropolis* and *Bacillus subtilis*, respectively, resulted in a high level of pre-emergence rot, which may be related to their ability to produce pectinolytic enzymes.

One strain, characterized as *Serratia plymuthica* (A30), provided a consistent high level of blackleg disease control in repeated greenhouse experiments, even under conditions very favorable for disease development. *S. plymuthica* strain A30 possesses different features that may play a role in antagonism of *Dickeya* sp. IPO2254; the strain produces antibiotics and surfactants, it is motile and produced auxins.

Protection was found after vacuum infiltration of intact tubers and tubers from which part of the stolon end had been mechanically removed, under warm (28 °C) and humid (80% relative humidity) conditions favorable for blackleg disease development. Strains of *S. plymuthica* have been frequently used to control fungal pathogens of plants, but not to our knowledge to control phytopathogenic bacteria (De Vleesschauwer & Hofte, 2007). Potato plants, subsequently-inoculated with *S. plymuthica* strain A30 and the pathogen, were protected against systemic colonization by the pathogen and consequently also against disease development. The tubers were vacuum- infiltrated with a high inoculum dose ($10^{10} – 10^{11}$ cfu ml^{-1}) of the antagonist to ensure relatively high densities in the tuber periderm. Results indicate the ability of *S. plymuthica* strain A30 as an antagonist to control of blackleg caused by biovar 3 *Dickeya* sp. on potato.

S. plymuthica A30 is potentially a good candidate for developing a commercial potato crop protection product. *S. plymuthica* is classified into risk group 1 according to DSMZ (German Collection of Microorganisms and Cell cultures), meaning that the species is not expected to pose a risk for humans and environment, and to date no human or animal-related pathogenicity factors for *S. plymuthica* have been described. Our strain A30 strain does not produce prodigiosin, a red pigment (Czajkowski, unpublished results), an antifungal and anti-eukaryotic compound that can be produced by *S. marcescens* (risk group 2); *S. plymuthica* strain A30 strain is also susceptible to a number of antibiotics routinely

used in medicine to treat bacterial infections in humans (Czajkowski, unpublished results); Finally, a commercial product named Rhizostar (E-nema GmbH Raisdorf, Germany) based on *S. plymuthica* HRO-C48 and active against *Rhizoctonia solani* and *Verticillum dahliae* is presently available on the market in Europe (European patent 98124694.5) indicating the possibility of using strains belonging to *S. plymuthica* species as biocontrol agents in agriculture.

To fully explore the usefulness of *S. plymuthica* strain A30 strain in practice, additional studies are required on effectiveness and consistency of control in the field, including population dynamics in the potato ecosystem, application timing, production and formulation and eco-toxicological risks. Work with deletion (knockout) mutants is now being conducted to understand the molecular basis of the antagonistic activity of *S. plymuthica* strain A30 against *Dickeya* sp. IPO2254.

ACKNOWLEDGMENTS

The project was financed by STW Foundation (Technologiestichting STW, The Netherlands) via grant no. 10306 "Curing seed potato from blackleg causing bacteria". We thank P. S. van der Zouwen (PRI, The Netherlands) for technical assistance, S. Jafra (University of Gdansk, Poland) for helpful discussion and M. C. M. Perombelon (ex. SCRI, UK) and Mrs. L. J. Hyman (ex SCRI, UK) for their comments on the manuscript and their editorial work.

Chapter 7

Studies on the interaction between the biocontrol agent, *Serratia plymuthica* A30, with blackleg causing *Dickeya* sp. (biovar 3) in potato (*Solanum tuberosum*)

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Plant Pathology (submitted)

ABSTRACT

Interactions between *Serratia plymuthica* A30 and the blackleg causing biovar 3 *Dickeya* sp. were examined. In a potato slice assay, *S. plymuthica* A30 inhibited tissue maceration caused by *Dickeya* sp. IPO2222 when co-inoculated at a density at least 10 times greater than that of the pathogen. In greenhouse experiments, population dynamics of the antagonist and of the pathogen *in planta* were studied by dilution plating and confocal laser scanning microscopy (CLSM) using fluorescent proteins-tagged strains. Pathogen-free minitubers were vacuum-infiltrated with DsRed-tagged *Dickeya* sp. IPO2222 and superficially treated during planting with a water suspension containing GFP-tagged *S. plymuthica* A30. A30 reduced the blackleg incidence from 55% to 0%. Both the pathogen and the antagonist colonized the seed potato tubers internally within one day post inoculation (dpi). Between 1 and 7 dpi, the population of A30 in tubers increased from 10^1 to ca.10^3 cfu g^{-1} and subsequently remained stable until the end of the experiment (28 dpi). Populations of A30 in stems and roots increased from ca. 10^2 to ca.10^4 cfu g^{-1} between 7 and 28 dpi. Dilution plating and CLSM studies showed that A30 decreased the density of *Dickeya* sp. population in plants. Dilution plating combined with microscopy allowed the enumeration of strain A30 and its visualization in the vascular tissues of stem and roots and in the pith of roots as well as its adherence to and colonization of the root surface. The implications of these finding for the use of *S. plymuthica* A30 as a biocontrol agent are discussed.

INTRODUCTION

Blackleg and soft rot bacterial diseases caused by *Dickeya* and *Pectobacterium* species can result in important losses in seed potato production in Europe (Toth et al., 2011). The importance of *Dickeya* spp. as a potato pathogen has been increasing recently (Sławiak et al., 2009, Toth et al., 2011). This increase has been associated with the presence of a new genetic clade of *Dickeya* spp., which appeared recently in Europe and which could not be classified in one of the known six species described so far (Tsror et al., 2008, Slawiak et al., 2009). The new clade probably constitutes a new *Dickeya* species, which has provisionally been called "*D. solani*" (Toth et al., 2011). Its occurrence has been reported in potato in several European countries including The Netherlands, Finland, Poland, Germany, Belgium, France, United Kingdom and Sweden as well as in Israel (Toth et al., 2011).

Control measures for *Dickeya* and *Pectobacterium* species in potato are limited (Czajkowski et al., 2011b). They include the use of certified seed derived initially from pathogen-free minitubers, hygienic measures to avoid introduction and dissemination of the bacterial pathogens, avoidance of wounding of tubers and field soil drainage to avoid oxygen depletion which can impair the tuber resistance to rotting. However, despite an integrative strategy involving these control measures, an acceptable reduction of blackleg and soft rot problems has not been achieved consistently.

Tuber treatments to reduce bacterial inoculum are rarely used in practice. Physical treatments, such as hot water treatments, hot air and radiation, as well as chemical control agents may reduce superficial bacterial populations on tubers, but have little effect on internally located bacteria. *Dickeya* spp. are vascular pathogens capable of colonizing vascular tissues following root or stem infections (Czajkowski et al., 2010a, Czajkowski et al., 2010b). Consequently, relatively high populations of *Dickeya* spp. are frequently found at the stolon end of progeny tubers (Czajkowski et al., 2009). Not surprisingly disinfection with chemical and physical treatments is not effective against these internal populations. At present, no systemic bactericides are available which could eliminate these bacteria inside plant vascular tissues.

As an alternative to seed tuber disinfection procedures, use of antagonistic bacteria has been attempted but generally with little consistent success mainly because of inability to invade and survive within the host tissues. However, endophytic bacteria that were isolated from within surface-sterilized plant tissues were able to colonize plants systemically when applied artificially (Lodewyckx et al., 2002). They are by definition nonpathogenic to and exert no adverse effect on the host plant while interacting with pathogens present (Hallmann et al., 1997). It has already been demonstrated that some endophytes can act as antagonists and that their presence can have a direct positive effect on plant fitness (Adhikari et al., 2001, Chen et al., 1995).

Recently, we have isolated and described an endophytic antagonistic *Serratia plymuthica* strain A30 which was active against biovar 3 *Dickeya* spp. *in vitro* and on potato plants under greenhouse conditions (Czajkowski et al., 2011a). The antagonist was isolated from rotting tissue of superficially disinfected tubers wrapped in plastic foil to induce tuber decay. The strain had been selected on the basis of *in vitro* production of antibiotics against *Dickeya* spp. and has been extensively characterized in *in vitro* tests for other features that are potentially involved in antagonism: production of biosurfactants, motility and growth under aerobic and anaerobic conditions at relatively low (10 °C) temperatures. In

replicated greenhouse experiments, *S. plymuthica* A30 reduced blackleg symptom expression caused by biovar 3 *Dickeya* spp. by 100% and colonization of stems by the pathogen by 97% after co-inoculation of tubers by vacuum infiltration (Czajkowski et al., 2011a).

In this study we aimed to acquire knowledge on the interactions between *Serratia plymuthica* A30 and biovar type 3 *Dickeya* sp. (strain IPO2222), in potato plants as a preliminary step in the possibility of commercial exploitation of the biocontrol agent (Cook, 1993). This will involve understanding the antagonistic mechanism, ecology of the biocontrol agent, survival in the environment and its ability to colonize internally and superficially potato plants.

The control of *Dickeya* spp. by *S. plymuthica* A30 was investigated in a potato slice assay at different inoculum densities. The potato slice assay was also used to study the population dynamics of the pathogen and the antagonist on/in tuber tissue. In repeated greenhouse experiments, tubers were treated with a suspension of the antagonist just before covering tubers with soil, to simulate a seed tuber application procedure in the field. The possibility of the strain to colonize potato plants after tuber treatments was studied, to determine the potential of the antagonist for control of biovar 3 *Dickeya* spp. in internal plant tissues. To enable visualization of bacteria *in planta* in vascular tissues with microscopical techniques, the biovar 3 *Dickeya* sp. strain was tagged with plasmid-based red fluorescent protein (DsRed) and *S. plymuthica* A30 with green fluorescent protein (GFP).

MATERIALS AND METHODS

Bacterial strain and growth media used

S. plymuthica A30 (Czajkowski et al., 2011a) and biovar 3 type strain *Dickeya* sp. IPO2222 (Slawiak et al., 2009) were grown at 28 °C for 24-48 h on tryptic soya agar (TSA) (Oxoid) or nutrient agar (NA) (Oxoid) prior to use. Liquid cultures were prepared in nutrient broth (NB) (Oxoid) and/or tryptic soya broth (TSB) (Oxoid), grown at 28 °C for 24 h with agitation (200 rpm). Strains of GFP-tagged *S. plymuthica* A30 and DsRed-tagged *Dickeya* sp. IPO3012 (derived from wild type strain A30 and IPO2222, respectively) were grown using the same media but supplemented with 40 μg ml^{-1} of tetracycline (Sigma) (NAt, TSAt, NBt, TSBt, respectively). When plant extracts were analyzed, growth media were

supplemented additionally with cycloheximide (Sigma) to a final concentration of 200 µg ml^{-1} to prevent fungal growth.

Generation of GFP-tagged S. plymuthica A30 and DsRed-tagged Dickeya sp. *IPO2222 strains*

Plasmids pRZ-T3-*gfp* and pRZ-T3-*dsred* (Bloemberg et al., 2000) were used for generation of GFP-tagged *S. plymuthica* A30 and DsRed-tagged biovar 3 *Dickeya* sp. IPO3012 respectively. The plasmids carrying genes coding for fluorescent proteins under constitutive promoters, were introduced in bacterial cells by electroporation as described in (Czajkowski et al., 2010a). Briefly, 50 µl suspensions of competent bacterial cells of A30 or IPO2222 (containing approx. 10^{11}-10^{12} colony forming units (cfu ml^{-1}) were mixed with 100 ng μl^{-1} plasmid DNA and electroshocked at 2.5 kV for 1-2 sec at 4°C using a Bio-Rad Gene Pulser 200/0.2 (Biorad, Hercules, CA, USA). After electroporation, bacterial cells were resuscitated in 500 µl of NB at 28 °C for 1 h with shaking (200 rpm). Hundred microliters of the transformed cells were plated on TSAt and incubated for 24-48 h at 28 °C before selection of GFP or DsRed fluorescent transformants.

Growth of tagged bacterial strains relative to their wild type parental strains

Relative growth of DsRed-tagged *Dickeya* sp. strain IPO3012 and wild type strain IPO2222 and GFP-tagged *S. plymuthica* A30 strain and wild type A30 was determined under aerobic conditions using as inoculum 100 µl overnight cultures containing ca. 10^{9}-10^{10} cfu ml^{-1} in 20 ml of NBt or NB diluted 50 times in NBt or NB. Bacteria were grown at 28 °C with a shaking rate of 200 rpm and growth rates were determined by measuring the OD_{600} over a period of up to 24 h.
Growth of the wild type IPO2222 and tagged IPO3012 under anaerobic conditions, created by adding 5 ml of liquid paraffin to 30 ml of the bacterial suspensions in PEB (Perombelon & Van der Wolf, 2002), was also determined as described above except that the cultures were not agitated during incubation.

Ability of DsRed-tagged IPO3012 to macerate potato tuber tissue

Bacterial suspension of IPO3012 was diluted in Ringer's buffer (Merck) to ca.10^{6} cfu ml^{-1}. Potato tubers of cultivar Agria (Agrico, The Netherlands) were rinsed under running tap water, followed by washing twice with 70 % ethanol for 5 min

and again twice for 1 min with demineralized water. Tubers were dried with tissue paper and cut into 0.7 cm transverse slices. Three 5 mm deep wells per slice were made with a 5 mm diameter sterile cork borer and filled with 50 µl of the bacterial suspension. Three potato slices derived from three different tubers were used per treatment per strain. The slices were incubated at 28 °C for 72 h in a high humidity box and the diameter of rotting tissue around inoculated wells was measured after 72 h incubation at 28 °C. The experiment was repeated twice and the growth rate results for the two strains analyzed and compared.

Inhibition of wild type IPO2222 by GFP-tagged A30 in an overlay plate assay

The ability of GFP-tagged *S. plymuthica* A30 relative to wild type strain A30 to inhibit growth of IPO2222 was compared in an overlay plate assay with IPO2222 as the indicator strain. Fifty µl of an overnight culture of strain IPO2222 (approx. 10^9 cfu ml^{-1}) in NB was mixed with 5 ml of soft agar (NB supplemented with 0.7% agar) pre-warmed to 45-50 °C, and poured onto TSA plates. After agar had solidified, one aliquot of 2.5 µl of an overnight culture of A30 or GFP-tagged A30 in NB and NBt respectively (approx. 10^9 cfu ml^{-1}) was spotted on the surface of the agar plate (2 replicated plates). Plates were incubated for 24 – 48 h at 28 °C. The diameter of the clear 'halo' (indicating IPO2222 growth inhibition) around the inoculated spot was measured.

Control of Dickeya sp. IPO2222 by GFP-tagged A30 relative to the wild type strain A30 on potato slices

The ability of GFP-tagged A30 to protect potato tuber tissue against maceration by IPO2222 was evaluated in a potato slice assay as described above. GFP-tagged A30 strain, the wild type A30 strain and IPO2222 were grown overnight in NBt or NB at 28 °C. Bacterial cultures were centrifuged (5 min, 6000 x g), washed twice with 1/4 Ringer's buffer and re-suspended in sterile water to the original volume. Wells of the tuber were filled up with 50 µl of the suspension containing 10^8 cfu ml^{-1} of GFP-tagged A30 or 10^8 cfu ml^{-1} of A30 and 10^6 cfu ml^{-1} of IPO3012. Three potato slices derived from three different tubers were used per treatment. As negative control, 50 µl of sterile water was used instead of bacterial suspensions, and for the positive control 50 µl containing 10^6 cfu ml^{-1} of *Dickeya* sp. IPO2222 were used. The slices were incubated at 28 °C for 72 h in a humid box and the

experiment was independently repeated one time. The protection effect of GFP-tagged A30 on potato tissue was measured by comparing the average diameter of rotten potato tissue around co-inoculated wells with the average diameter of rotten potato tissue around wells of the positive control.

Population dynamics studies of GFP-tagged A30 and DsRed-tagged Dickeya sp. IPO3012 on potato slices

In order to study the population dynamics of the GFP-tagged *S. plymuthica* A30 and the DsRed-tagged *Dickeya* sp. IPO3012 on potato slices a similar experimental set up as above was used. This time tuber wells were filled with a 50 µl suspension containing 10^{10} cfu ml^{-1} of GFP-tagged A30 and 10^{8} cfu ml^{-1} of IPO3012 and the experiment was repeated twice. Population densities of GFP-tagged A30 and IPO3012 on potato slices were determined: ca. 2 g of tuber tissue from 3 wells per tuber per treatment taken at random were collected daily and crushed in 4 ml of 1/4 strength Ringer's buffer in a Universal Extraction bag (BIOREBA) using a hammer. 100 µl of undiluted and 1000 times and 10000 times diluted tuber extracts were mixed with liquefied NA, cooled down to 48 °C and supplemented with tetracycline (NAt) to a final concentration of 40 µg ml^{-1}, and poured into the wells of 24-well plate (Greiner). After agar had solidified, the plates were covered with parafilm and incubated at 28 °C for 24-48 h for growth of bacterial colonies. GFP and DsRed tagged colonies were counted under an epifluorescence stereo microscope (Leica Wild M32 FL4) equipped with a mercury high pressure photo-optic lamp (Leica Hg 50W/AC) and a GFP and RFP plus filters.

Density dependence of the control of Dickeya sp. IPO2222 by GFP-tagged A30 on potato slices

The effect of GFP-tagged A30 strain density on tuber tissue rotting caused by *Dickeya* sp. IPO2222 was studied in a similar experimental set up as above with minor modifications. Tuber wells were co-inoculated with a 50 µl suspension containing different densities of A30 (0, 10^{4}, 10^{5}, 10^{6}, 10^{7} and 10^{8} cfu ml^{-1}) and 10^{6} cfu ml^{-1} of IPO2222 and as control, wells were inoculated with 50 µl suspension containing of 10^{6} cfu ml^{-1} of IPO2222 *Dickeya* sp. in water. The potato slices were incubated under the same conditions as described above. The experiment was independently repeated one time. The effect of GFP-tagged A30 on maceration of

potato tissue was determined by comparing the average diameter of rotting potato tissue around co-inoculated wells with that for the positive control.

Greenhouse experiments

Greenhouse experiments were conducted in June - July (experiment 1) and September – October (experiment 2) in 2010. In each experiment, four treatments were applied to potato tubers: (a) tubers vacuum infiltrated with DsRed-tagged *Dickeya* sp. IPO3012 (positive control), (b) tubers vacuum infiltrated with DsRed-tagged *Dickeya* sp. IPO3012 and surface inoculated with a suspension of GFP-tagged *S. plymuthica* A30, (c) tubers surface inoculated with a suspension of GFP-tagged *S. plymuthica* A30, and (d) tubers vacuum infiltrated with water (negative control),

Inoculation of potato tubers with GFP-tagged S. plymuthica A30 and DsRed-tagged Dickeya sp. IPO3012

Suspensions of DsRed-tagged *Dickeya* sp. IPO3012 containing 10^6 cfu ml^{-1} were prepared in sterile demineralized water. *Dickeya* spp-free minitubers of cv. Kondor (Dutch Plant Inspection Service for agricultural seed potatoes (NAK), Emmeloord, The Netherlands) were used. The minitubers were immersed in the bacterial suspension and vacuum infiltrated for 10 min at -800 mBar in a desciccator followed by 10 min incubation at atmospheric pressure. Minitubers infiltrated with sterile demineralized water only, served as negative controls. All tubers were dried in a flow cabinet overnight. Suspensions of GFP-tagged A30 containing 10^{10}-10^{11} cfu ml^{-1}were prepared in sterile demineralized water. Negative control tubers and DsRed-tagged IPO3012 vacuum infiltrated tubers were inoculated by adding 50 ml of A30 suspension over the tuber surface just before planting in 5 L plastic pots containing moist sandy soil (2.9% of organic mater, 0.2% $CaCO_3$, pH 6.4) freshly collected from a potato field in Wageningen (51°57′52″N 5°39′47″E), The Netherlands. The pots were kept unwatered for 24 h after planting and subsequently were watered daily to field capacity. Pots were kept in the greenhouse under a 16/8 h photoperiod regime, at ca. 70 % relative humidity and at ca. 28 °C for 4 weeks (28 days) in a random block design of the pots (3 blocks containing 10 pots for each treatment – 40 pots in total per block). At each sampling time, 10 plants inoculated with IPO3012 (positive control), 10 plants inoculated with sterile water (negative control), 10 plants sequentially inoculated with *Dickeya* sp.

IPO3012 and GFP-tagged *S. plymuthica* A30, and 10 plants inoculated with GFP-tagged A30 were sampled.

Symptom development

Plants were visually inspected weekly for development of symptoms: non-emergence, wilting and chlorosis of leaves, black soft rot at the stem base, aerial stem rot, haulm desiccation and plant death.

Quantification of DsRed-tagged Dickeya sp. *IPO3012 and GFP-tagged S. plymuthica A30 in potato plants by pour plating*

At each time point, 10 plants per treatment were sampled 1, 7 and 28 days post inoculation (dpi). Seed tubers were collected and processed individually. They were washed under tap water to remove soil particles, sterilized in 70% ethanol for 1 min, washed three times with water for 1 min, soaked in 1% sodium hypochlorite (commercial bleach) for 4 min and finally washed for 4 min three times with water. Each tuber was suspended in twice its weight in 1/4 Ringer's buffer supplemented with 0.02% dieethyldithiocarbamic acid (DIECA) as an antioxidant. Tubers were then crushed in a Universal Extraction Bag (BIOREBA) using a hammer. 100 µl of the undiluted and 10^{-1} and 10^{-2} dilutions were mixed with 300 µl of NAt pre-warmed at 48 °C, and poured into the wells of a 24-well plate (Greiner). After agar had solidified, plates were wrapped with parafilm and incubated at 28 °C for 24-48 h. before screening for GFP and/or DsRed positive colonies as described before using an epifluorescence stereo microscope equipped with GFP and RFP plus filters.
All shoots (including leaves) per plant were collected and processed as a composite sample as well as the whole root system. At 7 dpi, all shoots were sampled as a composite sample per plant and at 28 days composite samples of 2 cm long stem cuttings taken 5 cm above the ground level were analyzed per plant. Both shoot and root samples were sterilized and bacterial density determined by pour-plaiting as described above for seed tubers.

Sampling of potato plants for confocal laser scanning microscopy (CLSM)

For microscopy, plant samples were collected 7 and 28 dpi (roots and shoots): 8 roots, 5 – 10 cm long, and 3 whole stems including leaves both randomly taken per

plant. Each root was cut into 2-3 cm long segments and each stem into 0.25 - 0.5 cm thick fragments. Fragments were embedded in molten NA at 48 °C containing 40 µg ml^{-1} of tetracycline and 200 µg ml^{-1} of cycloheximide in petri dishes. After the medium had solidified, the plates were sealed with parafilm and incubated for 1-2 days at 28 °C. Plant samples were removed from the agar plates, washed briefly in demineralized sterile water and examined under the confocal laser scanning microscope (CLSM).

Four roots per plant raised from tubers surfacially treated with GFP-tagged *S. plymuthica* A30 (treatment c) were processed without surface sterilization and without embedding directly after sampling to monitor bacterial populations on the root surface.

To visualize plant cells, 405 nm (excitation) ultraviolet laser with a 450 nm filter (emission) was used. For excitation of the GFP and DsRed in bacterial cells, 495 nm (blue) laser with 505 nm emission filters and 532 nm (green) lasers with 610 nm emission filters were used, respectively. Photographs were taken with a Leica Digital System (Leica) combined with a Leica CLSM microscope using 10x and 63x water immersion objectives.

Statistical analyses

Data were analyzed accordingly to the experimental design used, i.e. experiment replication in time, four treatments per replication, three different sampling time points and ten plants for each treatment and time point. The visual inspection of symptom development was a dichotomous score, e.g. no symptoms were observed or the emergence of blackleg and/or pre-emergence tuber rot was assessed. Data were analyzed with a generalized linear model (GLM) assuming data to arise from a binomial distribution. The logit link was used to stretch the binomial to normal distribution Bacterial count data were analyzed using a linear mixed model with replicates taken randomly in time. To approximate normality, counts were log transformed, adding a value of 1 to each value to deal with zero values. Effects were considered significant at the P=0.05. Pair-wise differences were obtained using the t-test. All analyses were performed with the statistical software package GenStat (Payne et al., 2008)

RESULTS

Construction of marker strains tagged with GFP or DsRed and their performance compared to the wild type strains

Transformation of *S. plymuthica* A30 with pRZ-T3-*gfp* and *Dickeya* sp. IPO2222 with pRZ-T3-*dsred* plasmids resulted in 43 and 29 transformants, respectively. One colony with high fluorescence was collected for each of the bacteria. Four repeated transfers of transformants on NAt plates with overnight incubation at 28 °C showed stable expression of GFP or DsRed. The presence of pRZ-T3-*gfp* in GFP-tagged A30 and pRZ-T3-*dsred* in IPO2222 (IPO3012) was demonstrated by plasmid DNA purification and agarose gel electrophoresis (data not shown).

GFP-tagged A30 and IPO3012 displayed similar growth characteristics in liquid media as the wild type A30 and IPO2222 strains, respectively, indicating that the growth of the strains was not affected either by the presence of the pRZ-T3 plasmids or by expression of fluorescent (GFP or DsRed) proteins (data not shown).

The ability of the IPO3012 and of wild type strain *Dickeya* sp. IPO2222 to macerate potato tuber tissue showed that the diameters of the rotting tissue were not significantly different (data not shown).

When the ability to inhibit growth of IPO2222 and IPO3012 by GFP-tagged *S. plymuthica* A30 was compared with the wild type *S. plymuthica* A30 strain in an overlay plate assay, there was no significant difference between the diameter of the clear halos indicating that both *S. plymuthica* A30 and GFP-tagged A30 strains inhibited equally both *Dickeya* sp. strains. (data not shown).

Testing the ability of the GFP-tagged *S. plymuthica* A30 and the wild type strain A30 to protect potato tuber tissue from maceration by IPO2222 and IPO3012 showed that there were no differences in the diameter of the rotting tissue (data not shown).

Density effect of GFP-tagged S. plymuthica A30 on tuber maceration by Dickeya **sp.**

The effect of inoculum density of GFP-tagged *S. plymuthica* A30 on its ability to protect potato tuber tissue against maceration caused by *Dickeya* sp. IPO3012 when co-inoculated was tested in a potato slice assay. Maceration of tuber tissue by IPO3012 was completely inhibited at a density of 10^8 cfu ml $^{-1}$ of GFP-tagged *S.*

plymuthica A30 (Fig. 1). Inhibition was less but was still significant at densities of 10^7 and 10^6 cfu ml^{-1}, but no significant inhibition was noted at 10^5 cfu ml^{-1} and 10^4 cfu ml^{-1}.

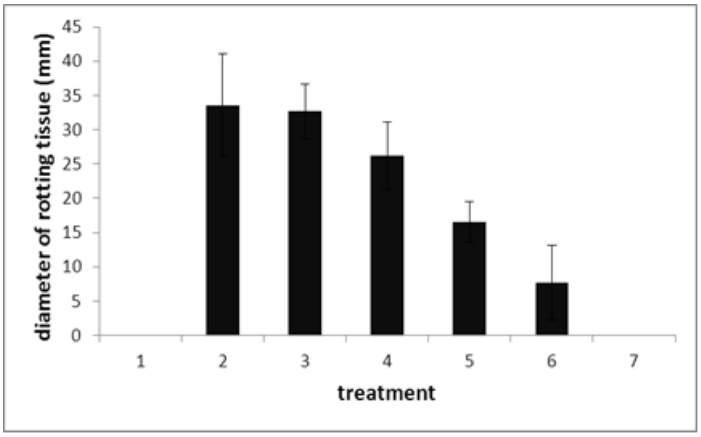


Figure 1. Reduction of the maceration ability of *Dickeya* sp. IPO2222 co-inoculated with GFP-tagged *S. plymuthica* A30 in potato tuber slices: Effect determined by measuring the diameter of rotting tissue (in mm) after 72 h incubation at 28 °C in a humid box. Wells of potato slices were filled up with 50 µl of sterile water (negative control) with 50 µl bacterial suspension in water containing 10^6 cfu ml^{-1} of *Dickeya* sp. IPO2222 (positive control) or with 50 µl of bacterial suspension in water containing 10^6 cfu ml^{-1} of *Dickeya* sp. IPO3012 together with different densities of *S. plymuthica* A30 (0, 10^4, 10^5, 10^6, 10^7 and 10^8 cfu ml^{-1}). Three potato slices containing 3 wells each and derived from three different tubers were used per treatment. The experiment was independently repeated one time and the results were averaged. legend: 1. negative control (water), 2. positive control (10^6 cfu ml^{-1} *Dickeya* sp. IPO2222), 3. 10^6 cfu ml^{-1} *Dickeya* sp. IPO2222 + 10^4 cfu ml^{-1} *S. plymuthica* A30, 4. 10^6 cfu ml^{-1} *Dickeya* sp. IPO2222 + 10^5 cfu ml^{-1} *S. plymuthica* A30, 5. 10^6 cfu ml^{-1} *Dickeya* sp. IPO2222 + 10^6 cfu ml^{-1} *S. plymuthica* A30, 6. 10^6 cfu ml^{-1} *Dickeya* sp. IPO2222 + 10^7 cfu ml^{-1} *S. plymuthica* A30, 7. 10^6 cfu ml^{-1} *Dickeya* sp. IPO2222 + 10^8 cfu ml^{-1} *S. plymuthica* A30 (vertical lines represent standard error)

Population dynamics of GFP-tagged S. plymuthica A30 and Dickeya sp. *IPO3012 on potato tuber slices*

Population dynamics of *S. plymuthica* A30-GFP and *Dickeya* sp. IPO3012 after inoculation singly or jointly on potato slices showed that after 3 days, densities of *Dickeya* sp. IPO3012 on control slices without A30 added, increased from 10^7 cfu g^{-1} to 10^{11} cfu g^{-1} with progressive rotting of the potato slices. In contrast, populations of GFP-tagged *S. plymuthica* A30 on control slices without added *Dickeya* sp. decreased rapidly from $10^7 – 10^8$ cfu g^{-1} at 0 dpi to $10^1 – 10^2$ cfu g^{-1} at

2 dpi and 0 cfu g^{-1} at 3 dpi. No rotting of potato slices was observed, although after 3 days, a slight brown discoloration of tuber tissue was found, not visible in the water control slices. Joint inoculation of tuber slices with GFP-tagged A30 strain and IPO3012 resulted in a decrease in population densities for both bacterial species. No GFP-tagged A30 or DsRed tagged IPO3012 bacteria were recovered from inoculated potato slices at 3 dpi (Fig. 2) and no rotting of the slices was observed (data *not* shown).

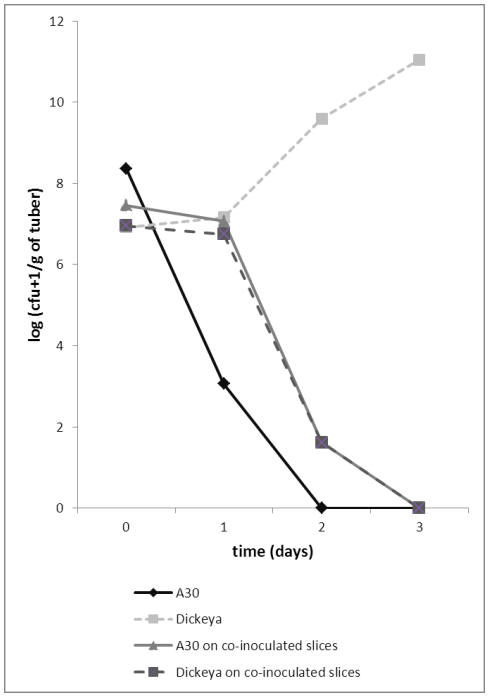


Figure 2. Population dynamics of GFP-tagged *S. plymuthica* A30 and *Dickeya* sp. IPO3012 on potato slices. Potato slices were inoculated either with GFP-tagged *S. plymuthica* A30, DsRed-tagged *Dickeya* sp. IPO3012 or co-inoculated with both strains. At time 0, 1, 2 and 3 days post inoculation, plant material was collected from the inoculated wells and crushed in the presence of 1/4 Ringer's buffer. Serial dilutions of plant extract were poor plated in NAt and green and red fluorescent colonies were counted. Experiment was independently repeated one time and results were pooled. Results from six independent samples per treatment and per time point were averaged.

Greenhouse experiments

Disease development

Treatment (a) In plants grown from minitubers inoculated with DsRed-tagged *Dickeya sp.* IPO3012 the first symptoms appeared 7 dpi, when shoots were ca. 5 – 7 cm and roots were ca. 8-12 cm long. The pathogen severely affected sprouting and plant development: non-emergence (approx. 20 – 30%) was due to rotting of seed tubers inside soil. Deterioration of shoots and typical blackleg symptoms, *i.e.* wilting and chlorosis of leaves as well as stem wet rot, first developed at 7 dpi. By 28 dpi, 60% and 50 % of inoculated plants in experiment 1 and 2, respectively, showed characteristic blackening and soft rotting of the stem basis.
Treatment (b) Incidences of non-emergence and of blackleg symptoms were significantly reduced in plants grown from seed tubers inoculated with DsRed-tagged *Dickeya sp.* IPO3012 and treated with GFP-tagged *S. plymuthica* A30 strain before planting. In Experiment 1, at 7 dpi, only 10% of the plants showed pre-emergence seed tuber rot and stunted stem growth and no blackleg symptoms developed even at 28 dpi. In Experiment 2, none of the inoculated plants showed pre-emergence tuber rot and blackleg symptoms at any time.
Treatments (c & d) None of the plants grown from seed tubers inoculated with water and with GFP-tagged *S. plymuthica* A30 showed any non-emergence and blackleg symptoms during the entire course of both experiments.

Population dynamics of GFP-tagged *S. plymuthica A30 and* DsRed-tagged *Dickeya* sp. *IPO3012 in planta*

Population dynamics of GFP-tagged *S. plymuthica* A30 and DsRed-tagged *Dickeya* sp. IPO3012 in plants have been evaluated at 1 day post inoculation (dpi) in tubers only, and at 7 and 28 dpi in tubers, roots and shoots in all four treatments. Population dynamics of bacteria in the three plant parts were examined by NAt pour plating. In none of the water inoculated control plants (treatment (d) were GFP and Dsred-tagged bacteria nor blackleg symptoms found.

Bacterial populations inside seed tubers

In seed tubers of A30 treated plants (treatment (c)), relatively low populations (average ca. 10^1 cfu g^{-1}) of the bacteria were detected at 1 dpi (Fig. 3A). Populations increased in 7 days to $10^2 – 10^3$ cfu g^{-1} and remained at this level till 28

dpi. With sequentially treated plants (treatment (b)), *S. plymuthica* A30 populations in tubers at 1 dpi were on average 10^3 cfu g^{-1} but decreased to 10^2 cfu g^{-1} in the next 6 days and remained at this level till 28 dpi. In contrast, *Dickeya* sp. populations had decreased significantly from 10^4 cfu g^{-1} at 1 dpi to on average 1 cfu g^{-1} or less at 28 dpi. With *Dickeya* sp. treated plants (treatment (a), populations of 10^4 cfu g^{-1} of the bacteria were detected at 1 dpi and at 28 dpi populations had declined only slightly to $10^3 – 10^4$ cfu g^{-1}.

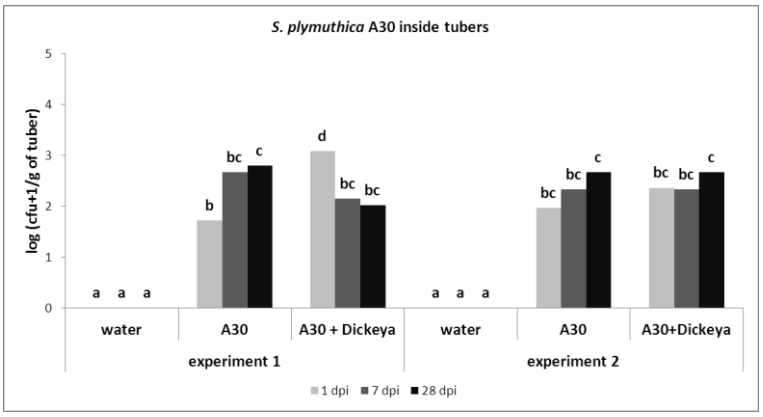
Bacterial populations in roots

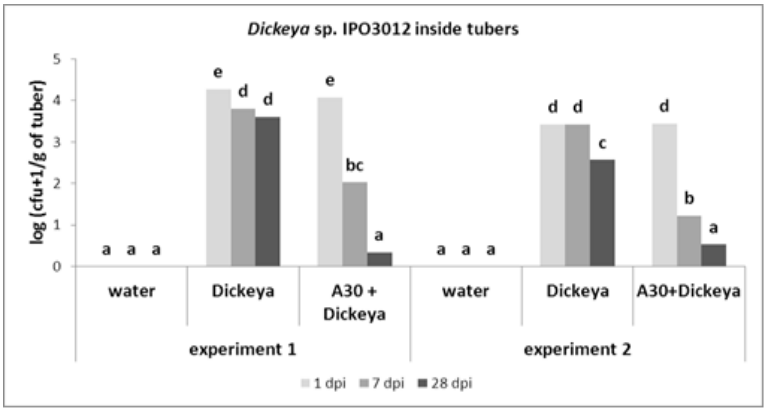
Bacterial populations in roots were analyzed only at 7 and 28 dpi, as at 1 dpi no roots had developed yet. In treatment (c) at 7 dpi, *S. plymuthica* A30 was present inside roots at a density of 10^2 cfu g^{-1} (Fig. 3B). At 28 dpi, the population had increased to $10^3 – 10^4$ cfu g^{-1}. In roots of plants of the sequentially inoculated with A30 and IPO3012 (treatment (b)), population dynamics of A30 followed a similar patter as above. In contrast, no *Dickeya* sp. was detected in roots at 7 dpi and at 28 dpi, only low populations averaging to 1 cfu g^{-1} were detected. In the *Dickeya* sp. IPO3012 treated plants (treatment (a)), low *Dickeya* sp. populations (< 10^1 cfu g^{-1}) were found at 7 dpi. Populations increased slightly to 10^1 - 10^2 cfu g^{-1} at 28 dpi.

Bacterial populations in shoots

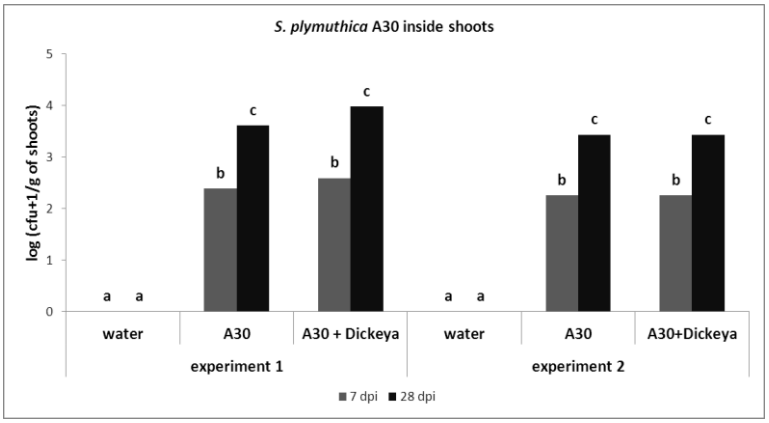
Bacterial populations in shoots were analyzed only at 7 and 28 dpi, as at 1 dpi shoots had not yet been formed (Fig. 3C). In *S. plymuthica* A30 treated plants (treatment (c)), at 7 dpi the bacterium was already present in shoots at a density of 10^2 cfu $g^{-1}$and at 28 dpi, the size of the population had increased 10 times. In sequentially inoculated plants with GFP-tagged *S. plymuthica* A30 and DsRed-tagged *Dickeya* sp. IPO3012 (treatment (b), the population dynamics of A30 again followed a similar trend as above. At 7 dpi less than 10 cfu g^{-1} of *Dickeya* sp. were present and none was detected at 28 dpi., Stems of plants treated with *Dickeya* sp. IPO3012 only (treatment (a)) at 7 dpi had low densities of the bacteria, 5 - 10 cfu g^{-1}. Populations increased to a density of 10^2 - 10^3 at 28 dpi.

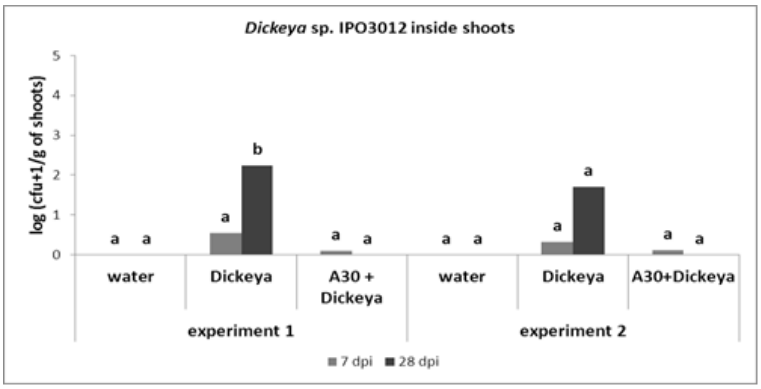
A


S. plymuthica A30 inside tubers
log (cfu+1/g of tuber)
water
A30
A30 + Dickeya
water
A30
A30+Dickeya
experiment 1
experiment 2
1 dpi
7 dpi
28 dpi

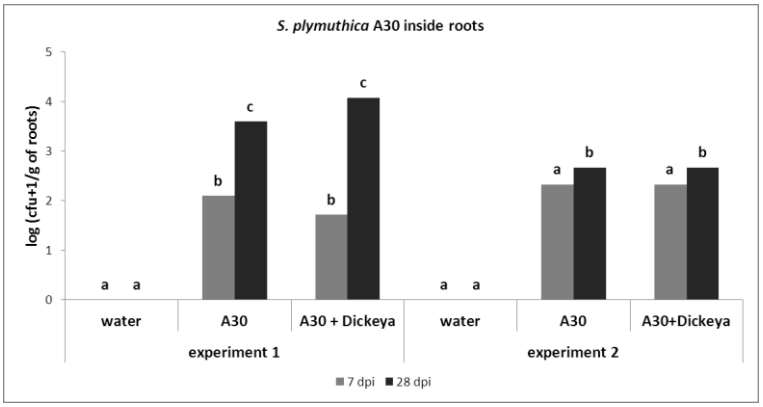

Dickeya sp. IPO3012 inside tubers
log (cfu+1/g of tuber)
water
Dickeya
A30 + Dickeya
water
Dickeya
A30+Dickeya
experiment 1
experiment 2
1 dpi
7 dpi
28 dpi

B


S. plymuthica A30 inside shoots
log (cfu+1/g of shoots)
water
A30
A30 + Dickeya
water
A30
A30+Dickeya
experiment 1
experiment 2
7 dpi
28 dpi



C



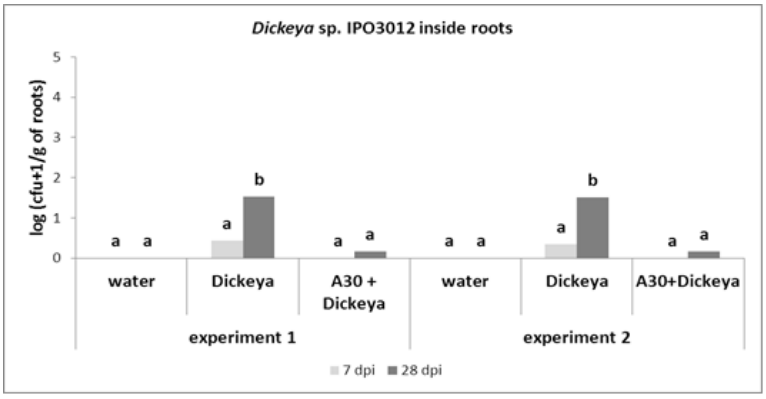


Figure 3. Population dynamics of GFP-tagged *S. plymuthica* A30 and DsRed-tagged *Dickeya* sp. IPO3012 in seed tubers (A), stems (shoots) (B), and in roots (C) sampled 1 (tubers only), 7 and 28 days (tubers, roots and shoots) post inoculation (dpi) in experiment 1 and 2. The whole seed tuber and all roots were sampled per plant at each time point. At 7 dpi, all shoots were sampled as a composite

sample per plant and at 28 days composite samples of stem cuttings taken 5 cm above the ground level were analyzed per plant. The average values are shown from ten plants per time point. Statistical analysis was done per subsample and per time point (n=10). Values followed by identical characters are not significantly different (P=0.05)

Plant colonization examined by confocal laser scanning microscopy

Stems and roots were analyzed with a CLSM at a magnification of x640 and x1000. Results (Fig. 4) showed that at 7 dpi both bacterial species were present inside roots in plants grown from minitubers sequentially inoculated with GFP-tagged *S. plymuthica* A30 and DsRed-tagged *Dickeya* sp. IPO3012 (treatment (b)). Green (*S. plymuthica* A30) and red (IPO3012) fluorescent cells were found inside xylem vessels and between protoxylem cells of the vascular tissue of roots and also in the medulla and cortex of the pith, both intra- and intercellularly. In stems, green and red fluorescent bacterial cells were observed inside and between xylem vessels and protoxylem cells of vascular tissue. At 28 dpi only green fluorescent cells were observed in roots and stems, indicating that GFP-tagged *S. plymuthica* A30 but not IPO3012 was present.

At 28 dpi, in DsRed-tagged *Dickeya* sp. inoculated (control) plants (treatment (a)), red fluorescent bacteria were present inside and between pith cells of roots and inside and between xylem vessels of stems. Similarly, in plants inoculated with GFP-tagged *S. plymuthica* A30 (treatment c), green fluorescent bacteria were present inside and between parenchyma cells of roots and in xylem vessels of stems. In none of plant parts inoculated with water (treatment d) green and/or red fluorescent bacteria were found.

The ability of GFP-tagged *S. plymuthica* A30 to colonize roots of potato plants was tested by analyzing randomly selected roots from plants at 28 dpi using CLSM. All roots of plants grown from A30 inoculated tubers (treatment (c)) were superficially colonized by green fluorescent cells. GFP-tagged bacteria occurred in clumps or patches on the root surface, interspersed by areas where bacteria were absent or in which only low densities were present (Fig. 5). In none of water control plants GFP-tagged bacteria on root surface were detected.

DISCUSSION

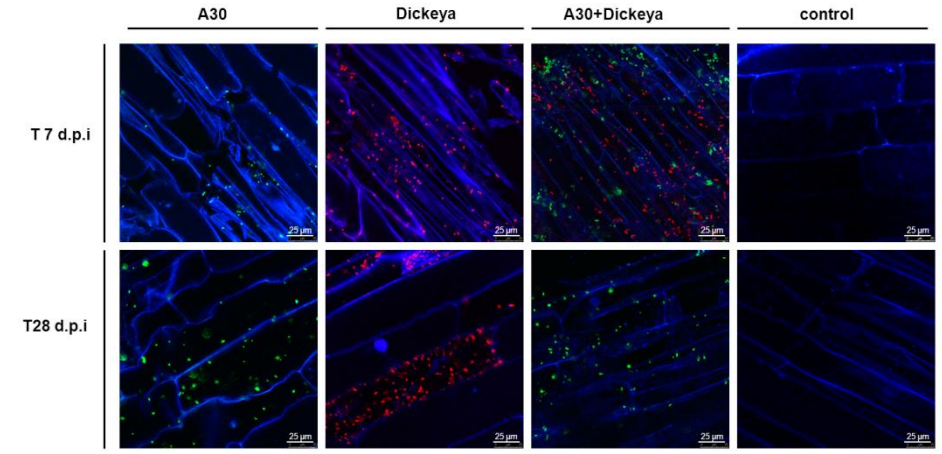
Previously we have shown that when, seed tubers were vacuum co-infiltrated with *Dickeya* spp. and high densities of *S. plymuthica* A30 and then planted in compost, blackleg level caused by *Dickeya* sp. IPO3012 was reduced by

100% and the incidence of stems colonization fell by 97% (Czajkowski et al., 2011a). In this study, experimental conditions were chosen to be more realistic for a field application: seed tubers were treated by superficial wetting with a suspension of the antagonist prior to planting in potted field soil. This tuber treatment method is similar to that commonly used when fungicides such as monceren (pencycuron) are applied to seed tubers to control *Rhizoctonia solani* (Wicks et al., 1995). The results obtained here suggest that *S. plymuthica* A30 has been still effective in controlling blackleg and soft rot caused by the test strain used. Blackleg incidence was reduced from 55% in the control *Dickeya* sp. inoculated treatment to 0% . The effectiveness of strain A30 appears to be independent from the way tubers were treated and whether grown in compost or field soil.

S. plymuthica strains have been frequently found in association with plants. They have been isolated from the rhizospheres of wheat, oat, cucumber, maize, oilseed rape and potato (Åström & Gerhardson, 1988), as endophytes from the endorhiza of potato (Berg et al., 2005) and also found in onion, carrot, lettuce, *Brassica* spp. leaves as well as in the phyllosphere of spring wheat (De Vleesschauwer & Hofte, 2007). They has been used extensively before for biocontrol of fungal diseases (De Vleesschauwer & Hofte, 2007) but not to our knowledge for the control of bacterial pathogens. *S. plymuthica* rhizosphere isolates have been frequently used to control soil-borne fungal pathogens of plants. In contrast *S. plymuthica* strains isolated from internal plant tissues were rarely used in biological control (Bowen & Robvira, 1974, Brown, 1974, Whipps, 2001) (Åström & Gerhardson, 1988, Weller, 1988).

S. plymuthica A30 was initially isolated from rotting tissue of surface-sterilized potato tubers, which suggests that it is an endophyte rather than a commensal from the tuber surface (Czajkowski et al., 2011a). When applied to seed tubers, it readily colonized tubers as well as roots and stems of the growing plant. Results obtained by both dilution plating and confocal laser scanning microscopy showed that within one day post application, the bacterium was present inside surface sterilized seed tubers. Colonization of internal seed tuber tissues so quickly could be attributed to penetration in lenticels. Seven days after planting, it was found in large numbers in the vascular tissue of roots and stems (Fig. 4). A30 may have entered roots via openings that occur during lateral root formation, like many bacterial species do (Huang & Allen, 2000). Presence of wounds or degradation of the root tissue does not appear to be required for root colonization (Huang, 1986). An increase of A30 population numbers with time indicates that *S. plymuthica* actively grew inside plants (Fig 3.).

A



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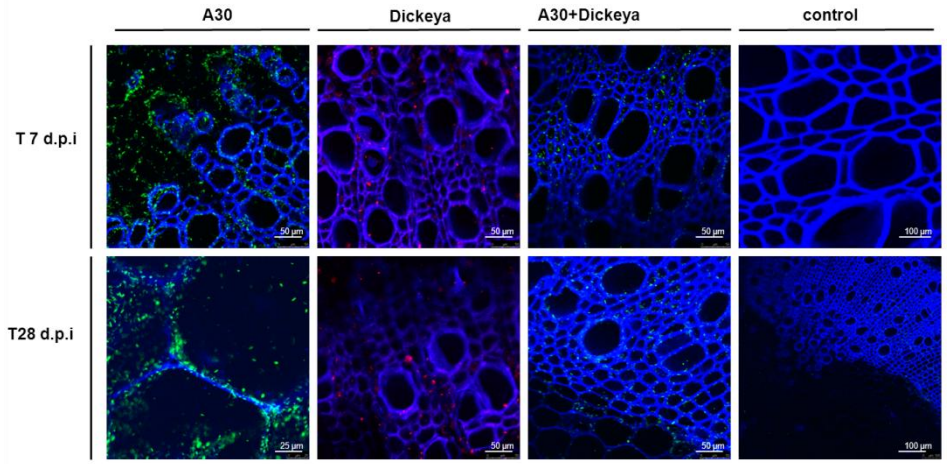


Figure 4. Internal colonization of surface sterilized potato roots (A) and stems (B) by GFP-tagged *S. plymuthica* A30 and DsRed-tagged *Dickeya* sp. IPO3012, 7 and 28 days post inoculation analyzed with confocal laser scanning microscopy. Samples were taken from plants from which tubers were inoculated at planting with GFP-tagged *S. plymuthica* A30 (*A30*), from plants raised from potato minitubers vacuum infiltrated with DsRed-tagged *Dickeya* sp. IPO3012 (*Dickeya*) and after sequential-inoculation of the minitubers with both strains (*A30+Dickeya*). For control, potato minitubers were vacuum infiltrated with sterile water (*control*). Samples were embedded in NAt (nutrient agar supplemented with 40 µg ml^{-1} of tetracycline) and incubated for 2 days at 28 °C.

Dickeya spp. as well as *Pectobacterium* spp. in rotting mother tubers are translocated via the vascular system up the growing stems (Czajkowski et al., 2010a) (Perombelon, 1974). In addition, the bacteria are also released into wet soil and find their way to and penetrate the root system of both the mother and neighboring potato plants resulting in both instances in systemic infection of plants including the progeny tubers. The ease by which A30 is able to colonize the roots and stems of potato plants, explains its ability to protect the plants against infection by *Dickeya* spp. from soil.

The basis for A30 antagonist effect is not clear. It is known that the A30 strain produces antibiotics against biovar 3 *Dickeya* spp. (Czajkowski et al., 2011a), and preliminary results suggest that A30 mutants defective in antibiotic production/secretion did not prevent tuber maceration when tested *in vitro* (Czajkowski, unpublished results). In addition, A30 and the pathogen are located in the same niche *in planta* and the antagonism depends possibly also on the ability to compete for nutrients. However, other factors cannot be excluded, including induction of systemic resistance of potato plants against biovar 3 *Dickeya* sp. Strains of *S. plymuthica* were reported to induce systemic resistance in various crops; *S. plymuthica* R1GC4 stimulates defense mechanisms against fungal pathogens in cucumber (Benhamou et al., 2000) and *S. plymuthica* IC270 stimulates rice plants defense against *Magnaporthe oryzae* causal agent of rice blast disease (De Vleesschauwer et al., 2009).

The efficacy of A30 to control *Dickeya* sp. appears to be density dependent. In a potato slice assay, GFP-tagged *S. plymuthica* A30 was able to prevent potato tissue maceration by *Dickeya* sp. if applied at a minimum density which was 10 - 100 times higher than the density of *Dickeya* sp. (Fig 1). However, a considerable reduction of tuber rotting was still observed at lower densities. It is generally accepted that the biocontrol agent must be applied at a higher density than the pathogen to achieve an satisfactory level of protection (Parke, 1990). What is surprising is the drop in numbers of the antagonist in tuber slice assays within 3 days when co-inoculated with *Dickeya* sp. or on its own (Fig. 2). The conditions on cut tuber slices could be somehow detrimental to the bacterium due to the presence of phenolics and other wound metabolites.

The density dependence of the efficacy in the slice assay may be related to the exploitation of a quorum sensing mechanism by A30. It is known that in *S. plymuthica* HRO-C48, acyl-homoserine lactone (AHLs) based quorum sensing (QS) signaling is involved in the regulation of important biocontrol mechanisms, including protection of cucumber against *Pythium apahnidermatum* and induction

of systemic resistance in bean and tomato plants against *Botrytis cinerea* (Pang et al., 2009), QS participates also in motility and indole-3-acetic acid and hydrolytic enzyme production in HRO-C48 strain (Müller et al., 2009). *S. plymuthica* A30 is known to produce AHLs (Czajkowski et al., 2011a) and therefore it can be speculated that the strain uses the QS mechanism in the same way as strain HRO-C48. The population density of *S. plymuthica* A30 inside roots, tubers and shoots was stable for at least 28 days at a level 10^3 – 10^4 cfu g^{-1} after tuber application . This density in internal plant tissue seems to be sufficient to trigger the quorum sensing mechanism (von Bodman et al., 2003)

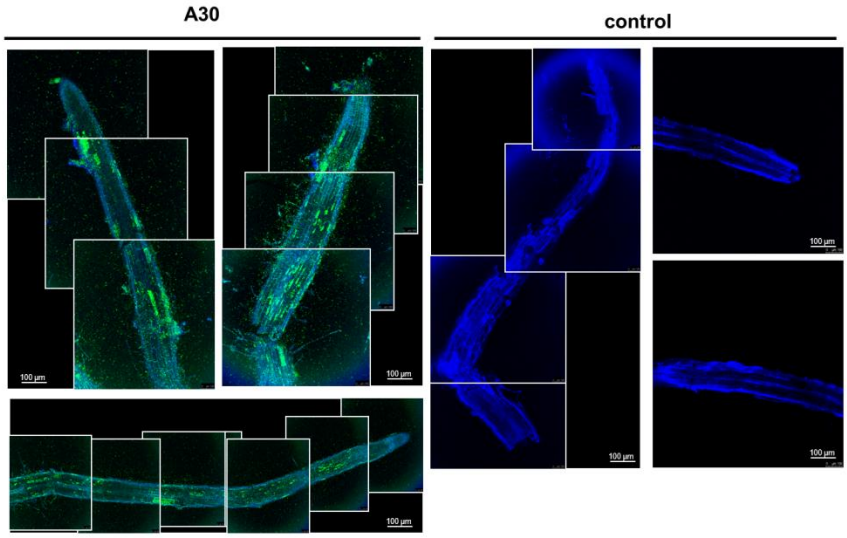


Figure 5. Colonization of the potato root surface by GFP-tagged *S. plymuthica* A30 (*A30*), 28 days post treatment of seed tubers. Roots were freshly collected and briefly washed in sterile tap water to remove soil particles. Plant samples were analyzed with a confocal laser scanning microscopy. For control, roots collected from negative control plants were used (*control*). For counter staining of plant cells, UV light was used.

At present the inoculum density of A30 able to protect tubers against *Dickeya* sp. in the field is unknown. In the greenhouse experiments relatively high densities of 10^{11} – 10^{12} cfu ml^{-1} of the antagonist were used for tuber application, but possibly lower densities can be used in the field trials if bacterial preparation is formulated to increase inoculum stability. For commercial reasons, it would be

necessary to decrease the density to a more realistic level, such as $10^6 – 10^9$ cfu ml^{-1} which are commonly used in commercial applications with formulated bacteria (Kloepper & Schroth, 1980, Vidhyasekaran & Muthamilan, 1995).

In conclusion, although the results obtained in this study are promising for bio-control of blackleg caused by the virulent *Dickeya* sp. biovar 3 using *S. plymuthica* A30 as an antagonist, there is still considerable work to be done to achieve a viable commercial application. Aspects which require further examination especially are: formulation of a stable bacterial preparation, optimization of application procedures, longevity of the applied antagonist in soil at least long enough to bridge the gap between planting and shoot and root growth and possibly also in subsequent crop generations, effectiveness when using standard size seed tubers with well-set skin in a wide range of cultivars and under different edaphic and environmental conditions. Finally, elucidation of the antagonism mechanism could be of value for use in other pathogen host combinations.

ACKNOWLEDGMENTS

The project was financed by STW Foundation (Technologiestichting STW, The Netherlands) via grant no. 10306 “Curing seed potato from blackleg causing bacteria”. We thank P. S. van der Zouwen (PRI, The Netherlands) for technical assistance and M. C. M. Perombelon (ex. SCRI, UK) and L. J. Hyman (ex SCRI, UK) for their highly valuable comments on the manuscript and their editorial work.

Chapter 8

Summarizing Discussion

This thesis focuses on the ecology and pathogenicity of biovar 3 *Dickeya* sp. provisionally called "*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 "*D. solani*" from infected tubers.

NEW INFECTION ROUTES IN RELATION TO PATHOGENESIS OF BIOVAR 3 *DICKEYA* SPP.

Potato seed tubers are the main source of blackleg and soft rot inoculum and the predominant route for long-distance spreading of *Dickeya* and *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 *Dickeya* spp. and *Pectobacterium carotovorum* subsp. *carotovorum* but not with *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 cfu g^{-1}) of pectinolytic bacteria were also found in peels, these densities calculated per gram of tuber tissue were low (approx. 10^1 cfu g^{-1}) compared to the densities in stolon ends (10^2 – 10^3 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 EFFECTIVNESS 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.

SUMMARY

Potato blackleg caused by pectinolytic *Pectobacterium* and *Dickeya* species is a bacterial disease creating serious economic losses in (seed)potato production worldwide. Effective management to control blackleg is absent and validated, cost-effective detection protocols for blackleg bacteria do not exist. This situation results partially from the lack of knowledge of the ecology of blackleg pathogens in general, and *Dickeya* spp. in particular. Since 2000 an increasing frequency of *Dickeya* spp. in seed potatoes in Europe has been observed which is connected to emergence of a new species belonging to biovar 3 but unclassified inside known and described six *Dickeya* species. This species was provisionally named "*D. solani*" and isolated from potato in The Netherlands, Finland, Poland, Germany, Belgium, France, United Kingdom and Israel.

The objectives of the study described in this thesis were to acquire knowledge on the ecology (i. e. distribution of *Dickeya* spp. in seed potato tubers, role of the soil-borne inoculum in disease incidence, role of the aerial haulm infection for the blackleg incidence in progeny tubers) of a new genetic clade of biovar 3 *Dickeya* spp.; and to find and characterize in detail the valuable biocontrol agent(s) (isolated from rotten potato tissue) to cure seed potato tubers from blackleg caused by biovar 3 *Dickeya* spp. ("*D. solani*").

Direct isolation of viable bacterial cells using (selective) plating techniques combined with molecular (16S rDNA and Rep-PCR), serological (DAS-ELISA) and biochemical (biovar determination) characterization of the isolates were used to evaluate the presence of *Dickeya* and *Pectobacterium* spp. in different plant parts and tissues. Bacterial population dynamics *in planta* was studied in greenhouse experiments. For the studies on colonization of potato tissues by biovar 3 *Dickeya* spp. ("*D. solani*") and *Serratia plymuthica* A30, bacterial strains were transformed with plasmid-based genes coding for red or green fluorescent proteins (DsRed and GFP, respectively) that were constitutively expressed in bacterial cells. Epifluorescence stereo microscopy and confocal laser scanning microscopy were used to visualize bacterial presence in different tissues of potato tubers and plants.

Studies on the distribution of *Dickeya* (and "*D. solani"*) and *Pectobacterium* species in seed potatoes revealed that the pathogens were distributed unevenly inside tubers and that the stolon end tuber part harbored the highest bacterial inoculum, whereas deeper located tissues were usually not colonized. Studies on the importance of soil-borne biovar 3 *Dickeya* sp. ("*D.*

solani") inoculum for systemic colonization of potato plants and latent infection of progeny tubers unveiled that *D. solani* is able to systemically colonize progeny tubers from soil via the roots and to cause true blackleg symptoms in infected plants after soil infestation.

Studies on the role of haulm infections for latent contamination of progeny tubers by *D. solani* presented that the injection of bacteria into stems can result in the downward vascular translocation and a colonization of underground plant parts and finally, latent contamination of progeny tubers.

Potato leaves inoculation with a biovar 3 *Dickeya* sp., showed degradation of the inoculated plant material and spreading of the internal inoculum to the petiole and axil and finally to the main stem but bacteria were not detected in the underground plant parts. Studies on characterization of the bacteria antagonistic to biovar 3 *Dickeya* sp. ("*D. solani*") allowed to obtain knowledge of the presence of such strains in rotten potato tissue and on possibility of their used in the biocontrol of *Dickeya* spp. in potato.

Selection of a candidate strain (*Serratia plymuthica* A30) that was effective in protecting potato plant tissue from blackleg was performed on the basis of *in vitro* and greenhouse tests. *In vitro* tests proved that *S. plymuthica* A30 possess different mechanisms by which it can control *Dickeya* spp. (i. e. production of antibiotics and biosurfactants). The interaction of *D. solani* and *S. plymuthica* A30 under greenhouse conditions was investigated in detail using artificially co-inoculated tubers and by application of A30 to the soil. It was showed that both micro-organisms interact *in situ* and that *S. plymuthica* A30 is able to decrease or eradicate *Dickeya* sp. inoculum from infected potato plants by competition un the same niches.

In conclusion, a new biovar 3 *Dickeya* spp. strain was present in high densities in stolon end of naturally infected progeny tubers. The bacterium efficiently colonized various plant tissues after artificial inoculation of soil or haulms and was able to systemically colonize entire potato plant in relatively short time.

For biocontrol of biovar 3 *Dickeya* spp. in potato, a bacterial strain characterized as *S. plymuthica* A30 tested in *in vitro* and under greenhouse conditions proved to have great potential for controlling blackleg in conditions favorable for disease development and when *Dickeya* sp. was applied in the high

inoculum. The A30 strain effectively protected potato plants against blackleg in greenhouse experiments, both in vacuum infiltrated tuber and when applied as superficial treatment during planting. Treatment with A30 reduced the disease incidence to 0%. *S. plymuthica* A30 was able to colonize the plants internally, and combat internal infections with *D. solani*.

SAMENVATTING

De aardappelziekten zwartbenigheid en natrot worden veroorzaakt door bacteriën van het geslacht *Pectobacterium* en *Dickeya*. Deze bacterieziekten worden gezien als het belangrijkste probleem in de pootaardappelteelt en veroorzaken wereldwijd grote economische schade aan (poot)aardappelen. Er zijn geen effectieve middelen om zwartbenigheid te bestrijden en er zijn geen resistente aardappelcultivars bekend. Er wordt getracht de ziekten te beheersen door keuring van pootgoed en door hygiënische- en teeltmaatregelen die introductie en verspreiding van deze ziekteverwekkers tegengaan. Hiervoor is, echter, kennis nodig over de ecologie van *Dickeya* en *Pectobacterium* soorten.

Sinds 2000 is er in Europa in aardappelpootgoed een toename waargenomen van *Dickeya*. De schade wordt veroorzaakt door een nieuwe biovar 3 stam die niet kon worden geclassificeerd binnen de zes beschreven *Dickeya* soorten. Deze soort wordt voorlopig "*D.solani*" genoemd en is gevonden in aardappelplanten afkomstig uit Nederland, Finland, Polen, Duitsland, België, Frankrijk, Engeland en Israel.

Het doel van deze studie was: (i) het verkrijgen van inzicht in de verdeling van *Dickeya* spp. in dochterknollen, (ii) het analyseren van infectieroutes die kunnen leiden tot systemische verspreiding in planten en besmetting van dochterknollen, (iii) het vinden en karakteriseren van geschikte biologische middelen om aardappelpootgoed te vrijwaren van zwartbenigheid veroorzaakt door "*D. solani"*.

In de studies naar de kolonisatie van aardappel weefsels door biovar 3 *Dickeya* spp. ("*D. solani*"), en *Serratia plymuthica* A30, werd gebruik gemaakt van bacteriestammen die getransformeerd waren met plasmide-gebaseerde genen die coderen voor een rood of groen fluorescerend eiwit (GFP en RFP, respectievelijk) en constitutief tot expressie komen in bacteriële cellen. Naast uitplaattechnieken, werden epifluorescentie stereo-microscopie en confocale laser scanning microscopie gebruikt om de bacteriën te visualiseren in plantenweefsels of grond.

Onderzoek naar de verdeling van "*D. solani"* en *Pectobacterium* soorten in aardappelknollen toonde aan dat de pathogenen onregelmatig verdeeld zijn in het knolweefsel. Stolonen bevatten relatief hoge bacteriedichtheden terwijl het periderm relatief lage dichtheden bevatte. Dieper gelegen weefsel werd meestal niet gekoloniseerd.

Onderzoek naar infecties van biovar 3 *Dickeya* sp. (*"D. solani"*) vanuit de grond toonde aan dat " *D. solani* "in staat was om wortels te infecteren en vandaar

uit systemisch het vaatsysteem van de plant te koloniseren, inclusief dat van de dochterknollen. Wortelinfecties vanuit de grond resulteerden zelfs in ziektesymptomen. Ook infectie van stengels leidde tot systemische verspreiding van de bacterie in de plant. Er vond translocatie van de bacterie in de houtvaten plaats tegen de stroom in tot in de ondergrondse delen van de plant, waardoor uiteindelijk een latente besmetting van de dochterknollen plaatsvond. Inoculatie van aardappelblad met een biovar 3 *Dickeya sp.* gaf wel degradatie van het bladweefsel te zien en verspreiding van het inoculum naar de bladsteel en hoofdstengel, maar er vond geen verspreiding naar ondergrondse delen plaats.

Voor onderzoek naar de potentie van antagonisten voor bestrijding van *Dickeya* soorten werden er bacteriën geïsoleerd uit rottend aardappelknolweefsel. De bacteriën werden getoetst *in vitro* op antagonisme tegen *"D. solani"* op basis van de aanmaak van antibiotica of productie van sideroforen. Relatief vaak werden bacteriën geïsoleerd die behoorden tot de groep van opportunistische humaan pathogenen. Deze werden niet voor verder onderzoek gebruikt. Eén stam (*Serratia plymuthica* A30), die antibiotica tegen "*D. solani"* produceerde, bleek in kasexperimenten een effectieve bescherming te geven tegen zwartbenigheid, zowel bij vacuüminfiltratie als bij oppervlakkige behandeling van knollen. Behandeling met A30 verminderde de ziekte incidentie tot 0%. *S. plymuthica* A30 was in staat de plant intern te koloniseren; ook interne infecties met *D. solani* konden daardoor effectief bestreden worden.

STRESZCZENIE

Bakteryjne choroby ziemniaka (*Solanum tuberosum* L.): czarna nóżka (ang. blackleg) i mokra zgnilizna (ang. soft rot) powodowane przez bakterie z rodzaju *Pectobacterium* i *Dickeya*, są przyczyną dużych strat ekonomicznych w produkcji sadzeniaków na całym świecie. Eliminacja możliwości infekcji polega na stosowaniu zabiegów higienicznych oraz stosowaniu certyfikowanego materiału siewnego, wolnego od patogenów. Wykorzystywane do kontroli czarnej nóżki i mokrej zgnilizny rutynowe metody detekcji bakterii *Pectobacterium* spp. i *Dickeya* spp. przez narodowe stacje ochrony roślin są niewystarczające, często drogie, o niskim poziomie skuteczności i dużym ryzyku błędów. Brak dobrych i sprawdzonych metod detekcji bakterii z rodzaju *Pectobacterium* i *Dickeya* spp. wynika między innymi z ograniczonej wiedzy o ekologii tych patogenów, ich przeżywalności w środowisku, zajmowanych niszach i podłożu procesów chorobowych.

Do roku 2000 w Europie, główną przyczyną występowania czarnej nóżki ziemniaka były bakterie z rodzaju *Pectobacterium* (najczęściej *P. atrosepticum*, także z udziałem *P. carotovorum* subsp. *carotovorum*) a infekcje powodowane przez *Dickeya* spp. miały mały, często bardzo ograniczony udział w patogenezie. Od roku 2000 notuje się duży wzrost udziału bakterii z rodzaju *Dickeya* spp. w zachorowaniach roślin ziemniaka na czarną nóżkę i mokrą zgniliznę. Sytuacja ta jest najprawdopodobniej spowodowana pojawieniem się nowego gatunku *Dickeya* spp. należącego do biowaru 3, wcześniej nie obserwowanego w Europie. Ten nowy gatunek bakterii określono jako *D. solani*, a jego obecność do tej pory stwierdzono w uprawach ziemniaka w Holandii. Polsce, Finlandii, Niemczech, Belgii. Francji, Wielkiej Brytanii i Izraelu, a ostatnio w także w Szwecji, Gruzji, Armenii i na Ukrainie. Obecność *D. solani* w wielu europejskich krajach i częste straty w uprawach ziemniaka wskazują, że bakterie *D. solani* są bardzo wirulentne i świetnie przystosowane do europejskich warunków środowiskowych (w tym do zmian temperatury i wilgotności). Uważa się obecnie, że *D. solani* stanowi poważne zagrożenie dla produkcji sadzeniaków w Europie a straty finansowe w produkcji sadzeniaków spowodowane przez czarną nóżkę szacuje się na miliony euro rocznie.

Przedmiotem tej pracy doktorskiej było poznanie ekologii bakterii *D. solani* (między innymi, ich udziału w infekcji bulw sadzeniaków, dystrybucji bakterii w tkankach bulwy, roli populacji glebowych w rozwoju czarnej nóżki, roli zakażeń liści i łodyg ziemniaka w patogenezie czarnej nóżki i obecności bakterii w bulwach potomnych) oraz wykorzystaniu tej wiedzy do opracowania efektywnej

biologicznej metody ochrony ziemniaka przed zakażeniem z wykorzystaniem bakteryjnych endofitów i bakterii glebowych izolowanych ze zgniłej tkanki bulw ziemniaka

Doświadczenia na bulwach ziemniaków wykazały, że w naturalnie zainfekowanych bulwach, bakterie nie są rozmieszczone równomiernie w całej bulwie, ale, że najliczniejsze populacje bakterii z rodzaju *Pectobacterium* i *Dickeya* spp. zlokalizowane są w tkance przewodzącej zakończeń stolonów. Tkanka przewodząca i inne tkanki bulwy oddalone od zakończeń stolonów nie są natomiast skolonizowane przez bakterie albo populacje bakterii w nich występujące są bardzo małe.

W doświadczeniach badających rolę populacji glebowych w patogenezie czarnej nóżki wykazano, że bakterie *D. solani* po aplikacji do gleby były zdolne do infekcji korzeni zdrowych roślin ziemniaka i wywoływania typowych symptomów chorobowych czarnej nóżki w krótkim czasie. Wykazano także, że bakterie te są zdolne do przemieszczania się w tkance przewodzącej z korzeni do łodyg i liści oraz, co bardzo istotne, do infekowania bulw potomnych poprzez tkankę przewodzącą. Zakażenie gleby przez *D. solani* powodowało systemiczną infekcje roślinach.

W doświadczeniach badających rolę zakażeń łodyg i liści w patogenezie czarnej nóżki roślin ziemniaka i ich udziale w patogenezie mokrej zgnilizny ziemniaków potomnych wykazano, że bakterie *D. solani* są zdolne do przemieszczania się z łodyg do korzeni i bulw w tkance przewodzącej.

W doświadczeniach badających możliwość wykorzystania bakterii izolowanych ze zgniłej tkanki bulw ziemniaka do biologicznej kontroli roślin wykazano, że bakterie antagonistyczne są obecne w gnijących tkankach ziemniaka i efektywnie kontrolują zakażenia powodowane przez *D. solani* w warunkach *in vitro* i w roślinach. W wyniku tych badań wyizolowano i scharakteryzowano antagonistyczną bakterię *Serratia plymuthica* A30, którą następnie wykorzystano w eksperymentach szklarniowych. Izolat A30 był zdolny do efektywnej kolonizacji wewnętrznych tkanek ziemniaka, w tym tkanki przewodzącej i ochrony roślin przed zakażeniem. Zainfekowanie bulw ziemniaka *D. solani* i izolatem A30 powodowało obniżenie zachorowalności roślin ziemniaka na czarną nóżkę. Bakterie *S. plymuthica* A30 były zdolne do ochrony roślin ziemniaka przed zakażeniem także po ich aplikacji do gleby w trakcie wysiewu bulw. Przeprowadzone badania wykazują, że bakterie *S. plymuthica* A30 mogą stanowić nowy obiecujący biologiczny środek ochrony roślin przeciwko zakażeniom *D. solani* powodującymi powstawanie czarnej nóżki i mokrej zgnilizny.

ACKNOWLEDGEMENTS

This thesis describes the work done in the last three and a half years. To be able to finish this study and to be in the place I am right now, many people have helped me, and without their support and encouragement, love and friendship, inspiration and endorsement it would not be possible to finish this journey. Here I would like to take the opportunity acknowledge some of them.

First of all I would like to thank **STW Technologiestichting** for support with my fellowship and financing the project "Curing seed potato from blackleg causing bacteria".

I would like to thank all the people from the Business Unit Bio-interactions and Plant Health (**Petra, Trudy, Marieke, Els V., Els N. Carin L., Lia, Helen, Marjon and Odette, Kees B., Cees W., Joeke, Jurgen, Cor, Leo, Pieter B. and Pieter K., Jan B.**), Plant Research International, Wageningen for the every-day support, help in my project and for creating great working place and friendly atmosphere. Here especially I would like to thank **Carin** and **Patricia** for everything good I have received from them.

Dear **Patricia**, thank you also very much for the time we spent together in the lab, for your great support in the everyday lab life, for ordering thousands of things for me, for helping me in the experiments and for our discussions and also for accepting to be my Paranimph.

Then I would like to thank **Waldo** for his enormous help with statistics, for constructive comments and data analyses.

I am very grateful to **Michel Perombelon** (ex. SCRI, UK) for his highly valuable comments on my project and his editorial work on my manuscripts.

I am grateful to **Piet Boonekamp** for constant positive feedback on my research, for ideas and discussions, for creating good and open atmosphere in BU Bio-interactions and Plant Health.

I would like to acknowledge **Gerda, Elly and Gerrie** from Terrestrial Microbial Ecology Group and NIOO-KNAW for perfect organization of everything and for great and constant support.

Here, I would like really thank **Theo** for pleasant and stimulating scientific and non-scientific discussions about almost all possible subjects.

A very special thanks are addressed to **Ineke** for the perfect organization of everything, for her kindness for answering questions and solving problems.

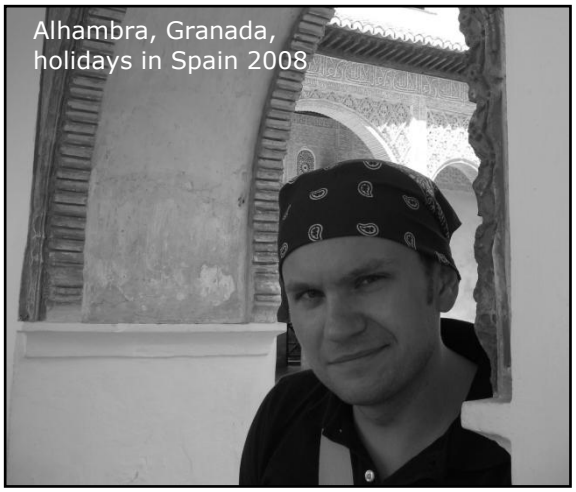
I would like to thank **Paolina** for very many things, particularly that she agree to by my Paranimph.

To my Parents-in-law. Drodzy Moi, nie mógłbym wyobrazić sobie lepszych teściów niż Wy. Wasz dom był i jest zawsze otwarty dla mnie, a Wasze ciepło, przyjaźń i wsparcie powodowało i powoduje, że bycie z Wami jest zawsze ogromną radością. Dziękuje Wam za zrozumienie, wsparcie i zaufanie.

To my Parents, to My Mum and My Dad. Kochani Moi, mam świadomość, że żadne słowa nie oddadzą tego co chce Wam powiedzieć i jak bardzo podziękować Wam za wszystko. Ta praca doktorska jest dedykowana Wam i tak ja napisałem na jednej z pierwszych stron tej tezy, to dzięki Wam jestem kim jestem. Udowodniliście mi, że jeżeli tylko czegoś naprawdę pragnę jestem w stanie to osiągnąć, daliście mi siłę i mądrość, nauczyliście wytrwałości i pogody ducha, pokazaliście co jest dobre a co złe i, że zawsze ostateczny wybór należy do mnie. Wszystko co osiągam, osiągam dlatego ze mam wasze wsparcie i zrozumienie nawet w tych niełatwych dla Was decyzjach. Wiem ze jesteście, kochacie mnie i wspieracie we wszystkim co robię a także, że jesteście ze mnie dumni, że robicie tysiące innych rzeczy żeby moje życie było szczęśliwe i spełnione – dziękuję Wam za wszystko.

To Sylwia. Słonko Moje kochane, ta książka jest także Twoją książką, tak jak moje życie jest także Twoim. Dziękuję Ci za wszystko.

ABOUT THE AUTHOR



Robert Czajkowski was born on 20th of April 1981 in Gdynia, Poland. In 2000 he finished his secondary education in First General-Education High School in Gdynia. In 2004 Robert finished the BSc education at Intercollegiate Faculty of Biotechnology (IFC), University of Gdansk and Medical University of Gdansk, Poland and in the same year Robert started the MSc study at IFC. In June 2006 he defended his MSc thesis entitled "Biochemical and genetic analyses of signal molecules-degrading factor from *Ochrobactrum* sp. A44". During this period, in 2004-2005 Robert visited the University of Nottingham, Nottingham, United Kingdom where he worked on genetic characterization of *Ochrobactrum* sp. A44 under the guidance of Prof. Dr. Paul Williams. In 2006, Robert received an EU Marie Curie EST fellowship and during 2006-2007 he worked in the Max Planck Institute for Terrestrial Microbiology, Marburg, Germany on the MAP kinase cascade components in *Ustilago maydis* under supervision of Prof. Regine Kahmann. In 2007, Robert received a Institute Fellowship at Wageningen University and Research Centre - Plant Research International (PRI-WUR), Wageningen, The Netherlands where he worked till 2009 under supervision of Dr. Jan M. van der Wolf on ecology of *Dickeya* and *Pectobacterium* species in potato. In 2009, Robert started a STW-founded PhD project. His project entitled "Curing seed potatoes from blackleg causing bacteria" was a co-operation between Netherlands Institute of Ecology (NIOO-KNAW), Heteren/Wageningen and PRI-WUR, Wagenignen, The Netherlands with Prof. Dr. Johannes A. van Veen as thesis promotor and Dr. Jan M. van der Wolf as a co-promotor. The results of this project are summarized in the presented thesis. After completion of PhD, Robert continues his scientific work in the same project in WUR-PRI/NIOO as a postdoc fellow.

Publications

(publications marked with ***** are donated as chapters of this thesis)

1. *** R. Czajkowski**, W. J. de Boer, J. A. van Veen and J. M. van der Wolf. Studies on the interaction between the biocontrol agent, *Serratia plymuthica* A30, with blackleg causing *Dickeya* sp. (biovar 3) in potato (*Solanum tuberosum*) ***Plant Pathology*** (submitted)

2. *** R. Czajkowski**, M. C. M Perombelon, J. A. van Veen and J. M. van der Wolf. Control of blackleg and tuber soft rot of potato caused by *Pectobacterium* and *Dickeya* species: a review. ***Plant Pathology*** (2011) DOI: 10.1111/j.1365-3059.2011.02470.x

3. *** R. Czajkowski**, W. J. de Boer, J. A. van Veen and J. M. van der Wolf. Characterization of bacterial isolates from rotting potato tuber tissue showing antagonism to *Dickeya* sp. biovar 3 in vitro and *in planta*. ***Plant Pathology*** (2011) DOI: 10.1111/j.1365-3059.2011.02486.x

4. *** R. Czajkowski**, W. J. de Boer, J. A. van Veen, and J. M. van der Wolf (2010). Downward vascular translocation of a green fluorescent protein-tagged Strain of *Dickeya* sp. (biovar 3) from stem and leaf inoculation sites on potato. ***Phytopathology*** 100: 1128-1137

5. *** R. Czajkowski,** W. J. de Boer, J. M. van der Wolf, (2010). Systemic colonization of potato plants by soil-borne, GFP-tagged strain of *Dickeya* sp. biovar 3. ***Phytopathology*** 100: 134-142.

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17. **R. Czajkowski**, S. Jafra – Enzymatic degradation of acyl-homoserine lactones and it possible use in biocontrol and inhibition of infection's development. ***Biotechnologia*** 2, 49-64, 2006 (*in Polish*)

Patents

1. J. M. van der Wolf, **R. Czajkowski**, J. A. van Veen – Agents of biological control of bacterial plant pathogens – ***UK Patent Application*** RAW/P132853GB

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