IDENTIFICATION OF THE BARLEY PHYLLOSPHERE AND CHARACTERISATION OF MANIPULATION MEANS OF THE BACTERIOME AGAINST LEAF SCALD AND POWDERY MILDEW

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ABSTRACT

In the context of increasing food insecurity, new integrated and more sustainable crop protection methods need to be developed. The phyllosphere, i.e. the leaf habitat, hosts a considerable number of microorganisms. However, only a limited number of these are pathogenic and the roles of the vast majority still remain unknown. Managing the leaf-associated microbial communities is emerging as a potential integrated crop protection strategy. This thesis reports the characterisation of the phyllosphere of barley, an economically important crop in Scotland, with the purpose of developing tools to manipulate it.

Field experiments were carried out to determine the composition of the culturable bacterial phyllosphere. The leaf-associated populations were demonstrated to be dominated by bacteria belonging to the *Pseudomonas* genus. Two bacterial isolates, *Pseudomonas syringae* and *Pectobacterium atrosepticum*, hindered the growth of *Rhynchosporium commune*, the causal agent of the leaf scald, but promoted the development of powdery mildew symptoms, caused by *Blumeria graminis* f.sp. *hordei*. However, using a molecular fingerprinting technique, namely T-RFLP, the global community was shown to be significantly richer and more diverse than indicated by the culture-based methods, thus increasing the complexity of interactions taking place in the phyllosphere.

Various factors were found to affect the structure of the phyllobacteria significantly. Under controlled conditions, a root-associated symbiont, *Piriformospora indica*, was shown to increase the plant fitness and shift the abundance of the most common bacteria. In the field, both agro-chemical treatments

tested, conventional fungicide and an elicitor mixture, increased the bacterial diversity, but fungicide application resulted in a higher yield and better protection against diseases. Finally, the plant genotype also affected the phyllosphere structure. Mutations in the leaf epicuticular waxes led to significant changes in the bacterial diversity and differentially affected disease in the field. A negative correlation between bacterial diversity and scald infection was also observed.

LIST OF PUBLICATIONS ARAISING FROM THIS WORK

Gravouil C., Fountaine J.M., Dickinson M.J. and Newton A.C. (2012) Ecological tolerance: Changing our approach to crop protection. In *Proceedings Crop Protection in Northern Britain 2012*, pp. 95-100.

Newton A.C., **Gravouil C.** and Fountaine J.M. (2010) Managing the ecology of foliar pathogens: ecological tolerance in crops. *Annals of Applied Biology* 157: 343-359.

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LIST OF ABBREVIATIONS

1-D: Simpson Index
AHL: acyl-homoserine lactone
AMF: arbuscular mycorrhizal fungus
ANOVA: analysis of variance
ARISA: automated ribosomal intergenic spacer analysis
Avr: avirulence
BABA: beta-aminobutyric acid
BCA: biological control agent
BX: benzoxanoid
cfp: cyan fluorescent protein
cfu: colony forming unit
cis-JA: cis-jasmonate
CLa: cuticle layer
CP: cuticle proper
crRNA: chloroplastic rRNA
Ct: cycle threshold
CTAB: cetyltrimethylammonium bromide
DGGE: denaturating gradient gel electrophoresis
dpi: days post inoculation
E: evenness
EC: epidermal cell
EDTA: ethylenediaminetetraacetic acid
EPS: extracellular polysaccharide
ESEM: environmental scanning electron microscopy
ETI: effector-triggered immunity
ETS: effector-triggered susceptibility
EW: epicuticular wax
FAM: 6-carboxy-fluorescein
GABA: gamma-aminobutyric acid

GC: guard cell

GC-MS: gas chromatography-mass spectrometry

gfp: green fluorescent protein

g_{FW}: gram of fresh weight

GS: growth stage

H: Shannon Index

H14: *Rhynchosporium* nursery field

HEX: hexachloro-6-carboxy-fluorescein

HGCA: Home-Grown Cereals Authority

HR: hypersensitive response

IAA: indole-3-acetic acid

JA: jasmonic acid

LB: Luria-Bertani

LF: Lab Field

lsd: least significant difference

MiCA: microbial community analysis

mrRNA: mitochondrial rRNA

NA: nutrient agar

nd: not determined

OD_{600}: optical density at 600 nm

OTU: operon taxonomic unit

P: probability

PAMP: pathogen-associated molecular patterns

PC: principal component

PCA: principal component analysis

PDB: potato dextrose broth

PPB: potassium phosphate buffer

PTI: PAMP-triggered immunity

PVP: polyvinylpyrrolidone

PVPP: polyvinylpolypyrrolidone

qPCR: quantitative PCR

QS: quorum sensing

QTL: quantitative trait locus

R gene: resistance gene

rDNA: ribosomal DNA

RFLP: restriction fragment length polymorphism

rfu: relative fluorescence unit

RISA: ribosomal intergenic spacer analysis

RLS: ramularia leaf spot

rRNA: ribosomal ribonucleic acid

S: richness

SA: salicylic acid

SB: spring barley

SD: standard deviation

SDW: sterile distilled water

SDS: sodium dodecyl sulphate

SEM: standard error of the mean

SLB: southern leaf blight

ST: stoma

T1SS: type I secretion system

T2SS: type II secretion system

T3SS: type III secretion system

T4SS: type IV secretion system

T6SS: type VI secretion system

TBDR: TonB-dependent receptor

TBE: Tris-borate-ethylene diamine tetraacetic

T-DNA: transfer DNA

TRF: terminal restriction fragment

T-RFLP: terminal-restriction fragment length polymorphism

USDA: United States Department of Agriculture

WB: winter barley

WT: wild-type

CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION AND DEFINITIONS

1.1.1 Koch's Postulates

The aetiology, i.e. the identification of the cause of a disease, is historically based on Koch's postulates (Koch, 1884). They describe four requirements that a microbe has to meet in order to be identified as the causal agent of a disease:

- The microorganism must be found in abundance in the affected host and should not be found in healthy organisms;
- It must be isolated from a diseased host and grown in pure culture;
- The isolated microbe should cause disease, when infected in a healthy host;
- The inoculated microorganism must be re-isolated from the experimentally infected host and identified as identical to the original disease causal agent.

These postulates have laid the cornerstone for consistency in the identification of causal agents. Once the pathogens have been identified, epidemiologists can then study the pathogen's life cycle to develop appropriate protection strategies.

However, Koch's postulates quickly showed their limitations and are not valid anymore. They could not be used for the identification of certain causal agents, including (i) non-culturable pathogens, for instance phytoplasmas (Hodgetts *et al.*, 2007), (ii) disease complexes of similar species such as *Fusarium* (Parry *et al.*, 1995) and (iii) opportunistic pathogens (e.g. *Trichoderma* spp. (Harman *et al.*, 2004)). Even though molecular biology has helped with the development of disease aetiology (Falkow, 1988), there is still nowadays no accepted paradigm (Inglis, 2007).

Furthermore, disease causal agents are constantly challenged at the microscopic scale by other microorganisms. Hosts are highly colonised with a large

variety of microorganisms, called a microbiome, constituting with the host a super-organism. Understanding disease causal agents requires to overcome Koch's postulates and look at diseases as a complex system with multiple active factors (e.g. associated microbes) rather than a one-on-one closed system.

1.1.2 Plants as Super-Organisms

A super-organism is an organism consisting of multiple organisms. The composition, roles and functions of the plant-associated microbiome is greatly dependant on various factors such as the plant life-style (perennial or annual) or its habitat.

1.1.2.1 Definitions of the Plant Microbiome

The plant microbiome can be roughly divided into two based on the organs that it relates to. The rhizosphere corresponds to the below-ground parts of the plant (roots, rhizome, tubers...), commonly referring to the root habitat; whereas the phyllosphere is the above-ground parts of the plant (flower, bud, stem, leaf...) and commonly refers to the leaf habitat. The surface only of these environments is named the rhizoplane and phylloplane respectively. Even though the microbiome is divided into two, a certain continuum between the two exists and some soil-associated microorganisms are able to colonise the phyllosphere from the rhizosphere (Chi *et al.*, 2005; Kutter *et al.*, 2006)

Plant-associated microorganisms are often separated into two groups: epiphytes that grow on the surface of organs and endophytes that grow within. Functionally, epiphytes are described as microbes either removed from the plant surface by washing or killed by ultraviolet (UV) radiation or disinfectant. Endophytes are those remaining. However, as stated by Beattie and Lindow (1999) "the terms 'epiphytic' and 'endophytic' may be more accurately viewed as two ends

of a spectrum reflecting the growth patterns of leaf-associated [microbes] than as two distinct groups of organisms", because during their plant colonization, microbes can go through a series of egression and ingression phases. Hence, the characterisation of a microbe as epiphytic or endophytic has to be treated with caution, but here epiphytes and endophytes will be defined as two separate groups based on the microbial removal technique.

1.1.2.2 The Abundance of Plant-Associated Microorganisms

The plant-associated microbiome greatly influences nutrition and defences of the host. Most studies have focused on the rhizosphere rather than the phyllosphere. The interactions between microbes and the plant can lead to the death of the host (i.e. pathogenic interactions), or both can live together synergistically (mutualism) or antagonistically (parasitism) (Newton *et al.*, 2010a).

The rhizosphere has been known for a long time to be an environment rich in microorganisms. A gram of soil can contain up to 10^9 bacterial cells and the richness varies greatly depending on the soil, reaching up to 10,000 taxa in undisturbed forest soils (Roesch *et al.*, 2007). However, the world leaf area has been estimated as around $6.4x10^8$ km^2 (Morris and Kinkel, 2002). The most common organisms in the phyllosphere are the bacteria with an estimated average of 10^6 to 10^7 per cm^2. Hence, the worldwide bacterial leaf-associated population could be as large as 10^{26} cells (Morris and Kinkel, 2002).

1.1.2.3 The Known Roles of Plant-Associated Microorganisms

The phyllosphere is a rich environment and complex too, including bacteria, archaea, filamentous fungi, yeast, oomycetes, algae and less frequently protists and nematodes (Lindow and Brandl, 2003). Studies of leaf-associated microorganisms have almost exclusively focused on plant pathogens, driven by economic interests.

However, over the years, the interest for other plant-associated microorganisms has greatly widened the scope of research.

Using microbes to control pathogens has emerged as integrated means of disease control in recent years (Kiss, 2003). These biological control agents (BCAs) can kill or hinder pests and pathogens through a vast array of mechanisms (Section 1.3.4.1). Some BCAs are commercially available such as the AQ10 Biofungicide® from Ecogen Inc. (USA), for which commercialisation is also authorised in Europe. The active fungus, *Ampelomyces quisqualis* isolate M-10, kills the hyphae of the barley powdery mildew agent (*Blumeria graminis* f.sp. *hordei*) via mycoparasitism (Kiss, 2003). Nearly 200 bacterial and fungal species have shown biocontrol properties against air-borne pathogens (Nicot *et al.*, 2011). However, the registration of a BCA is a long process in the European Union (EU) compared to the United States of America (USA) (Hauschild and Speiser, 2007) (Table 1.1). The commercialisation of only 25 microbial BCAs was authorised in 2007 and the consistency of BCA's effectiveness is still questioned (Nicot *et al.*, 2011).

Table 1.1: Registration time (in months) of biocontrol products and their associated active ingredients in the European Union (EU) and in the United States of America (USA).

	Registration time (months)	
Organism (Product)	EU	USA
Bacillus subtilis (Serenade®)	81	14
Coniothyrium minitans (Contans®)	57	15
Gliocladium catenulatum (Prestop®)	67	13
Paecilomyces fumosoroseus (Preferal®)	85	60
Spodoptera exigua NPV (Spodex®)	121	12

Recent food-borne disease out-breaks (Brandl, 2006) have led to the investigation of the survival of human pathogens in the phyllosphere. Food-borne diseases have been reported by the United States Department of Agriculture (USDA) to cost several billions of dollars every year. Despite increased and more accurate control, a significant number of cases are reported every year. In the USA alone, in 2009, around 1,300,000 *Salmonella* cases were recorded. The associated costs were estimated at over two billion dollars (USDA, 2011). The bacterium *Salmonella enterica* serovar *typhimurium* has been shown to be able to colonise salad leaves and actively invade leaf tissues (Kroupitski *et al.*, 2009). It can either penetrate through stomata, sensing light-produced compounds, or colonise the host roots and progress to the phyllosphere via the xylem (Kutter *et al.*, 2006). In addition, it has been shown to overcome the innate immune system of *Arabidopsis thaliana* resulting in wilting, chlorosis and eventually death (Schikora *et al.*, 2008).

The phyllosphere hosts various types of organisms of economic interest, but many more microbes such as saprophytes and commensals also colonise the leaf habitat actively. Little is known about the direct and indirect interactions between these microbes with the host and microbes of more economic interest.

1.1.3 Crop Protection

1.1.3.1 Innate Plant Defences

Plants are sessile and have to cope with numerous and various biotic and abiotic stresses. They have evolved a vast array of defences to fight back or tolerate such attacks. As a result, pathogens and pests have evolved other mechanisms to counteract and/or evade the triggering of plant defences (Chisholm *et al.*, 2006). The current model for defence establishment and evolution in plants is referred to as the "zig-zag-zig" model (Figure 1.1) (Jones and Dangl, 2006).

Briefly, plants have evolved mechanisms for recognition of conserved pathogen-associated molecular patterns (PAMPs) to induce PAMP-triggered immunity (PTI). Because of the resulting increased selection pressure, pathogens have developed means to manipulate host immunity with effectors that target key recognition components of the plants defences, either directly or indirectly (Dangl and Jones, 2001), eventually leading to effector-triggered susceptibility (ETS). Plants subsequently bred-in resistance (*R*) genes that specifically recognised the effectors (called avirulence (Avr) proteins), resulting in effector-triggered immunity (ETI) and leading to a hypersensitive response (HR). As selection pressure increased again on the pathogen, new or mutated effectors that are not recognised by the host, emerge and the ETS-ETI competition starts again (Jones and Dangl, 2006).

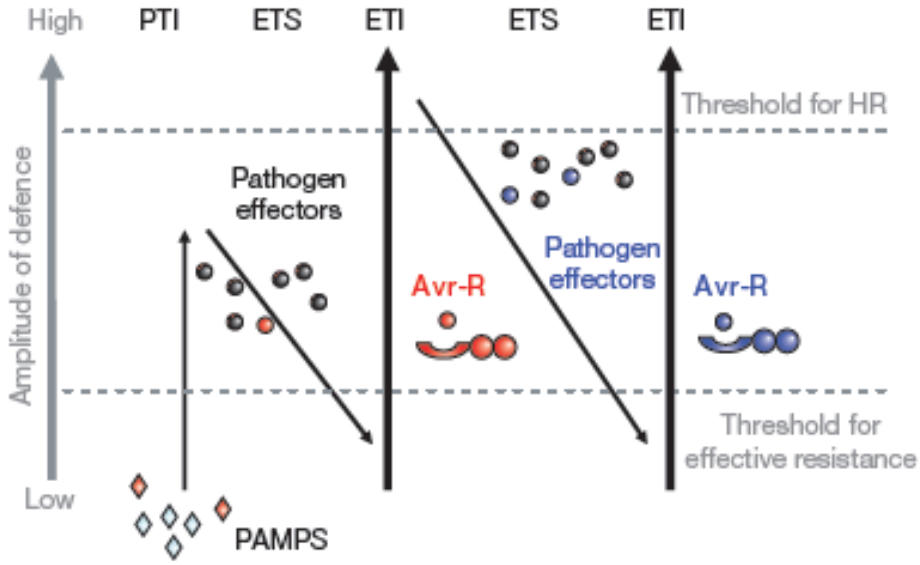

Figure 1.1: The "zig-zag-zig" model for plant defences, from Jones and Dangl (2006). The recognition of conserved pathogen-associated molecular patterns (PAMPs) by the plant leads to the PAMP-triggered immunity (PTI). Pathogens evolved effectors to counteract plant defences, resulting in the effector-triggered susceptibility (ETS). Subsequently, plants evolved resistance (*R*) proteins, which recognise specific pathogenic effectors (called avirulence (Avr) proteins) and induce the effector-triggered immunity (ETI) causing hypersensitive response (HR), i.e. necrosis. As selection pressure increases on the pathogen, it can evolve new or mutated effectors that are not recognised by the plant and eventually trigger ETS again. The selection pressure shifts on the plant to evolve new *R* proteins.

The trigger threshold of PTI or ETI is dependent on the type of microorganism but also on the recognition and signal- transmission ability of the host. Therefore, the spread of causal agents and symptom apparition greatly varies with different host (Hill and Purcell, 1995).

1.1.3.2 Crop Protection Strategies and Limits

Crop protection is achieved through three main strategies: (i) increasing the innate crop resistance by breeding (e.g. introducing new *R* genes), (ii) directly targeting the pathogen by applying agro-chemical treatments, and (iii) reducing the pathogenic inoculum and infection by phytosanitary methods.

A breeding programme is a long process, but the development of molecular biology has greatly improved the search for new *R* genes (Varshney *et al.*, 2005). However, introducing *R* genes singly or in groups into crops has increased the selection pressure on pathogens and the latter can often quickly evolve and break the newly inserted resistance. For example, the Pentland Dell potato cultivar, containing a pyramiding of three *R* genes, was commercialised in 1963. By 1967, blight infection caused by *Phytophthora infestans* reached up to 75% on this cultivar in England and Wales (Fry, 2008). Plant breeders also have to face the emergence of super-virulent pathogens, such as the Ug99 strain of *Puccinia graminis* causing stem rust on wheat (Singh *et al.*, 2008), or the re-emergence of pathogens, thought to have been eliminated, such as *Ramularia collo-cygni* causing ramularia leaf spot (RLS) on barley (Walters *et al.*, 2008a).

A vast number of different fungicide chemicals target many metabolic and structural key elements of the life cycle of fungal pathogens (Table 1.2). However, fungal pathogens develop tolerance and resistance to these chemical with time (Table 1.3) (Brent and Hollomon, 2007). For example, the rice blast pathogen *Magnaporthe*

oryzae developed resistance to melanin biosynthesis inhibitors, following a point mutation of the target enzyme, scytalone dehydrogenase (Kaku *et al.*, 2003). Furthermore, the EU is extending the list of banned pesticides (Regulation (EC) No. 1107/2009), pressurising agro-chemical companies to search for new active molecules. As alternatives, research has been undertaken on assessing the use of elicitors (or priming agents), which have no direct effect on pathogens but can offer good protection under field conditions (Walters *et al.*, 2008b). Elicitors are PAMP-like compounds such as polysaccharides, or plant phytohormone analogues. The priming process consists of inducing the gene expression of transduction cascades involved in plant defences, without inducing the plant defences. Hence, they prepare the plant for a faster establishment of defences. The time scale of defence deployment was shown to be a limiting factor in compatible interactions (Tao *et al.*, 2003).

Table 1.2: List of fungicides' targets and modes of action with examples of active compounds.

Target		Example of chemical family (active ingredient)
Mitosis	Mitosis and cell division	Benzimidazole (thiabendazole)
	Cell division	(zoxamide)
Metabolism	Respiration	Strobilurin (azoxystrobin)
	Nucleic acid synthesis	Acylalanine (metalaxyl)
	Protein synthesis	(cyprodinil)
	Signaling	(quinoxyfen)
Integrity	Sterol synthesis	Triazole (prothioconazole)
	Lipids and membranes	1,2,4-Thiadiazole (etridiazole)
	Cell wall synthesis	Peptidyl pyrimidine nucleoside (polyoxin)
	Cell membrane permeability	(propamocarb)
Other	Multi-site contact activity	Chloronitrile (chlorothalonil)
	Unknown	(cymoxanil)

Table 1.3: List of various fungicide resistance occurrences on cereals, adapted from Brent and Hollomon (2007). Each fungicide example is characterised with its commercialisation date, the disease target and the number of years before resistance was detected.

Date	Fungicide or fungicide class	Years of usage	Main crop diseases (causal agent)
1964	Organo-mercurials	40	Cereal leaf spot and stripe (*Pyrenophora* spp.)
1971	2-Amino-pyrimidines	2	Barley powdery mildew (*Blumeria graminis* f.sp. *hordei*)
1971	Kasugamycin	6	Rice blast (*Magnaporthe oryzae*)
1976	Phosphorothiolates	9	Rice blast (*Magnaporthe oryzae*)
1982	Sterol Demethylation Inhibitors (DMI)	7	Barley powdery mildew (*Blumeria graminis* f.sp. *hordei*)
1985	Carboxanilides	15	Barley loose smut (*Ustilago nuda*)
1995	Quinone outside Inhibitors	2	Many target diseases and pathogens
2002	Melanin Biosynthesis Inhibitors (Dehydratase) (MBI-D)	2	Rice blast (*Magnaporthe oryzae*)

All previously described crop protection strategies tend to one common goal: the destruction of pathogens, generally disregarding the natural micro-flora. However, because of the limitations of conventional crop protection, new integrated methods are being sought. Introducing a BCA does not always offer protection in the field, because of many ecological (competitors, presence of the pathogen needed etc.) and environmental factors. Hence, utilizing the innate microbial population as a sustainable and long-lasting defence against pathogens has emerged as a potential new strategy (Newton *et al.*, 2010b). As previously stated in Section 1.1.2.3, little is known about the phyllosphere ecology, so interest had focused on this particular habitat.

1.2 THE LEAF-ASSOCIATED MICROBIAL RICHNESS

1.2.1 The Pioneering Phyllosphere Studies

Since the 1950's, study of the phyllosphere has gained considerable attention and from the early 1970's, regular international symposia have been held, from which proceedings were published (Preece and Dickinson, 1971). These early studies did not only focused on plant pathogens, but investigated new non-invasive techniques and biological control activities of plant-associated microorganisms, defined the phyllosphere ecology, characterised saprophyte-plant interactions as well as the physics and the chemistry of the leaf surface (Blakeman, 1981; Fokkema and Van den Heuvel, 1986). However, all were based on culturable methods and knowledge of culturable microorganisms.

One of the first extensive studies on the phyllosphere ecology was a study of culturable organisms over three years on olive tree leaves (*Olea europaea* L.) (Ercolani, 1991). The epiphytic bacterial community was found to be largely dominated by *Pseudomonas syringae* and other less abundant species such as *Xanthomonas campestris*, *Pantoea agglomerans* (formerly *Erwinia herbicola*) and *Acetobacter aceti*. A total of 25 bacteria were identified at the genus or species level and the dominant genera showed fluctuating dynamics over growing seasons. The variations in population sizes were caused largely by the considerable fluctuations in the physical and nutritional conditions of the phyllosphere (Ercolani, 1991).

Thompson *et al.* (1993) observed similar microbial structures associated with the sugar beet (*Beta vulgaris*) leaf habitat: microbial populations were dominated by a few species throughout the season. However, compared to Ercolani's data (1991), the phyllosphere composition had a greater richness of culturable leaf-associated microbes, with 78 bacterial species and 37 named and 12 unnamed genera, as well as

fungi. The most common bacteria were *Pseudomonas* spp., *Pantoea agglomerans* and *Arthrobacter oxydans*. The most prevalent fungi belonged to the *Cladosporium* and *Alternaria* genera.

1.2.2 The Molecular Approach

The development of molecular techniques has enabled greater insight into the microbial composition of the phyllosphere (Figure 1.2). Using molecular phylogenetic analysis based on conserved ribosomal RNA (rRNA) genes (usually 16S rRNA gene for bacteria and 18S rRNA gene for fungi), the leaf-associated microbial communities were shown to have a higher complexity than previously described, because of the presence of numerous viable but non-culturable microorganisms (Yang *et al.*, 2001): indeed the most common bacteria detected with denaturating gradient gel electrophoresis (DGGE) did not include the *Pseudomonads* observed by Ercolani (1991) and Thompson *et al.* (1993). Furthermore some 16S rDNA sequences had less than 90% similarities with database entries, suggesting that they represented undescribed species as they are not culturable (Yang *et al.*, 2001).

Other techniques based on DNA amplification with fluorophore-labelled primers and automated separation of ribotypes (hereafter referred to as operon taxonomic units (OTUs)) enabled the detection of a greater microbial richness than the usage of culturable methods had (Koopman *et al.*, 2010). The terminal-restriction fragment length polymorphism (T-RFLP) procedure generates DNA fragments of variable length after restriction digest. The automated ribosomal internal transcribed spacer analysis (ARISA) method separates microbial species based on the variable length of the intergenic spacer region (i.e. between the 16S and 23S rRNA genes).

Second-generation DNA sequencing techniques have enabled the whole plant-associated microbiome to be studied in more depth. All studies to date have

been undertaken using the pyrosequencing Roche 454® technology, based on live recording of light emitted by a luciferase after detecting free pyrophosphate, a byproduct of a nucleotide incorporation. The bacterial phyllospheres of many crop species have already been analysed including tomato, spinach and grapevine (Leveau and Tech, 2011; Lopez-Velasco *et al.*, 2011; Telias *et al.*, 2011), trees (Redford *et al.*, 2010; Glenn *et al.*, 2011) and a carnivorous plant (Koopman *et al.*, 2010).

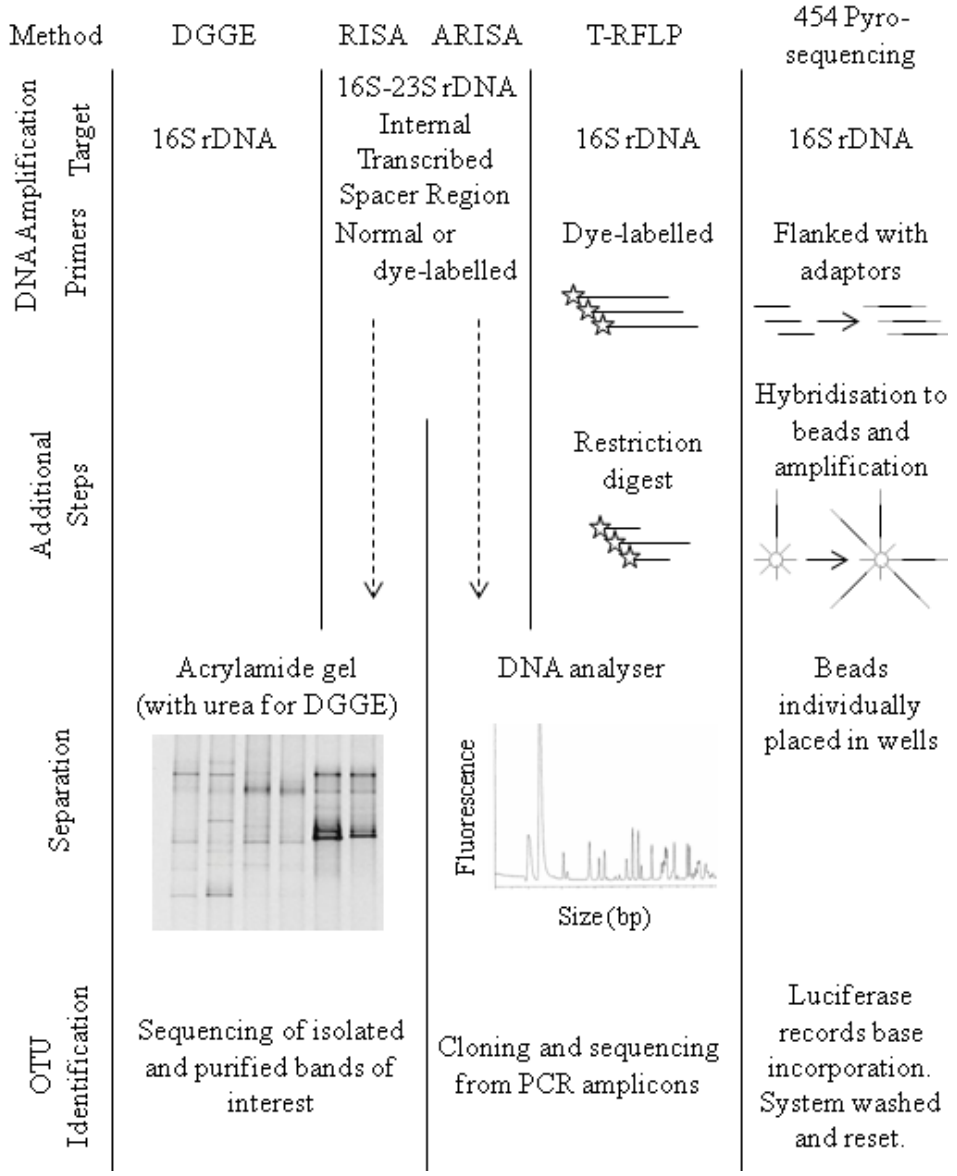

Figure 1.2: Schematic of four DNA fingerprinting techniques to monitor microbial ecology, from DNA amplification to operon taxonomic unit (OTU) identification and includes denaturating gradient gel electrophoresis (DGGE), [automated] ribosomal intergenic spacer analysis ([A]RISA), terminal-restriction fragment length polymorphism (T-RFLP) and 454 pyrosequencing.

All molecular ecology studies previously quoted have always focused on a snapshot of the phyllosphere. Even though pyrosequencing results in a great number of microbial sequences, the phyllosphere can be colonised at different times by many other different microorganisms, that can be detected with other methods such as ARISA and T-RFLP (Koopman *et al.*, 2010). Furthermore, a great number of these sequences are unique and they must be treated with caution. A better degree of microbial identification and understanding of their roles and functions could be achieved by coupling high-throughput DNA analysis with with metagenomics microbial gene (Burke *et al.*, 2011) or proteomics analyses (Delmotte *et al.*, 2009), allowing to determine regulated genes and expressed proteins.

1.3 MICROBIAL COLONISATION OF THE PLANTS

Many factors have an impact on the phyllosphere microbial populations: (i) the environment, (ii) the plant itself and (iii) the presence of microbial competitors. As a result, microorganisms have developed a broad range of strategies to survive, tolerate or take advantage of these factors.

1.3.1 The Leaf Structure and Colonisation

1.3.1.1 Physiology of the Leaf

The first physico-chemical barrier that most microorganisms encounter in the phyllosphere is the epicuticular wax layer that constitutes the leaf surface (Figure 1.3). Epicuticular waxes cover the outer-most layer of the cuticle and all aerial parts of higher plants are covered with a cuticle. The cuticle is a complex hydrophobic structure and is composed of epicuticular wax, cutin and polysaccharides. All cuticular layers are more or less merged together. The inner-most cuticular region

also merges with the cellulose layer of the epidermis (Juniper and Jeffree, 1983; Shepherd and Griffiths, 2006).

The leaf surface is not a flat surface and presents plenty of ridges and grooves, associated with vein locations (Juniper and Jeffree, 1983; Monier and Lindow, 2005). Furthermore, special structures, such as stomata and trichomes, are commonly found on the leaf surface and are involved in gas exchange and stress resistance respectively. The cuticle provides structure to the leaf, limits evapo-transpiration and pathogen entry and reflects damaging solar radiation (Shepherd and Griffiths, 2006).

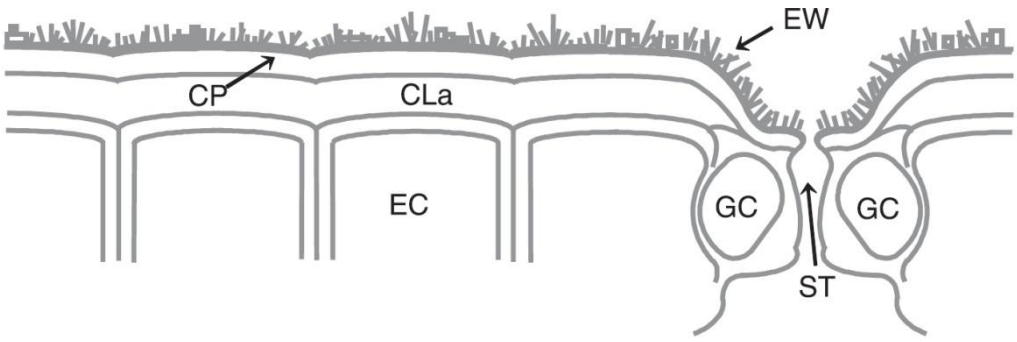

Figure 1.3: Cross section of the leaf surface from Shepherd and Griffiths (2006) with, from top to bottom, epicuticular waxes (EW), the cuticle proper (CP), the cuticular layer (CLa) and the epidermal cells (EC). Special structures occur on the leaf surface, such as stoma (ST): a gas-exchange pore, which opening is controlled by two guard cells (GC).

1.3.1.2 Spatial Heterogeneity of the Microbial Colonisation

The bacterial colonisation of the leaf surface follows a heterogeneous pattern. Using confocal microscopy, microorganisms expressing green and cyan fluorescent proteins (gfp and cfp respectively) have been observed aggregating in specific regions of the leaf surface (Monier and Lindow, 2005; Linsell *et al.*, 2011). Following the leaf topography, fungal and bacterial cells aggregated in protective

grooves (Figure 1.4 A and B). Bacteria also aggregated next to leaf protuberances (Figure 1.4 C and D).

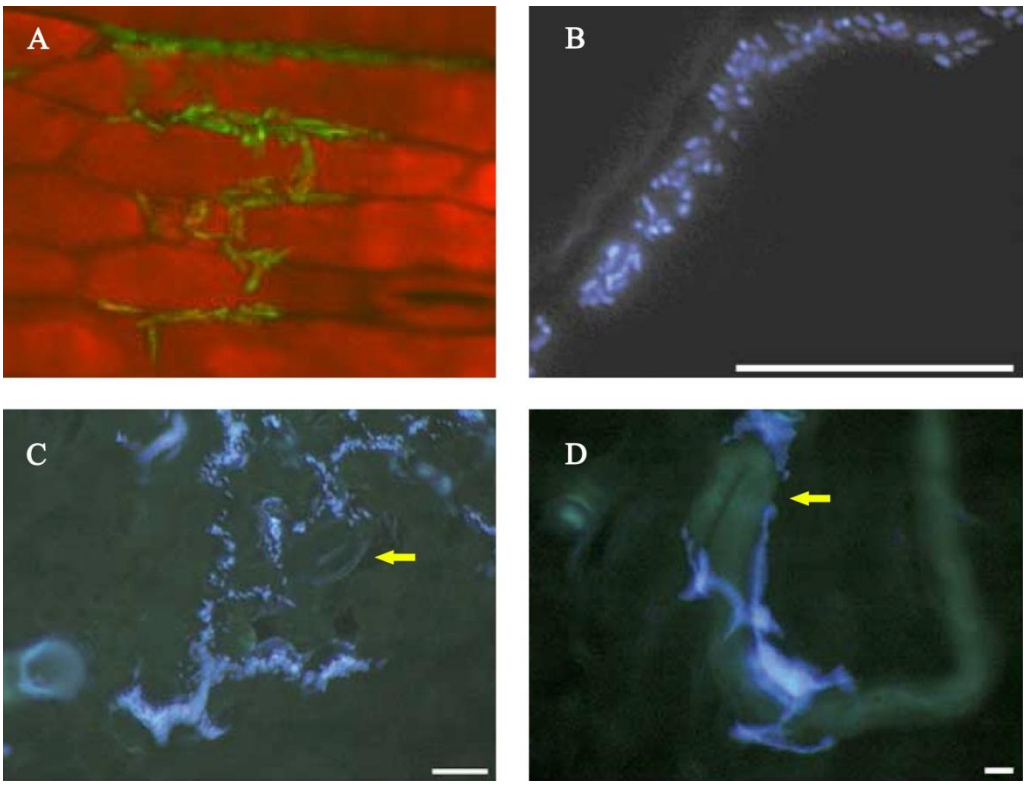

Figure 1.4: Localisation of microorganisms on the leaf surface, including gfp-expressing *Rhynchosporium commune* spores (A, from Linsell *et al.* (2011)) and cfp-expressing *Pantoea agglomerans* bacteria (B, C and D, from Monier and Lindow (2005)). Microorganisms aggregated in grooves (A, B) or next to leaf protuberances (indicated by arrows): stoma (C) and trichome (D). All scale bars represent 25 μm.

The abaxial side of the leaf is generally colonised by more microorganisms than the adaxial side (Andrews and Harris, 2000). It has better environmental conditions: lower exposure to solar radiation and higher relative humidity. As the epicuticular wax layer is thinner, plant compounds leak out more easily. Furthermore, there are more stomata and trichomes on this side of the leaf, which also increases the nutrient availability (Juniper and Jeffree, 1983).

1.3.2 The Hostile Environment

The leaf surface has long been considered as a biological desert, because of the harsh environmental conditions it is exposed to such as UV radiation, drought and high fluctuations of daily temperature (Andrews and Harris, 2000), but the phyllosphere was shown to be highly colonised with microorganisms.

Microbes living on the adaxial side of the leaf are directly exposed to UV. Ultraviolet radiation can cause irreversible and cytotoxic damage to DNA, such as the thymine-thymine dimers. Hence, bacteria have evolved genetic machineries to control their DNA integrity (Sinha and Hader, 2002). Using a bioluminescent assay, the induction of a DNA repair gene was monitored in *Pseudomonas aeruginosa* (Elasri and Miller, 1999). The presence of a bacterial biofilm was correlated with a significant reduction of the activity of the DNA repair gene, thus suggesting a protective role of the biofilm against UV radiation.

The plant canopy can provide shading to old leaves, but microbes have developed other strategies to limit UV damage. Many chromophore-producing microbes have been isolated from the leaf surface (Figure 1.5). These compounds were believed to promote tolerance to the UV. Even though this was proven in plate assays, bacterial strains deficient in chromophore biosynthesis showed similar colonisation to their wild-type (WT) equivalents on the leaves (Beattie and Lindow, 1995).

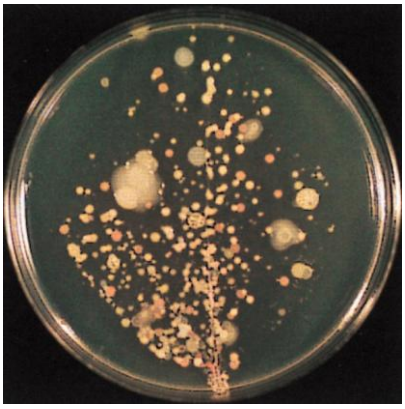

Figure 1.5: Bacterial growth from a print of a bean leaf on agar. The chromophore-producing *Pseudomonas* bacteria grew along the leaf veins (Hirano and Upper, 2000).

1.3.3 The Limiting Growth Factors

Although the phyllosphere is a harsh environment, water and nutrient availabilities are considered as the limiting factors for microbial growth (Mercier and Lindow, 2000). Following the inoculation of *Pseudomonas syringae* grown *in vitro* onto plant leaves, a quick shrinking in the average bacterial cell size was observed. This size reduction was also observed *in vitro* on nutrient deprived cells. Even though it may have appeared as evidence of stress, cells with a smaller size exhibited a better tolerance to the inoculation process on the leaf surface (Monier and Lindow, 2003).

1.3.3.1 Water Availability

In order to increase the water availability, two strategies are available to microbes: increasing water retention or finding new sources. Many leaf-associated bacteria can produce biofilms to protect themselves from the environment. One of the major and most studied components of these biofilms is the extracellular polysaccharides (EPS). Many reports have shown that EPS protect cells from osmotic shocks, pH shifts and UV radiation (Beattie and Lindow, 1999; Davey and O'Toole, 2000). However, EPS also modify the surrounding micro-environment by maintaining a highly hydrated layer protecting against desiccation (Sutherland, 2001; Monier and Lindow, 2005).

Molecules such as surfactants could also increase the water and the soluble nutrient availability in the near microbial environment. Many plant-associated bacteria produce surfactants of various types (Burch *et al.*, 2010). Because of their amphiphilic nature, surfactants interact well with the lipophilic waxes and facilitate the retention of water and soluble nutrients. Furthermore, surfactants have been hypothesised to facilitate bacterial movement on the leaf surface by creating water

films (Lindow and Brandl, 2003). Motility is essential to pathogens in plant infection (Hossain *et al.*, 2005) and provides a fitness advantage to epiphytes (Haefele and Lindow, 1987).

1.3.3.2 Nutrient Availability

Both strategies previously described also apply to the search for nutrients. Biofilm formation promotes nutrient scavenging, whereas surfactant production, associated with motility, enables microbes to access other nutrient resources. Microbes have evolved highly effective mechanisms to increase their nutrient uptake and outcompete other microbes as well.

Some bacteria have developed transporters with a very high affinity for plant carbohydrate. *Xanthomonas campestris* pv. *campestris* possesses numerous TonB-dependent receptors (TBDRs) (Blanvillain *et al.*, 2007). These receptors are located in the outer-membrane of Gram-negative bacteria and are involved in the active transport of iron siderophores and vitamin B12. They can also be associated with pathogenesis (Postle and Kadner, 2003). One TBDR in *X. campestris* pv. *campestris* associated with an inner transporter, a regulator and an amylosucrase was shown to be able to utilise sucrose with a very high affinity: its dissociation constant with sucrose was 1,500 to 3,000 lower than the dissociation constant between sucrose and an *Escherichia coli* sucrose porin, therefore binding better with sucrose (Blanvillain *et al.*, 2007).

Pathogenic and non-pathogenic bacteria can secrete significant amounts of auxin and indole-3-acetic acid (IAA) (Ortiz-Castro *et al.*, 2011). This phytohormone is a major component of *Agrobacterium tumefaciens* pathogenesis. Bacterial IAA production was demonstrated to offer a fitness advantage to epiphytes (Brandl and Lindow, 1998). The over-accumulation of IAA in *A. tumefaciens*-infected plants

leads to the formation of tumours. Other low IAA-producing bacteria were hypothesised to induce nutrient leakage from the plant, as low concentration of IAA induced a loosening of the plant cell wall (Vanderhoef and Dute, 1981).

1.3.4 Interactions with Other Organisms

1.3.4.1 Interactions between Microorganisms

Characterised interactions between microbes in the phyllosphere have been mostly limited to BCAs acting antagonistically to plant pathogens. They have a great economic value, as described in Section 1.1.2.3. The *Trichoderma* genus includes many species with biocontrol activity. However, they act through a large range of overlapping modes of action including parasitism, antibiosis and competition (Table 1.4) (Azcon-Aguilar and Barea, 1996; Howell, 2003; Kiss, 2003; Leveau and Preston, 2008).

Other microbes have been described as "helper" (Dewey *et al.*, 1999; Newton and Toth, 1999). During the axenic isolation of *Phaeosphaeria nodorum* (formerly *Septoria nodorum*), a few bacterial isolates were tightly associated with the fungus. Co-inoculation of a bacterial isolate with fungal inoculum on detached leaves resulted in an increase in symptom size on wheat leaf segments. By analogy with *Botrytis cinerea* on tomato leaves (Commenil *et al.*, 1998), the mechanism involved was thought to be due to lipase activity from the helper bacteria, as *P. nodorum* does not produce any (Dewey *et al.*, 1999). Located at the interface between a lipophilic substrate (e.g. epicuticular waxes) and a hydrophilic medium (e.g. a cell), lipases cleave insoluble glycerides into water-soluble molecules (Jaeger *et al.*, 1999). Lipases of *B. cinerea* were thought to be an important pathogenic factor in the early infection step on tomato leaves (Commenil *et al.*, 1998), but this finding was recently contradicted (Reis *et al.*, 2005).

Microbial aggregations can facilitate exchange of genetic information, known as horizontal gene transfer (Davey and O'Toole, 2000). Three mechanisms are involved: transformation, transduction and conjugation. The mobile genetic elements of the latter two mechanisms include bacteriophages, transposons and plasmids. All three are considered as major elements of bacterial and fungal evolution (van Elsas *et al.*, 2003; Richards *et al.*, 2011). However, *in situ* validation in plants is still lacking.

Table 1.4: List of modes of action and targets or effects with their identified mechanisms involved in *Trichoderma* spp. biological control (Howell, 2003).

Modes of Action	
Target / Effect	Active compounds
Mycoparasitism	
Pathogen hydrolytic enzymes	Extracellular enzymes (proteolytic enzymes)
Impair cell wall rigidity/integrity	Extracellular enzymes (β-1,3-glucanolytic system, chitinase)
Antibiosis	
Impair cell wall rigidity/integrity	Extracellular enzymes
Hinder or kill	Volatiles
Hinder or kill	Antibiotics (gliotoxin, gliovirin, peptaibols, peptaibiotics)
Competition	
Space in rhizosphere	
Nutrition: carbohydrate	
Nutrition: micro-element	Siderophore
Spore germination stimulant	
Plant Defences Induction	
Plant cell wall reinforcement	Callose deposition
Defence proteins	Pathogenesis-related proteins (chitinase)
	Peroxidase
Anti-fungal compounds	Terpenoids (gossypol and intermediates)
Adjunct Mechanisms	
Increase root and shoot growth	
Changes in nutritional status	Micro-element uptake improved
	Strong interaction with the N-fixing *Bradyrhizobium japonicum*

1.3.4.2 Interactions with the Host

As the microbes invade and modify the plant environment, the host can detect them and trigger defensive responses as described in Section 1.1.3.1. Hence microbes, pathogenic or not, have evolved mechanisms to avoid detection or alter host recognition. For example, Gram-negative bacteria can directly inject effectors into plant cells, using a secretion system referred to as the type III secretion system (T3SS). *Pectobacterium atrosepticum*, causal agent of potato soft rot on tubers and black leg on stems, possesses one T3SS participating in pathogenesis (Holeva *et al.*, 2004). In addition, many mutualistic *Rhizobium* species have a T3SS, which has been shown to be essential in the interactions with the host for nodule formation (Marie *et al.*, 2001). This secretion system has also been hypothesised to be used to interact with many other eukaryotic organisms (Preston, 2007). The T3SS also promotes survival of a *P. syringae* strain on non-host tomato (Lee *et al.*, 2012)

Many of the previously described mechanisms are dependent on the bacterial cell density (Liu *et al.*, 2008; Ortiz-Castro *et al.*, 2011). This phenomenon known as quorum sensing (QS) relies on the perception of signal molecules such as acyl-homoserine lactone (AHL). The more bacteria are present, the more AHL accumulate in the micro-environment. Once a particular threshold is reached, expression of particular genes is triggered. Bacteria can possess multiple QS machineries regulating each other in a complex network (Williams and Camara, 2009). The AHL signal molecules can also be recognised by other bacteria. *Salmonella enterica* strains unable to produce their own AHL, showed an up-regulation of AHL-dependent genes *in vitro* when in contact with AHL from *Pectobacterium carotovorum*, but did not do so *in planta* (Noel *et al.*, 2010). Conversely, evidence has emerged suggesting that bacteria can regulate their density-

dependent gene control by quorum quenching, i.e. by secreting enzymes degrading AHLs (Dong *et al.*, 2001). Plants can also alter microbial communication by degrading these AHLs (Dong *et al.*, 2001; Chevrot *et al.*, 2006) or sensing them and triggering plant defences (Schikora *et al.*, 2011). Plant-microbe interactions are clearly the result of complex and multi-level networks.

Beyond all the strategies developed by both plants and microbes to interact with each other, many other factors will affect the outcome of the interaction. The scope of interactions between plants and microorganisms is vast and flexible (Figure 1.6). The fungus *Ramularia collo-cygni*, causal agent of the ramularia leaf spot (RLS) on barley, is believed to promote plant health during its growth and trigger pathogenicity later in the season at the sexual stage (Newton *et al.*, 2010a). The ability of plants to mobilise their resources (Bolton, 2009), the environment (e.g. temperature (Wang *et al.*, 2009) and light (Kroupitski *et al.*, 2009; Roden and Ingle, 2009) as well as the plant circadian clock (Roden and Ingle, 2009) affect pathogenesis but potentially could influence all other leaf-associated microorganisms as well.

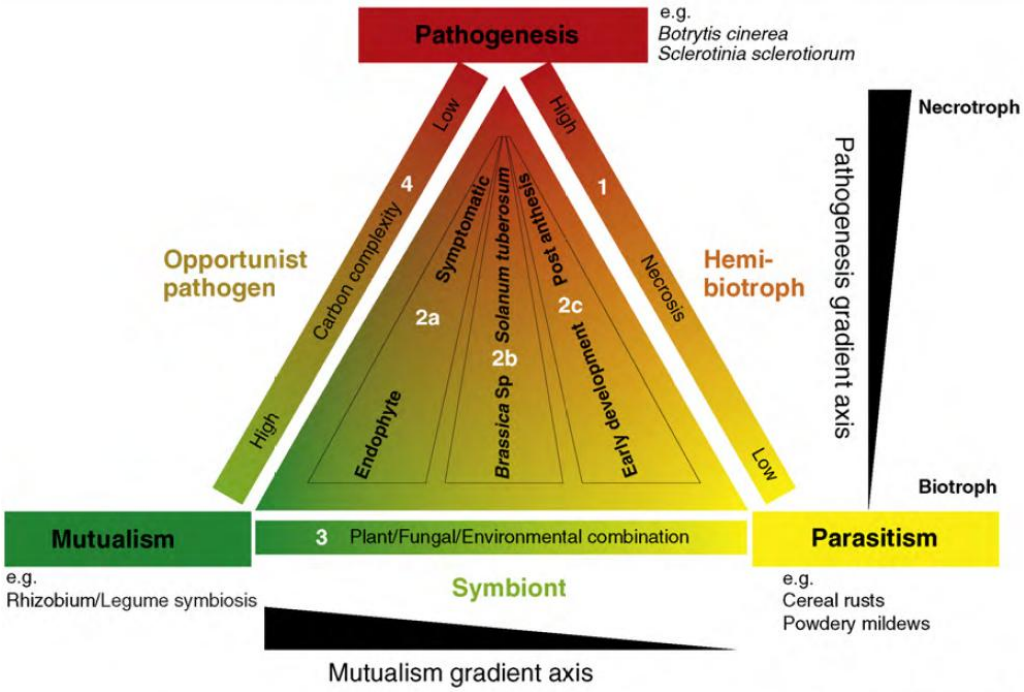

Figure 1.6: Scope of plant-microbe interactions with its three extreme tropisms: mutualism, pathogenesis and parasitism characterised by a vertical pathogenic gradient from biotrophic to necrotrophic and a horizontal symbiotic gradient from mutualistic to parasitic. Examples of microbes with variable trophic relationship include *Rhynchosporium commune* (1) and *Ramularia collo-cygni* on barley (2a), *Pectobacterium atrosepticum* on *Brassica* and *Solanum tuberosum* (2b), *Leptosphaeria maculans* on *Brassica napus* (2c), arbuscular mycorrhizal symbioses (3) and *Ceratobasidium cornigerum* on *Goodyera repens* (4). Their interactions with plants are further detailed by Newton *et al.* (2010a).

1.4 SHIFTS IN MICROBIAL POPULATIONS

1.4.1 Bacterial Ecology in the Phyllosphere

The microbial ecology is numerically estimated using three mains kinds of ecological estimations: richness, diversity and evenness. Richness corresponds to the number of different species present in one population. Diversity considers the structure of the population (i.e. dominated by one or each member evenly present), whereas evenness assesses the distribution (Figure 1.7). Ecological indices were originally determined to estimate macroorganism groups and should be used with care for microorganism studies (Blackwood *et al.*, 2007).

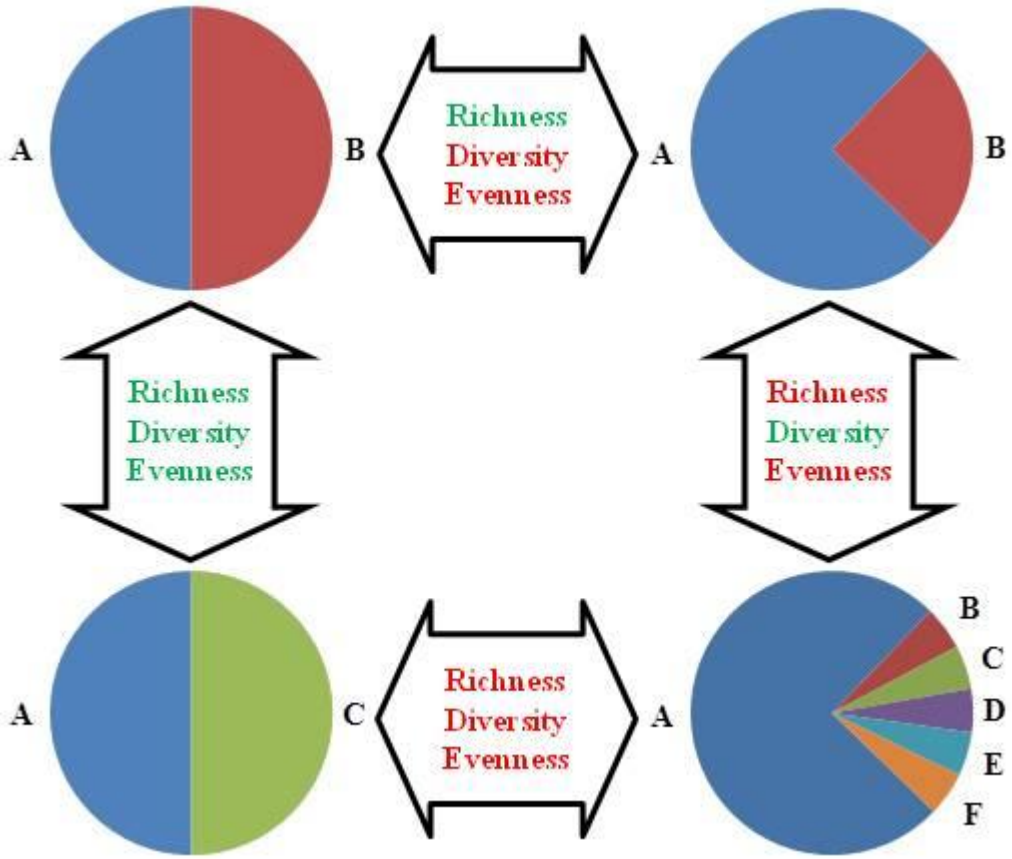

Figure 1.7: Schematic representation of the main ecological indices: richness, diversity and evenness. Four populations, composed of up to six OTUs (from A to F) present in various proportions, are compared and similar indices are in green, whereas different ones are in red.

The microbial populations of plant leaves are species specific (Lambais *et al.*, 2006) and even cultivar specific (de Costa *et al.*, 2006a). Overall, the diversity of culturable bacteria has been shown to be similar between leaves of the same age and the same time of year. However, they differed significantly on leaves of the same age at different times or on leaves of different age at the same time of year (Ercolani, 1991; Thompson *et al.*, 1993). More microbes could be recovered from old leaves than from young ones, but the number of different species was lower, suggesting that as leaves got older, only a few microbial species could remain viable and actively colonise the phyllosphere (Ercolani, 1991). Larger microbial populations on old

leaves could be explained by the fact that more nutrients leak out of old senescing leaves (Tukey, 1970) and that old leaves may be more protected against environmental challenges due to their position in the canopy. Patterns in bacterial colonisation have been observed with succession of bacterial species over time (Redford and Fierer, 2009).

1.4.2 Effects of Particular Microbes on the Phyllosphere Populations

Correlations between disease presence and modifications in the phyllosphere populations have been described so far as plant- and pathogen-specific. The powdery mildew causal agent on cucumber and Japanese spindle was shown to increase both diversity and richness of culturable epiphytic bacterial populations (Suda *et al.*, 2009). The driving forces of such modifications remain unclear but the promoted bacteria were shown to be able to degrade different substrates than the bacteria from healthy leaves, including polymers (α-cyclodextrin, Tween 40, Tween 80) or other substrates (carbohydrates: i-erythritol; amino acid: L-phenylalanine; phenolic compound: 4-hydroxy benzoic acid). In contrast, diseased leaves of the invasive plant *Eupatorium adenophorum* showed a significantly lower number of microbial species, using both culture-dependent and -independent techniques, compared to healthy leaves (Zhou *et al.*, 2010).

During plant-microbe interactions, some microorganisms produce or induce the production of high quantities of carbon- and/or nitrogen-rich compounds to sustain a nutritional advantage over other microbes. *Neotyphodium* endophytes are fungal symbionts of the meadow fescue. They increase protection against herbivore attacks by producing high levels of an alkaloid, loline (Zhang *et al.*, 2009a). Loline-containing plants possess a large population of *Burkholderia* bacteria able to catabolise loline, whereas these bacteria represent only a minority of the total

culturable bacteria population isolated from loline-free plants (Roberts and Lindow, 2010).

1.4.3 Effects of Plant Defences on the Phyllosphere Populations

Many phytohormones are involved in defence signalling (Bari and Jones, 2009), but the two most studied phytohormones are salicylic acid (SA) and jasmonic acid (JA). It is generally accepted that biotrophic pathogens are susceptible to SA-dependent defences, whereas necrotrophic pathogens are susceptible to JA-induced defences and these two phytohormones work antagonistically (Glazebrook, 2005). Both sets of defences influence the abundance and diversity of leaf-associated culturable bacterial communities on *A. thaliana* (Kniskern *et al.*, 2007; Traw *et al.*, 2007). Plants with constitutively SA-induced defences had a reduced endophytic population, whereas mutants deficient in JA-dependent defences had a greater epiphytic diversity. However, the microbial dynamics of particular bacteria were highly variable and no correlation with particular defence responses could be made.

1.4.4 Effects of Crop Protection on the Phyllosphere Populations

Agro-chemical treatments have various effects on non-target culturable microorganisms depending on the type and application rate of the pesticide and the type of microorganisms (Walter *et al.*, 2007). Application of streptomycin on maize leaves led to an increased bacterial diversity a few days after treatment (Balint-Kurti *et al.*, 2010). Similarly, application of cypermethrin, a synthetic pyrethroid behaving as a fast-acting neurotoxin against insects, led to an increased bacterial population on cucumber and pepper, with a significant effect on bacterial composition (Zhang *et al.*, 2008; Zhang *et al.*, 2009b). A dominance of Gram-negative bacteria was observed on treated cucumber leaves, as well as a significantly reduced fungal biomass (Zhang *et al.*, 2008). However, significant reduction of bacterial populations

(up to 10,000 fold) was observed on apple leaves with repeated application of pesticide, including cyprodinil, difenoconazole and dodine (Walter *et al.*, 2007) and a similar reduction was observed with fungicide treatment on barley leaves (Fountaine *et al.*, 2009).

1.5 MANAGING THE MICROBIAL POPULATION

The phyllosphere is a complex and dynamic habitat, where numerous microorganisms live together. The life style of one microorganism can differ during its life cycle on a host (Figure 1.6) (Kobayashi and Crouch, 2009; Newton *et al.*, 2010a). When looking at the overall phyllosphere population, only a few microbes are able to establish pathogenic interactions with the plant host, but they can be very costly for agriculture. As previously described in Section 1.1.3.2, conventional crop protection has many drawbacks. Manipulating the phyllosphere microbial population could offer a durable and integrated means to control disease by reducing the impact on yield over a short-term period and by decreasing the inoculum reservoir on a long-term scale (Newton, 2009; Newton *et al.*, 2010b)

1.5.1 Managing the Phyllosphere Genetically

Plant breeding could help to control the phyllosphere microbiological communities. Balint-Kurti *et al.* (2010) identified six quantitative trait loci (QTL) in maize associated with the control of epiphytic bacterial diversity. These loci colocalised with loci associated with susceptibility to southern leaf blight (SLB), caused by the *Cochliobolus heterostrophus* fungal pathogen. They came up with a model where the maize crop promoted a few suppressive bacteria to reduce its susceptibility to SLB. The bacterial diversity was linked to the activity level of an enzyme involved in the biosynthesis of the signal molecule gamma-aminobutyric

acid (GABA). The production of GABA is known to be induced by many environmental and biotic stresses (Chevrot *et al.*, 2006). Selecting for such traits could help in the future for developing integrated means to fight phytopathogens by promoting a natural suppressive microbiome.

Traits for manipulating the leaf-associated communities should be chosen wisely. Over the years, breeding of maize has naturally selected lines producing a higher level of benzoxanoids (BX), compounds with antifungal byproducts (Saunders and Kohn, 2009). A high level of BX correlated with a lower endophytic fungal population. However, many *Fusarium* species, including maize and human pathogens, appeared to be resistant to the BX antifungal byproducts. By selecting higher amounts of BX, the breeding process may have unintentionally favoured *Fusarium* species survival on the maize leaves, by reducing the competition with other natural fungal species sensitive to BX (Saunders and Kohn, 2009).

1.5.2 Managing the Phyllosphere with Combined Strategies

Pesticide application has been shown to greatly modify the phyllosphere structure and defence priming also affected the phyllosphere communities (Kniskern *et al.*, 2007). Some beneficial microorganisms are able to manipulate plant defences and induce systemic resistance (Marie *et al.*, 2001; Waller *et al.*, 2008). Hence, they may not necessarily be affected by elicitor treatment.

Protection by BCA usage is often time-limited and requires multiple applications. The pollinator vector technology offered an integrated possibility to combine biocontrol with pollination. Bumble bees were used as vectors to disseminate conidia of *Beauveria bassiana* under controlled conditions (Al Mazra'awi *et al.*, 2006). Tarnished plant bug and western flower thrip symptoms were significantly reduced on pepper plants using this method. Another strategy

consists of breeding beneficial microorganisms with their host. Endophyte-infected grasses are already patented (e.g. US Patent 6815591). Other endophytic fungi, such as the *Sebacinales*, have been shown to provide systemic resistance in major crops against various pathogens (Waller *et al.*, 2008) and offer great biotechnological opportunities (Oelmuller *et al.*, 2009). As a result, a combination of endophytes and elicitors could offer a sustainable and durable control against abiotic and biotic stresses (Newton *et al.*, 2010b).

1.6 THE PATHOSYSTEM

1.6.1 Barley (*Hordeum vulgare*)

Barley is the fourth most cultivated cereal in the world, behind rice, wheat and maize. In 2009, it was the 2nd most cultivated cereal in the United Kingdom (UK) and the 3rd most cultivated crop behind wheat and sugar beet but before potatoes. Scotland is the main barley growing region of the UK (Figure 1.8) (FAO, 2010). The principal uses of barley are for animal feed and human consumption, mainly in the production of alcoholic beverages (Newton *et al.*, 2011).

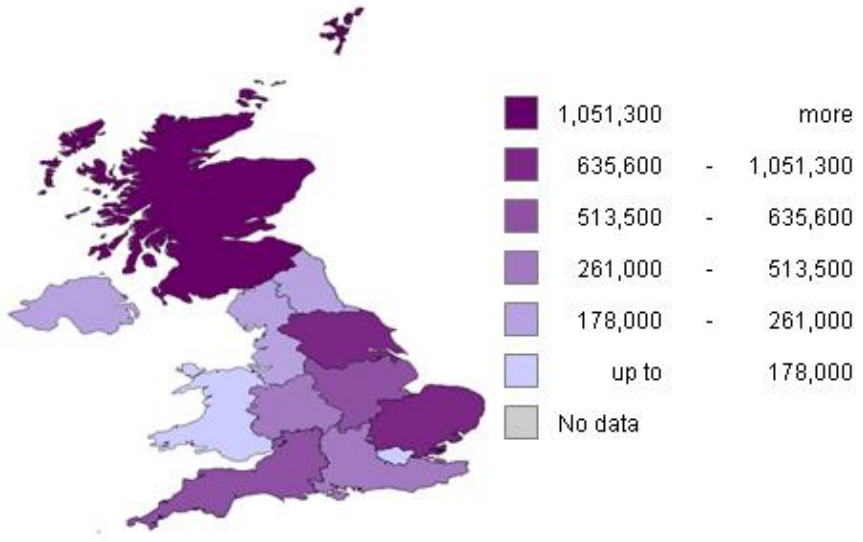

Figure 1.8: Production (in tons) of barley across the UK (FAO, 2010).

Worldwide, yield loss is estimated to be mostly caused by weeds (23%), fungal pathogens (15%), animal pests (7%) and viruses (3%) (Oerke and Dehne, 2004). The major fungal diseases in Scotland are barley leaf scald (also called blotch or rhynchosporium), powdery mildew and ramularia leaf spot (RLS), caused by *Rhynchosporium commune*, *Blumeria graminis* f.sp. *hordei* and *Ramularia collo-cygni* respectively. Other major diseases in the UK include net blotch, brown rust and eyespot (Figure 1.9). The national yield loss caused by *R. commune* has been estimated at around £4.8 million pounds per annum (HGCA, 2011).

Figure 1.9: Disease risk across the UK of the six major fungal diseases: rhynchosporium, ramularia leaf spot, net blotch, brown rust, powdery mildew and eyespot (HGCA, 2011). The disease risk is represented as low, intermediate and high by various colours: green, orange and red respectively.

1.6.2 Barley Leaf Scald Caused by *Rhynchosporium commune*

1.6.2.1 The Pathogen Life Cycle

The pathogen life cycle has been thoroughly described by Zhan *et al.* (2008). The primary source of infection lies in conidiospores (or conidia) resulting of asexual reproduction. They survive in plant stubble and debris from the previous harvest (Stedman, 1982). Surviving conidia keep the ability to infect plants for up to 30 weeks (Davis and Fitt, 1992). Infected seeds may also act as primary source of inoculation (Fountaine *et al.*, 2010).

The conidia germination rate varies with temperature and humidity and is optimal in temperate weather conditions, such as in the UK (Davis, 1990). It can occur on either sides of the leaf and on the seed coat of infected seeds (Brooks, 1928). The germination of conidiospores is suppressed in boundaries of lesions, possibly because of self-inhibitory compound (Davis, 1990). Furthermore, more than one isolate can be found in one lesion (Linde *et al.*, 2003). Around two germ tubes per conidia are usually formed and they follow the space between epidermal cells (Xi *et al.*, 2000). Under controlled conditions, conidial germination has been shown to occur within two days of infection on the leaf surface (Thirugnanasambandam *et al.*, 2011). After germination, germ tubes penetrate the plant cuticle via enzymatic degradation (Jones and Ayres, 1974). Occasionally, appressoria of variable shape have been observed under the microscope (Xi *et al.*, 2000). Once inside the plant, the fungus grows between the cuticle and the epidermal cells (Thirugnanasambandam *et al.*, 2011). This particular niche is rich in pectin, which is used via fungal pectic enzymes as a nutrient source by *R. commune*. The release of nutrients does not cause host death that is characteristic of a biotrophic phase. During this asymptomatic phase, no haustoria and no penetration of host cells have been observed.

Furthermore, the fungus was shown to be able to infect both susceptible and resistant barley cultivars (Thirugnanasambandam *et al.*, 2011).

However, if conditions are suitable for spore production, the epidermal cells collapse and there is formation of a stroma (i.e. a hyphal network where sporulation occur and symptoms appear). The collapse of the epidermal cells enables the fungus to reach the mesophyll tissue. Symptoms occur locally with the collapse of the mesophyll cells (Zhan *et al.*, 2008). This suite of events is caused by a change of the fungus life style to a necrotrophic phase. Spores are formed on both sides of the leaf (Brooks, 1928). The accumulation of conidia from the stroma leads to the cuticle to burst, exposing conidia to the environment and also causing symptoms locally (Howlett and Cooke, 1987). The local production of toxins, e.g. β-1-4-glucosides, is considered to be the cause of necrosis (Auriol *et al.*, 1978; Mazars *et al.*, 1990). Symtoms are also associated distantly with the diffusion of other fungal toxins, such as the necrosis-inducing peptides (Zhan *et al.*, 2008). However, spore formation was observed on resistant barley cultivars without symptom production (Thirugnanasambandam *et al.*, 2011).

Under controlled conditions, symptoms usually appear between 14 and 21 days post-infection (Thirugnanasambandam *et al.*, 2011). Leaves, auricles and leaf sheaths are usually infects first. The infection often occurs at the base of leaf junction, where conidia contained in water drops (rain or dew) may accumulate. Symptoms appear first as patches of water-soaked areas. With time, lesions turn darker and form dark brown lozenge-shape necrosis (Figure 1.10) and later become papery and white in the centre. Ears can also be infected but can appear symptomless (Lee *et al.*, 2001). Infected ears can result in a disease transmission to the next generation (Fountaine *et al.*, 2010). Seedlings grown from infected seeds can develop symptoms or remain

asymptomatic (Skoropad, 1959). As disease is polycyclic, secondary infection occurs but remains confined. Conidia are mostly dispersed via rain splash but can also be dispersed via direct leaf-leaf contact. Horizontal and vertical spread of conidia by rain splash does not exceed 70 and 50 cm respectively (Fitt *et al.*, 1988) Vertical spread is important to infect higher canopy and ear, which is the main factor impairing yield. Even though multiple evidences suggest that *R. commune* has a sexual phase, the teleomorph stage of the fungus has not been proven yet. The presence of *R. commune* has been detected in spore air-traps but at very low levels, suggesting a possible but unidentified air-borne dispersal of the disease (Fountaine *et al.*, 2010). The presence of two mating-type genes was detected in 1:1 ratio in many *R. commune* populations across the world and both mating types were also detected in one lesion (Linde *et al.*, 2003). However, no sexual ascospores were ever found (Salamati *et al.*, 2000; Fountaine *et al.*, 2010).

Figure 1.10: Typical symptoms of *Rhynchosporium commune* on field-grown barley crop. Picture kindly provided by Dr A.C. Newton from the James Hutton Institute (Dundee, UK).

1.6.2.2 Crop Protection Strategies

Control of *R. commune* is mainly achieved by breeding resistant cultivars and applying fungicides. Using phytosanitary techniques such as ploughing, crop rotation cultivation, delaying sowing date or changing the sowing rate, can also improve crop protection (Zhan *et al.*, 2008). Barley germplasm contains a large number of resistance traits. A total of 16 resistance alleles have been identified in barley and a large number of QTLs also confer partial resistance to leaf blotch. They are mostly localised on the chromosomes 3H, 6H and 7H (Zhan *et al.*, 2008).

As *R. commune* infects seeds, fungicide treatment on seeds is a valuable strategy to decrease early colonisation on seedlings. An early fungicide application is possible in spring to limit the early colonisation of the fungus on barley seedlings. The usual applications are undertaken in the field at two growth stages (GS), around GS30 and GS39. Both growth stages correspond to the beginning of stem elongation, which is a natural disease escape strategy, and the pre-inflorescence emergence period respectively (Zadoks *et al.*, 1974). The usage of fungicide mixtures reduces the risk of an early emergence of resistant pathogens (Cooke *et al.*, 2004).

Owing to the high level of diversity of pathogens, crop protection methods can be rendered ineffective within a few growing seasons, especially if they have been extensively used (Newton *et al.*, 2001). The diversity of *R. commune* populations in a field has been monitored using molecular markers, namely restriction fragment length polymorphism (RFLP) (Zaffarano *et al.*, 2006). About 60% of the variations in worldwide RFLP markers was found in one field. Introducing new resistant barley cultivars in a field resulted in a quick change of the pathotype diversity (Xi *et al.*, 2002). Using agro-chemicals, i.e. triazole or benzimidazole, also significantly affected the composition of the isolates diversity of

R. commune: fungicide-resistant isolates quickly became dominant (Taggart *et al.*, 1998). The ability of the fungus to complete its life cycle asymptomatically on both susceptible and resistant cultivars increases the difficulty to control the disease efficiently. By the time symptoms are visible, the fungus may have been through a few complete sporulation cycles, which could give it an adaptive fitness advantage. Pathogens isolated from resistant plants were more aggressive than similar isolates from less resistant plants (McDonald *et al.*, 1988).

The pathogen genetic structure is also affected by its ability to survive as a saprophyte. Changes in the composition of a mixture of eight pathogenic *R. commune* isolates infected on barley in the field were assessed during parasitic and saprophytic phases (Abang *et al.*, 2006). The frequencies of certain isolates were opposed during parasitic and saprophytic stages of the *R. commune* life cycle. This suggests that the isolates with a greater fitness during the saprophytic stage of their life cycle have an advantage compared to other isolates during the early infection of the host. Conversely, isolates with a more adapted parasitic life cycle have an advantage later in the season (Abang *et al.*, 2006).

New integrated crop protection methods are being sought. New genetic strategies are trying to identify plant QTLs affecting symptomless colonisation of the leaf by the fungus (Looseley *et al.*, 2012). The potential to manage the phyllosphere microbial ecology is investigated here.

1.7 AIMS AND OBJECTIVES

The phyllosphere is a complex environment where numerous and diverse microorganisms live and interact with each other and with the plant. The leaf pathogen *R. commune* causes significant yield loss every year on the barley crop,

especially in Scotland. Owing to its high diversity, conventional crop protection strategies have to be effectively managed and changed with time to hinder the emergence of resistance traits in the fungal pathogen. Treatments have a significant effect on the phyllosphere ecology and disrupt the innate microbial populations, which could be used to prevent the progress of the disease.

The aim of this work is to understand the interactions between the phyllosphere communities and *R. commune,* by:

- Developing a method to monitor the leaf-associated microbial populations from field-grown barley cultivars;
- Assessing the physiological effects of plants and the effect of agro-chemical treatments on microbial populations;
- Investigating the direct interactions occurring between leaf-associated bacteria and the *R. commune* fungus;
- Determining the systemic effects from a root-associated endophyte (*Piriformospora indica*) on leaf blotch.

The long-term objective of this study is the development of tools to monitor the innate plant microbial populations, in order to increase resistance to pests and diseases.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 PLANT GROWTH CONDITIONS

2.1.1 Field Experiments

2.1.1.1 Field and Cultivars Characteristics

To prevent seed-borne diseases, seeds were dressed with Raxil Pro® (active ingredients: prothioconazole, tebuconazole, triazoxide) according to the manufacturer's instructions (5 mL/kg of seed, Bayer, UK). Winter and spring barley (WB and SB) were sown in two different fields, both located on-site: the "Lab Field" (LF) and the "*Rhynchosporium* nursery" (H14). Both fields have grown barley continuously for over 10 years. Plots were sown in a split-plot design, with 360 seeds/m^2 sowing density (Appendix 10.1.1). In the LF, plots were present in triplicate and measured 5.6 m x 1.55 m, whereas in the H14 plots measured 2 m x 1.55 m and were usually present only in duplicate. A high natural *R. commune* inoculum level has been maintained in the H14 field over the years, by not applying fungicide, irrigating the crop during summer and limited ploughing. Fertilisers and herbicides were applied based on normal farming practices (Appendix 10.1.2).

A range of eight winter (WB) and eight spring (SB) barley cultivars was selected based on their different phenotypes and disease resistances (Table 2.1) and was grown in the field for three years (2008, 2009and 2010). The global resistance against leaf scald and powdery mildew was determined by the Home-Grown Cereals Authority (HGCA) using a scale ranging from 1 to 9, where 1 corresponds to highly susceptible and 9 to highly resistant. Some cultivars were old varieties, such as Maris Otter or Igri, whereas others were more recent: Leonie or Saffron. Finally, various phenotypes were selected: the majority of the varieties had two rows of seeds, whereas Manitou has six; and glossy mutants of Optic and Bowman were also sown.

Table 2.1: List of field-grown barley cultivars with description of variety characteristics and disease resistance against scald and powdery mildew, caused by *R. commune* (*Rc*) and *B. graminis* f.sp. *hordei* (*Bgh*). Resistance values were obtained from the latest (*) or older HGCA recommended lists, in which they were listed. When no numerical resistance record was available, the general resistance degree was described as susceptible (S) or resistant (R).

			Resistance	
	Cultivar	Characteristics	*Rc*	*Bgh*
Winter barley	Flagon *	6-row malting variety	7	7
	Igri	Old 2-row variety	S	S
	Leonie	2-row malting variety	R	R
	Manitou	6-row variety	R	R
	Maris Otter	Old 2-row variety	S	S
	Pearl *	2-row malting variety	6	6
	Saffron *	High yielding 2-row feed variety	4	3
	Sumo	High yielding 2-row feed variety	5	7
Spring barley	Bowman		S	S
	2015	Bowman glossy mutant	S	S
	2019	Bowman glossy mutant	S	S
	Cellar	Malting variety	4	9
	Chalice	Malting variety	5	9
	Cocktail	Feed variety	6	6
	Oxbridge *	Malting variety	7	7
	Optic *	High yielding malting variety	4	5
	48-82	Optic glossy mutant	S	S
	37-54	Optic glossy mutant	S	S

2.1.1.2 Crop Protection Treatments

Two kinds of crop protection treatments were applied in the LF: fungicide or elicitor, alone or combined (Table 2.2). All treatments were applied using a knapsack sprayer. Winter-type crops were treated at three growth stages: early spring (GS25), GS30 and GS39; whereas spring-grown crops were treated only twice (GS30 and GS39). Treatments at a particular GS were the same for both SB and WB.

Table 2.2: List of field-applied treatments, including fungicides and elicitors alone or combined, applied at three growth stages

Treatments		T0	T1	T2
Control		-	-	-
Fungicide	(FFF)	x (F)	x (F)	x (F)
Elicitors mix (t1) and fungicide (t2 and t3)	(EFF)	x (E)	x (F)	x (F)
Elicitors mix (t1 and t2) and fungicide (t3)	(EEF)	x (E)	x (E)	x (F)
Elicitors mix	(EEE)	x (E)	x (E)	x (E)
Bion		-	x	x
BABA		-	x	x
Cis-jasmonate		-	x	x
Activator 90		-	x	x
Maxicrop Triple		-	x	x
Saccharine		-	x	x

According to the SAC 2008 recommendations (Oxley and Burnett, 2008), fungicides were applied at each GS respectively as follows: cyprodinil (Kayak® 300 g active ingredient/L, 1.0 L/ha) combined with spiroxamine (Torch Extra®, 800 g active ingredient/L, 0.9 L/ha); prothioconazole (Proline®, 250 g active ingredient/L, 0.8 L/ha) combined with pyraclostrobin (Comet 200®, 200 g active ingredient/L, 1.25 L/ha); epoxiconazole (Opus®, 125 g active ingredient/L, 1.0 L/ha).

As described by Walters *et al.* (2011), elicitor treatments were used at the following concentrations: 1 mM acibenzolar-S-methyl (marketed as Bion® by Syngenta, CH), 1 mM beta-aminobutyric acid (BABA, Sigma-Aldrich, UK), 0.625 g/L cis-jasmonate (cis-JA, Sigma-Aldrich, UK), Maxicrop Triple® (according to manufacturer's instructions, Maxicrop®, UK) and 3 mM saccharine (Sigma-Aldrich, UK). All treatments were applied with a wetting agent (Activator 90, De Sangosse Ltd, UK).

2.1.1.3 Field Measurements

Disease symptoms of leaf scald and powdery mildew were assessed visually at regular intervals. Plots were scored using a logarithm scale ranging from 1 to 9, where 1 corresponds to 0% infection (no symptoms) and 9 to 100% (Newton and Hackett, 1994). At the end of the growing season, grain was harvested, left to dry and weighted. Yield was expressed in tonnes per hectare.

2.1.1.4 Sampling of Leaves

Leaves were sampled aseptically before and after each treatment across the growing season. The two most developed leaves were collected from seven plants in each plot. With the exception of during the isolation of culturable microorganisms, leaves were placed between tissue paper and stored in sealed plastic boxes at -20°C until needed.

2.1.2 Glasshouse Experiments

Seeds were placed to germinate on damp paper towels for three days in the dark at 18°C. Only pre-germinated seeds were selected for further experiments. Fifteen seeds were sown in a 10 x 10 x 12 cm black propylene pot filled with SCRI compost (Appendix 10.1.3). Barley was grown for two to three weeks in a spore-proof glasshouse (16 h daylight at 20°C; 8 h at night at 16°C) until emergence of the third leaf. No crop protection treatment was applied.

Glasshouse-grown barley seedlings were used to assess the effect of various microbes, as detailed in further sections, on disease resistance. All treatments were prepared in a Tween 80 suspension (0.01% v/v, Raymond Lamb Chemicals, UK) and applied using a hand-spray until run-off. Seedlings were subsequently left for two days in sealed transparent plastic bags with high humidity.

Treatment effect on disease was determined using a detached leaf assay with 8.0 x 4.5 x 2.0 cm clear plastic boxes (Stewart Solutions, UK) half-filled with water agar (0.5%) containing 1 mM benzimidazole (Sigma-Aldrich, UK) as described by Newton *et al.* (2001). Benzimidazole was used to slow down the leaf senescence. The middle segment of the second leaf was cut aseptically with a razor blade and placed adaxial side up on the water agar. Segments were left overnight in an environment controlled cabinet (Leec, model LT1201) with continuous light (200 $\mu mol.m^{-2}.s^{-1}$), and constant temperature (17°C), before being inoculated the following day with different disease causal agents.

2.2 MICROORGANISMS GROWING CONDITIONS

2.2.1 *Rhynchosporium commune*

The fungus *R. commune* was grown *in vitro* in 8.8 cm Petri dishes by smearing a chunk of mature hyphae with 300 µL of sterile distilled water (SDW) on a fresh CzV8CM plate (Appendix 10.2.1) (Newton and Caten, 1988). The isolate used (L2A) was originally isolated from on-site field. Plates were left in a cooled incubator (LMS, UK) in the dark at 18°C for two to three weeks. The isolate was stored at 4°C on CzV8CM agar slant.

Inoculum was prepared by scraping three weeks old plates, suspending it in 20 mL SDW and homogenizing for one minute. The homogenate was subsequently filtered through glass wool and centrifuged for 3 min at 700 g. The pellet was washed with 5 mL SDW and resuspended in 1 mL SDW. The inoculum concentration was determined using a haemocytometer with Neubauer rulings and diluted with SDW to a concentration of 10^5 spores/mL.

Inoculation for the detached leaf assay was undertaken as described by Newton *et al.* (2001). Briefly, the centre of leaf segments was abraded with a paint brush, which hairy tip was shortened. A volume of 10 μL of *R. commune* inoculum was then point-inoculated onto the abraded area. The wax layer would prevent the inoculum from staying on the leaf segment and its removal does not significantly interact with the assay's results (Newton *et al.*, 2001). From 12 days onwards, lesions started to appear. The emergence time on the lesion was determined, as well as the length of the lesion after emergence using dial callipers.

2.2.2 *Blumeria graminis* f.sp. *hordei*

The non-culturable fungus *Blumeria graminis* f.sp. *hordei* was originally isolated from leaves of naturally infected field-grown barley. It was subcultured every two to three weeks on detached leaf segments of a susceptible barley cultivar, Optic. Detached leaves were infected using an inoculation chamber. Briefly, the chamber consisted of a 1.20 m high circular aluminium column. After sterilisation with 70% ethanol (Sigma-Aldrich, UK), plates were left open at the bottom of the chamber. From the top, fresh spores were blown into the chamber and left for 10 min to settle. Plates were then closed and replaced in the same incubator as for the *R. commune* detached leaf assay.

Disease susceptibility was expressed as the number of infected leaves and the number of symptoms (*B. graminis* colonies) per leaf area unit (cm^2). Leaf area was estimated using the ImageJ software (Abrámoff *et al.*, 2004) by calculating the percentage of black from a black and white picture, where leaves are in black and background left white. Symptoms appeared around eight days post infection and their appearance was followed for 10 days.

2.2.3 Bacteria

Bacteria were grown on Luria-Bertani (LB) solid agar plates supplemented with antibiotics as appropriate (Appendix 10.2.3 and 10.2.5), incubated for two days at 28°C and stored in 30% glycerol (VWR BDH Prolabo, UK) stocks at -80°C.

Bacterial inoculum was prepared as follows: a single colony was placed in 5 mL liquid LB medium and cultured overnight at 28°C in a waterbath (OLS200 Grant, UK) under constant agitation. Overnight cultures were centrifuged at 2,000 g for three minutes (Biofuge pico, Heraeus, UK). The pellet was washed once with 5 mL SDW and resuspended in 1 mL SDW. The cell concentration was estimated by measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer (Jenway 6305, UK) of a 10-fold dilution and using the following formula: Cell concentration = $(1093.44 * OD_{600}) - 38.853) * 10^6 * 10$. The inoculum ($10^5$ cells/mL) was prepared in SDW for *in vitro* tests or in 0.01% Tween for *in planta* assays.

2.3 STATISTICAL ANALYSES

All statistical analyses were carried out using the GenStat software (version 13.1.0.4470, VSN International LTD). The normal distribution of samples was checked for each analysis. When values were not normal, they were transformed. Yield values were normalised to determine the effect of agro-chemical treatment or leaf surface mutation. One-way factorial analyses were carried out using the Chi-square test. Two-component analyses were calculated with the Student t-test, whereas analyses of multiple factors were achieved using analyses of variance (ANOVA). All statistical results were characterised by their probability (P) and least significant difference (lsd). All error bars of figures represent the standard deviation (SD) or the standard error of the mean (SEM) based on the number of samples (n).

CHAPTER 3: DEVELOPMENT OF A TECHNIQUE TO MONITOR MICROBIAL POPULATIONS OF BARLEY LEAVES

3.1 INTRODUCTION

The phyllosphere has been found to harbour many undescribed unculturable microorganisms, as demonstrated on citrus by Yang *et al.* (2001). The phyllosphere composition of various monocotyledons has been studied and includes maize (Kadivar and Stapleton, 2003; Balint-Kurti *et al.*, 2010), rice (de Costa *et al.*, 2006a; de Costa *et al.*, 2006b; Knief *et al.*, 2011) and wheat (Legard *et al.*, 1994; Gu *et al.*, 2010). However, little information is available on barley (Fountaine *et al.*, 2009). Most of the previously quoted studies have been carried out outside Europe and focused on one particular factor affecting the phyllosphere, such as UV radiation (Kadivar and Stapleton, 2003; Balint-Kurti *et al.*, 2010), the host genotype (de Costa *et al.*, 2006a; Fountaine *et al.*, 2009; Balint-Kurti *et al.*, 2010) or crop protection treatments (Fountaine *et al.*, 2009). Others investigated the roles and functions of leaf-associated bacteria (Knief *et al.*, 2011), with a particular interest in BCAs identification (de Costa *et al.*, 2006b; Fountaine *et al.*, 2009). The phyllosphere ecology was extensively investigated by Ercolani (1991) and Thompson *et al.* (1993) on citrus and sugar beet leaves respectively based on culturable techniques. Using molecular techniques, Redford and Fierer (2009) determined that there was a bacterial succession over the growing season in the cotton phyllosphere. No long-term molecular study of the microbial ecology of monocots is available.

Many analysis techniques have been used over the years, each with many issues to assess (Jacques and Morris, 1995). Culturable techniques are biased, as they exclude unculturable, non-viable and *in vitro* outcompeted microorganisms, which can represent large proportions of the total population (Wilson and Lindow, 1992;

Torsvik *et al.*, 1998). However, culturable microbes are valuable for further studies of interactions in axenic conditions. Molecular techniques offer good qualitative and semi-quantitative results. Methods such as DGGE (Kadivar and Stapleton, 2003) and RISA (Fountaine *et al.*, 2009), which separates amplicons via electrophoresis, show clear visual representations of the microbial diversity (presence/absence of bands) and easy identification of microbes of interest. Automated methods, e.g. ARISA (Ikeda *et al.*, 2004) and T-RFLP (Balint-Kurti *et al.*, 2010), offer better insight of the microbial relative abundance, but the identification of microbes of interest is more complicated.

Various genes can be used for ecological studies and specific populations can be targeted. For example, the nitrogen fixing bacteria have been monitored using the *nifH* nitrogenase gene (Rosado *et al.*, 1998). Global studies are usually done with conserved genes such as the 16S rDNA (George *et al.*, 2009), the internal transcribed spacer region (Ikeda *et al.*, 2004) or the 23S rDNA (Anthony *et al.*, 2000). The 16S rDNA has been mostly used and a great abundance of sequences have been gathered and are available in databases, such as the Microbial Community Analysis database (MiCA) (Shyu *et al.*, 2007). Many other parameters can influence the outcome of the profiling method and include, for example, the purine content and length of TRFs (Kaplan and Kitts, 2003), the PCR conditions (Sipos *et al.*, 2007; Rastogi *et al.*, 2010) or the statistical analysis approach (Blackwood *et al.*, 2007). The T-RFLP procedure from George *et al.* (2009) used to study the rhizosphere was adapted to monitor the barley phyllosphere.

The composition of the microbial epiphytes of different kingdom was firstly estimated using culturable techniques. After identification of bacteria and filamentous fungi, a database was established and expanded from available results in

the literature. This database was used to determine the gene of interest and the restriction enzyme for the molecular procedure chosen to monitor leaf-associated bacteria, the T-RFLP. Experimental validations were then undertaken on epiphytic and endophytic bacterial populations of field-grown leaves. Finally, results were compared with another molecular technique, RISA.

3.2 MATERIALS AND METHODS

3.2.1 Plant Material and Isolation of Microbes

Fresh leaves of Sumo grown under field conditions were sampled in six replicates as described in Section 2.1.1.4 in 2010 at the GS59. The removal of culturable epiphytes was undertaken using two techniques: sonication and washing. Leaves were placed with 10 mL of potassium phosphate buffer (PPB, pH = 7.0, Appendix 10.3.2) in a scintillation vial. The vials were left for 7 min in a sonicating bath (Clifton Ultrasonic Bath, NJ, USA). Similarly, five leaves were placed with 20 mL PPB in a 50 mL flask to be washed. Leaves had to be roughly chopped to fit in. The flasks were left for 1 h under gentle agitation. The removal efficiency of epiphytes was investigated by adding various chemicals to the PPB. The chemicals tested comprised different detergents (Tween, Triton and SDS at a concentration of 0.1% for all three, as well as 0.001% for Tween), a cation chelator (EDTA, 0.01 M) and a sterilising agent (hypochlorite NaOCl, 25 mg/L).

An aliquot of the washing/sonicating solution was diluted following a 10-fold serial dilution and 100 µL were spread on solid growth media: Nutrient Agar (Appendix 10.2.4) for bacterial isolation and CzV8CM with streptomycin for fungal isolation. Plates were incubated in the dark at 18°C for four and seven days respectively. The number of growing microorganisms was used to estimate the

number of colony forming units (cfu) per gram of fresh leaves. A random selection of 51 bacteria were then isolated and stored as described in Section 2.2.3. Yeast and filamentous fungi could be separately isolated. When possible a random selection of 50 isolates were subcultured and stored as described in Section 2.2.1.

For molecular characterisation, the rest of the washing/soniocating solutions were divided in four 2 mL microcentrifuge tubes and centrifuged for 40 min at 13,500 g at 4°C. The four pellets were resuspended in PPB, combined in one 2 mL screw-cap tube and centrifuged under the same conditions. The supernatant was discarded and the pelleted epiphytes were stored at -20°C for further molecular characterisation. Sonicated leaves were also stored at -20°C as well.

3.2.2 Identification of Epiphytes

The isolated bacteria were identified by sequencing their 16S rRNA gene (16F27 5' AGA GTT TGA TCC TGG CTC AG 3' and 1392R 5' ACG GGC GRT GTG TAC A 3'), whereas fungal identification was based on their 18S rRNA gene (0817F 5' TTA GCA TGG AAT AAT RRA ATA GGA 3' and 1536R 5' ATT GCA ATG CYC TAT CCC CA 3'). The polymerase chain reaction (PCR) had a volume of 50 μL and contained 0.4 μM of both primers, 0.2 mM of each nucleoside triphosphate (dNTPs, Invitrogen, CA, USA), 1 X of PCR buffer, 1 U of *Taq* DNA polymerase (Roche, DE) and 2 μL of microbial template. The PCR reaction was carried out on a PCR Express thermocycler (Hybaid, UK) as described: 94°C for 4.5 min, 30 cycles at 94°C for 30 sec, 57°C for 30 sec, 72°C for 1.5 min and a final extension step at 72°C for 10 min.

The bacterial DNA template consisted of a bacterial colony resuspended in 100 μL SDW, heated up at 100°C for 15 min to disrupt all cells and quickly centrifuged (13,500 g for 1 min) to pellet membranes and proteins. The fungal DNA

was extracted using a cetyltrimethylammonium bromide- (CTAB) based procedure. The mycelium was ground with liquid nitrogen using a mortar and pestle. The powder was mixed with 800 μL of CTAB buffer (Appendix 10.3.3) in a 2 mL microcentrifuge tube and incubated for 30 min at 65°C (Block Heater SBH130DC, Stuart, UK). An equal volume of chloroform-isoamyl alcohol (24:1, BDH-AnalaR, UK) was added. After a centrifugation step of 10 min at 1,300 g, the upper aqueous layer was transferred to a 1.5 mL tube with an equal volume of cold isopropanol (Fisher Scientific, UK). The mixture was vortexed and left overnight at -20°C. The DNA was pelleted by centrifugation (13,500 g for 30 min) and was washed twice with 500 μL 70% ethanol. The ethanol excess was air-dried and the DNA was suspended in 30 μL SDW.

The DNA amplification success was validated via electrophoresis on a Tris-borate-ethylene diamine tetraacetic- (TBE) based 1.5% agarose gel (Appendix 10.3.6). The remaining PCR products were cleaned using the Wizard PCR clean-up System (Promega, WI, USA) according to the manufacturer's instructions. The sequencing was carried out by the ABI3730 DNA analyser (Applied Biosystems, UK).

The identity of the culturable microorganisms was determined by comparing the sequenced rRNA gene to online databases using the BLASTN algorithm. Subsequently, the sequences were aligned to related species using the CLUSTALW algorithm. The non-overlapping ends of all sequences were trimmed and phylogenetic analyses were carried out with MEGA5 software, using the maximum-likelihood algorithm with a bootstrap value of 100.

3.2.3 Bioinformatic Analysis

A database of the 16S rDNA of leaf-associated bacteria was established from a selection of publications (Annex Table A.1). *In silico* T-RFLPs were visualised using the TRiFLe software (Junier *et al.*, 2008). A selection of restriction enzymes with a 4 bp recognition site was tested: *Aci*I, *Alu*I, *Bst*UI, *Cvi*QI, *Dpn*II, *Fat*I, *Hae*III, *Hha*I, *Mse*I, *Msp*I, *Taq*I and *Tsp509*I. The primer set used for the 16S rDNA database was the same as described in 2.4.2. The stringency conditions were set as low as possible.

3.2.4 Molecular Biology

The isolation of DNA from washing pellets and washed leaves was carried out using a phenanthroline-based method, but with different procedures. Washing pellets had very little DNA and required an efficient procedure, whereas leaf DNA could be extracted using a cruder procedure because of the excess of DNA. Both procedures were adapted from Fountaine *et al.* (2007).

3.2.4.1 Isolation of DNA from Epiphytes

Washing pellets were disrupted with 1 mm sterile stainless steel beads in the presence of 440 µL of phenanthroline-based extraction buffer (Appendix 10.3.5) by using a bead-beater (Qiagen TissueLyser, CA, USA) as follows: two burst of 1 min at 50 oscillations/sec with 2 min rest on ice in-between. After disruption, 400 µL of 2% sodium dodecyl sulphate (SDS, BDH Biochemical, UK) were added. Tubes were briefly vortexed and incubated for 30 min at 65°C. Subsequently, 800 µL of a 25:24:1 mixture of phenol, chloroform and isoamyl alcohol were added to each tube, which were centrifuged at 13,500 g for 10 min at 4°C. The supernatant was carefully transferred to a fresh 1.5 mL microcentrifuge tube containing 40 µL of 7.5 M ammonium acetate (Sigma-Aldrich, DE), 600 µL of cold isopropanol and 4 µL of

Glycoblue® (Ambion, UK). Tubes were left overnight at -20°C so that nucleic acids precipitated. Finally, DNA was pelleted (10 min at 13,500 g at 4°C), washed with 70% ethanol and resuspended in 30 µL SDW after evaporation of all remaining ethanol. DNA was quantified using a spectrophotometer (Nanodrop®, ND-1000, Thermo Scientific, Delaware, USA).

3.2.4.2 Isolation of DNA from Leaves

Leaves were manually crushed into fine powder in liquid nitrogen using a mortar and pestle. The phenanthroline-based extraction buffer and the SDS were added to the powder until a pourable mixture was obtained. A fraction of the mixture (500 µL) was transferred in a 2 mL microcentrifuge tube for DNA extraction. After incubating at 70°C for 20 min, 900 µL of 7.5 M ammonium acetate was added. The solution was gently mixed by inverting the tube and then placed on ice for 20 min. Following a centrifugation step (13,500 g, 15 min), the supernatant was transferred to a fresh 2 mL microcentrifuge tube containing 800 µL of cold isopropanol. Tubes were left for 15 min at room temperature and centrifuged for 15 min at 13,500 g. The supernatant was discarded and similarly to previously described, the DNA pellet was washed, resuspended and quantified.

3.2.5 Molecular Profiling of Bacterial Populations Using T-RFLP

3.2.5.1 Amplification Procedure

The T-RFLP technique is a semi-quantitative method based on 16S rDNA fragments of variable length because of the variable position of restriction sites. Forward and reverse fragments were labelled with different fluorescent-dyes: 6-carboxy-fluorescein (FAM) and hexachloro-6-carboxy-fluorescein (HEX) respectively. Both fluorophores, which are excited at the same wave lengths (488

nm) but emit differently: 518 nm for FAM and 556 nm for HEX (Figure 3.1), were detected by the ABI3730 DNA analyser.

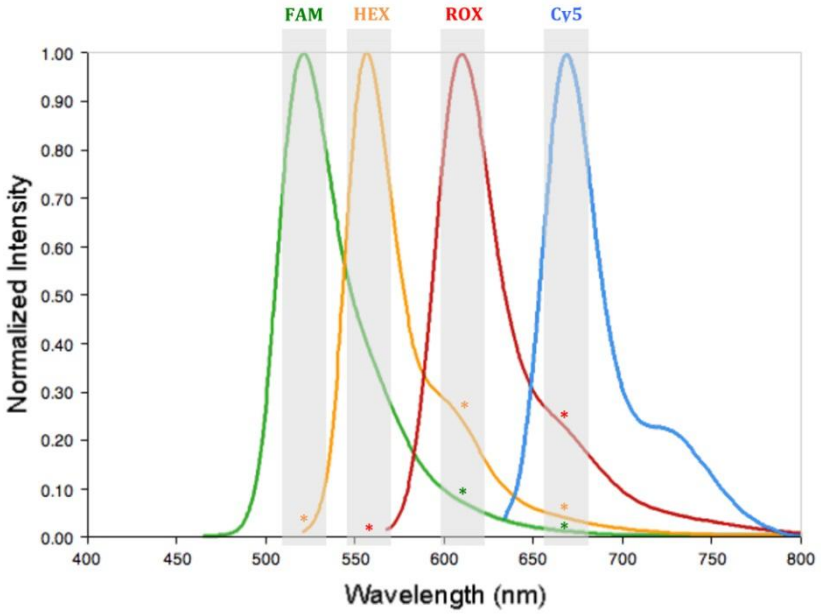

Figure 3.1: Emission spectra of four commonly used fluorophores in molecular biology: 6-carboxy-fluorescin (FAM), hexachloro-6-carboxy-fluorescin (HEX), 6-carboxy-X-rhodamine (ROX) and indodicarbocyanine (Cy5).

The PCR master mix consisted of 0.4 µM of both primers (16f27 5' FAM-AGA GTT TGA TCC TGG CTC AG 3' and 1392R 5' HEX-ACG GGC GRT GTG TAC A 3'), 1 X buffer, 2 mM $MgSO_4$, 0.25 mM dNTPs, 0.5 unit Platinum® *Taq* High Fidelity (Invitrogen, UK) and SDW (up to 25 µL), to which were added 10 ng of DNA template. The PCR reaction was carried out as described: 94°C for 4.5 min, 30 cycles at 94°C for 30 sec, 57°C for 30 sec, 68°C for 1.5 min and a final extension step at 68°C for 10 min (George *et al.*, 2009). After having checked the success of the amplification on a TBE-based 1% agarose gel with 1kb ladder (Promega, UK), 5 µL of amplicons were digested by 5 U of a restriction enzyme in a total volume of 6 µL for 2 h at 37°C and the reaction was stopped by heating samples at 65°C for 10 min.Three restriction enzymes, *AluI*, *HhaI* and *MseI* (Promega, UK), were tested.

The success of the restriction digest step was also checked on a TBE-based 2% agarose gel. Finally, 1 µL of DNA fragments were mixed with 9 µL of Hi-Di ™ formamide (Applied Biosystems, CA, USA) and 0.05 µL of LIZ 3730 size marker (Applied Biosystems, CA, USA) and loaded on the DNA analyser.

3.2.5.2 T-RFLP Analysis

Resulting T-RFLP electrophoregrams were uploaded into the GeneMapper software (version 3.7, Applied Biosystems, CA, USA). Profiles corresponded to series of peaks, called terminal restriction fragments (TRF), of various height expressed in relative fluorescence unit (rfu). Peak detection was carried out with the following settings: alignment of 3 bp bin size, detection TRF size frame from 50 to 500 bp, 50 rfu minimum peak fluorescence detection. Other settings were left as default. The peak fluorescence was determined as the area under the peak, rather than the peak height, because as TRFs migrate through the acrylamide column of the DNA analyser, late peaks are wider than the earlier ones. Each TRF represent one OTU, which can correspond to a single or multiple more or less related microorganisms.

The plant chloroplastic and mitochondrial rRNA genes (crRNA and mrRNA genes respectively) produced potentially detectable TRFs (Table 3.1). All fragment sizes were determined *in silico* from available sequences or by analogy with other grasses and cereals. The forward TRF of the crRNA gene was out of frame (1,049 bp), but the forward TRF of the mrRNA gene came out at 139 bp. Hence, the latter was manually removed for endophytic analysis. On the other hand, both crRNA and mrRNA genes shared a reverse TRF of a similar size (297 bp). The peak frequently showed a great fluorescence, particularly when looking at endophytic communities, causing an off-scale peak. This resulted in the appearance in an artefactual peak at

the same bin size on the forward TRF profile, because of a slight overlap in the fluorophore spectrum (Figure 3.1). Hence, the 297 bp TRF was systematically manually removed from both epiphytic and endophytic analysis.

Table 3.1: Theoretical TRF sizes (bp) of the 16S rRNA gene of chloroplasts and mitochondria from a selection of grasses and cereals (nd: not determined).

	Chloroplastic TRF size (bp)		Mitochondrial TRF size (bp)	
Organism	Forward	Reverse	Forward	Reverse
Barley (*Hordeum vulgare*)	1,049	297	nd	nd
Wheat (*Triticum aestivum*)	1,049	297	139	297
Sorghum (*Sorghum bicolor*)	1,049	297	139	297
Rice (*Oryza sativa* Indica Group)	1,049	297	139	297
Maize (*Zea mays*)	1,049	297	132	297
Brachypodium (*Brachypodium distachyon*)	1,050	297	nd	nd

Raw data were normalised using a 1% of total fluorescence threshold: all peaks below the 1% threshold were discarded. Remaining OTUs were expressed as relative abundance (peak fluorescence / total fluorescence) and used for multivariate analysis, namely the principal component analysis (PCA) using the GenStat software with correlation matrix algorithm. The identification of the OTUs involved in the separation of the bacterial communities was initially based on the loading vector value allocated for each principal component. For example, considering the first principal component, which is usually represented as the horizontal axis, OTUs with negative values are likely to be more common in populations localised on the left

hand side of the graph. Conversely, OTUs with positive loading vector values will be more common in populations placed on the right hand side.

Using the PAST software (Hammer *et al.*, 2001), a range of ecological indices were measured from the OTUs relative abundance (Table 3.2): the richness (S), the Simpson Index (1-D), the Shannon Index (H) and evenness (E). Comparison was carried out using ANOVA.

Table 3.2: Ecological indices used to characterise leaf-associated microbial populations. pi represent the relative abundance of each OTU i.

Ecological Indices	Description	Formula
Richness (S)	Represents the number of taxa (i.e. number of OTUs)	
Simpson index (1-D)	Ranges from 0 (population dominated by one taxon) to 1 (all taxa equally present).	$1 - \sum(p_i^2)$
Shannon index (H)	Varies from 0 for communities with only a single taxon to high values for communities with many taxa, each with few individuals.	$- \sum(p_i * \ln(p_i))$
Evenness (E)	Measures the ditribution of the microbes composing the communty	$\exp(H) / S$

3.2.6 Molecular Profiling of Bacterial Populations Using RISA

This gel-based method separates microbial species based on the natural variation of the internal transcribed spacer region, located between the 16S and 23S bacterial rDNA genes, whereas the T-RFLP is an automated method, where species-/genus-species fragments are obtained after restriction digest.

Each RISA PCR reaction was run in individual 0.2 mL PCR reaction tubes and contained 10 ng of DNA, 0.4 μM of each primers (1406F 5’ TGY ACA CAC

CGC CCG T 3' and 23SR 5' GGG TTB CAT TCR G 3'), 1X of GoTaq Green Master Mix (Promega, UK) and SDW up to a final volume of 25 µL. The PCR programme included an initial 2 min step at 95°C, followed by 30 cycles of 45 sec at 94°C, 1 min at 55°C, 2 min at 72°C and a final extension step of 7 min at 72°C (Fountaine *et al.*, 2009). Amplification products were separated on a 4 mm thick TBE-based 1.5% agarose gel and stained with RedSafe® (Chembio, UK). Samples migrated for 2 h 30 min at 125 V (Fountaine *et al.*, 2009).

Pictures were loaded in the ImageJ software (Abrámoff *et al.*, 2004) and the background subtracted with a 100 pixels rolling ball radius, in order to have a similar black background in all pictures. The plot lanes of the gels were carried out using the built-in gel analysis tools. The richness corresponded to the number of peaks.

3.3 RESULTS

3.3.1 Culturable Leaf-Associated Microbial Populations

Culturable microbes from barley leaves of Sumo grown under field conditions and sampled at GS59 were isolated using nine washing methods. The addition of surfactants or other chemical compounds to the extraction buffer significantly altered the effectiveness of the microbial isolation (Figure 3.2). Overall bacteria represented the most prevalent microbial group, followed by yeast and filamentous fungi. The use of SDW alone removed as many bacteria and yeast as the other best methods. Using the isotonic PPB alone either with the washing or sonication method, led to the recovery of significantly fewer yeast ($P < 0.001$) for both methods and fewer bacteria ($P = 0.006$) for the washing alone compared to using SDW. However, more filamentous fungi were recovered from the sonicated solution ($P < 0.001$).

Overall, the addition of detergent (Tween, Triton or SDS) to the buffer significantly improved the removal of microbes from the leaf surface, compared to washing the leaves with PPB only. All four treatments increased the number of recovered bacteria ($P = 0.006$) and filamentous fungi ($P < 0.001$). Significantly fewer yeast were isolated from the washing solutions containing Triton or SDS ($P < 0.001$). Using a lower amount of Tween (0.001%) in the extraction buffer significantly ($P < 0.001$) reduced only the number of isolated filamentous fungi compared to the extraction with a higher Tween concentration (0.1%). The EDTA significantly increased the number of isolated bacteria and filamentous fungi. Similarly, a low hypochlorite concentration enabled the isolation of more bacteria and filamentous fungi than the controls (Figure 3.2).

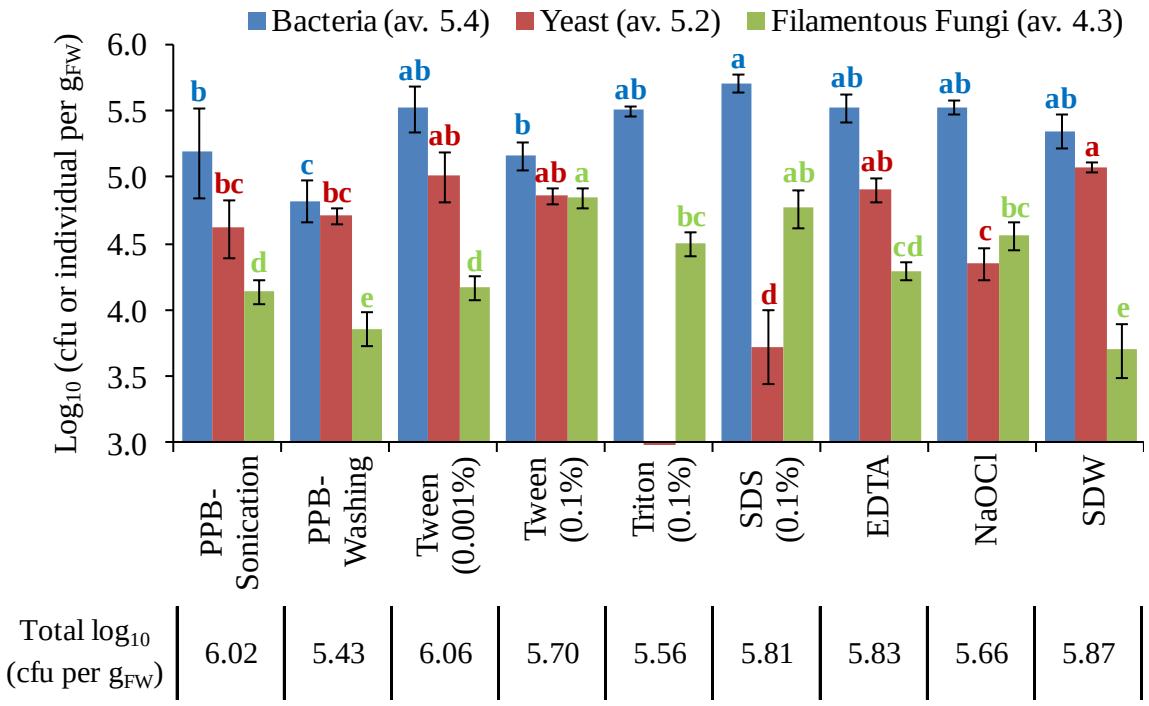

Figure 3.2: Number of epiphytic culturable bacteria, yeast and filamentous fungi per gram of leaf fresh weight expressed in logarithmic scale (total average in brackets) and isolated using nine different techniques. Treatments had a significant effect on the removal of bacteria ($P = 0.006$, lsd = 0.44), yeast ($P < 0.001$, lsd = 0.40) and filamentous fungi ($P < 0.001$, lsd = 0.28). Error bars represent the SEM (n = 9).

3.3.2 Optimisation of the T-RFLP

3.3.2.1 Bioinformatic Approach

A database of 182 different leaf-associated bacteria (Annex Table A.1) was established from seven publications (Annex Table A.3) that included various techniques (culturable and molecular identification) on different plants: *Arabidopsis thaliana*, cereals (wheat and maize), dicotyledonous crops (tomato and sugar beet) and trees (olive tree and citrus). The phyllosphere ecosystems from these different plants were all different in richness, but generally the *Proteobacteria* and more particularly the *Gamma-proteobacteria* were the most represented classes (Annex Table A.2).

The *in silico* T-RFLPs analyses were carried out using 60 leaf-associated bacteria (Annex Table A.1). Fragments from the 16S rDNA were generally grouped no matter which restriction enzyme was used (Figure 3.3). For example, the forward TRFs resulting from a digestion of the 16S rDNA with the *Alu*I restriction enzyme were roughly 80 and 220 bp long. Similar clustering could be observed with the reverse TRFs and when using the *Mse*I enzyme. Only the forward fragments of the 16S rDNA after *Hha*I digestion showed a certain variation in size.

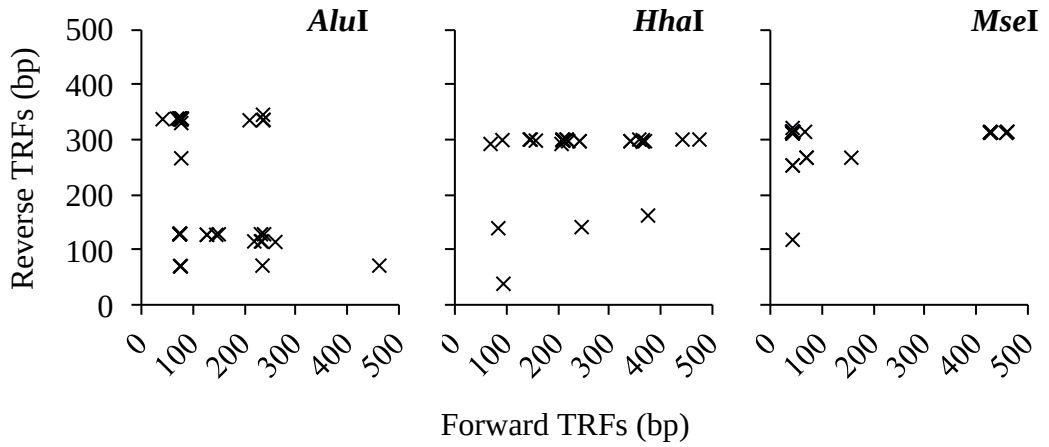

Figure 3.3: Range of forward and reverse TRFs obtained *in silico* from amplification of the 16S rRNA gene of a selection of 60 leaf-associated bacteria. The *in silico* digestion was performed with three different restrictions enzymes: *Alu*I, *Hha*I and *Mse*I.

3.3.2.2 Experimental Validation

The effectiveness of the *Hha*I enzyme to generate numerous OTUs of various size was experimentally confirmed by comparing it to the two other enzymes: *Alu*I and *Mse*I (Figure 3.4). The epiphytic bacterial richness of OTUs of forward TRFs was significantly greater ($P < 0.001$) when PCR products had been digested with *Hha*I rather than any other restriction enzymes, which was consistent with the *in silico* T-RFLP profiles (Figure 3.3).

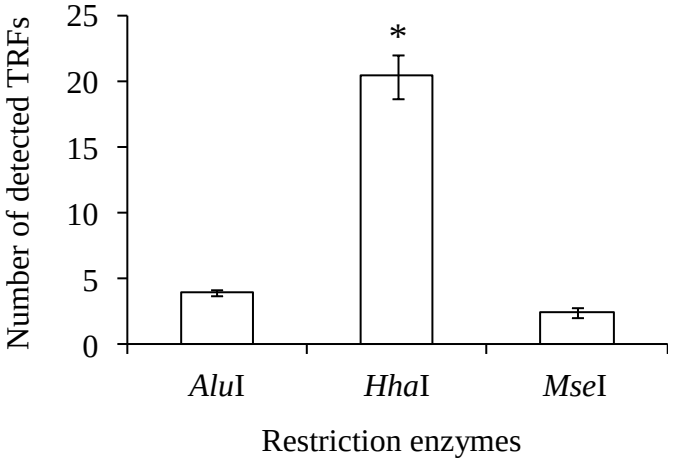

Figure 3.4: Experimental validation of the epiphytic bacterial richness of field-grown leaves from T-RFLP analyses using three restriction enzymes: *Alu*I, *Hha*I and *Mse*I (n = 10, P < 0.001%, lsd = 2.9). Error bars represent SEM.

Five leaves were used for the assessment of culturable microorganisms. However, a wider range of material, from one to 20 leaves, was tested using T-RFLP, to identify whether the sample size affected the structure of the bacterial communities. No significant difference of the bacterial richness, diversity or evenness was observed or differential clustering on the PCA plots (Table 3.3). Each of the 13 most abundant OTUs, which represented a total of 94% of the communities, were present in at least 60% of all samples (Table 3.4). Therefore, five leaves were kept as a standard for microbial removal.

Table 3.3: Ecological indices (richness, Simpson and Shannon diversity indices and evenness) of epiphytic communities isolated from sonicated washing solutions using from 1, 3, 5, 10 or 20 leaves.

Number of leaves	Richness (S)	Diversity (1-D)	Diversity (H)	Evenness (E)
1	14.3	0.797	1.992	0.514
3	14.0	0.802	2.032	0.550
5	14.0	0.771	1.924	0.500
10	14.7	0.823	2.104	0.562
20	15.3	0.843	2.236	0.613

Table 3.4: Relative abundance and occurrence of epiphytic OTUs from sonicated solutions using one to 20 leaves for microbial removal. Each abundance value is the average of three biological replicates. One OTU (62), marked with an asterisk, was significantly affected by the number of leaves (P = 0.048, lsd = 0.025).

	Relative abundance of OTUs isolated from various number of leaves						
OTUs	Av.	1	3	5	10	20	Occurence (out of 15)
230	0.321	0.332	0.313	0.383	0.315	0.259	15
197	0.175	0.183	0.183	0.206	0.179	0.121	15
77	0.089	0.136	0.070	0.083	0.094	0.061	15
368	0.060	0.020	0.119	0.006	0.103	0.054	11
205	0.060	0.090	0.053	0.021	0.063	0.072	15
365	0.041	0.004	0.015	0.019	0.025	0.142	9
83	0.039	0.025	0.020	0.071	0.026	0.052	9
93	0.036	0.036	0.034	0.037	0.036	0.036	15
90	0.031	0.029	0.031	0.032	0.032	0.029	15
63	0.025	0.016	0.016	0.007	0.024	0.063	10
62*	0.023	0.020	0.031	0.040	0.025		10
55	0.020	0.020	0.019	0.020	0.022	0.019	15
357	0.019	0.019	0.020	0.020	0.020	0.017	15
80	0.017	0.035	0.012	0.014		0.024	6
207	0.011		0.032			0.021	3
241	0.009	0.004	0.008	0.013	0.013	0.009	11
293	0.007	0.005	0.009	0.013	0.009		8
164	0.007	0.007	0.009	0.004	0.006	0.009	8
461	0.006	0.005	0.004	0.010	0.008	0.004	7
140	0.002	0.004				0.005	2
189	0.001	0.005					1
152	0.001					0.004	1
299	0.001	0.004					1
Number of OTUs detected	23	21	19	18	17	19	

3.3.3 Effects of the Removal Solution on the Phyllosphere Analysis

The characterisation of the culturable populations showed that the removal solution affected the number of epiphytes. Here, five out of the nine methods used for the characterisation of culturable populations (washing with water, washing with PPB, washing with PPB and Tween (0.001%), washing with PPB and SDS and sonicating with PPB) were further analysed using the T-RFLP method combined with the *Hha*I restriction enzyme. The molecular approach enabled the investigation of the structure of bacterial endophytic communities as well.

Analysis of the OTUs from forward TRFs showed that, according to the first principal component (PC1 = 11.4%), the communities isolated using water, PPB alone or sonication were different from the one isolated with buffer containing either surfactant (Figure 3.5 and Annex Table A.6). The relative abundance of the 62 and 365 OTUs were significantly reduced ($P < 0.001$ for both) in washing solutions containing surfactants, whereas it was increased for the 204 OTU ($P = 0.042$) (Figure 3.6). The relative abundance of the 57 OTU was also significantly higher ($P = 0.026$) in the solution containing Tween.

The numerical estimations of the phyllosphere ecology were also significantly altered by the treatment used for microbial isolation (Table 3.5). The bacterial diversity indices (Simpson and Shannon) were significantly reduced ($P = 0.008$ and $P = 0.025$ respectively) by adding SDS to the buffer to remove microorganisms from the leaf surface. A lower diversity means that these populations were dominated by few OTUs, which included the 204 OTU.

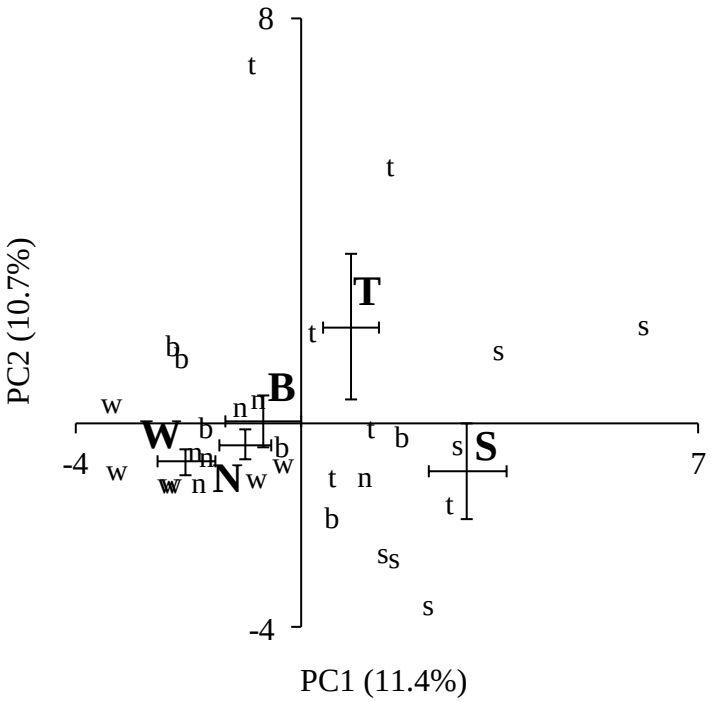

Figure 3.5: PCA (PC1 = 11.4% and PC2 = 10.7%) of epiphytic OTUs from forward TRFs isolated using five techniques: washing with SDW (w), washing with PPB (b), washing with PPB and SDS (s), washing with PPB and Tween (t) and sonicating with PPB (n). Bold uppercase data with SEM error bars correspond to the average of each treatment (n = 6).

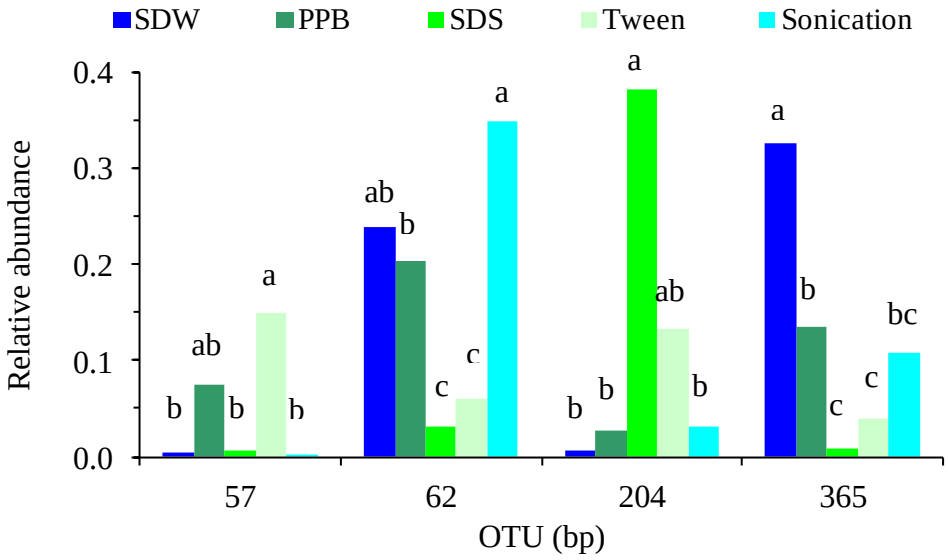

Figure 3.6: Treatments effects on the relative abundance of four OTUs significantly affected by the removal technique: 57 (P = 0.026, lsd = 0.10), 62 (P < 0.001, lsd = 0.14), 204 (P = 0.042, lsd = 0.27) and 365 (P < 0.001, lsd = 0.10).

Table 3.5: Ecological indices (richness (S), Simpson Index (1-D), Shannon Index (H) and evenness (E)) of epiphytic communities isolated with five different methods, estimated using OTUs from forward (top) and reverse (bottom) TRFs. Values correspond to the average of six biological replicates obtained from two field plots.

Forward TRFs

Treatment	Richness (S)	Diversity (1-D)	Diversity (H)	Evenness (E)
SDW	14.0	0.787[a]	1.971[a]	0.531
PPB	13.0	0.828[a]	2.068[a]	0.629
Tween	15.5	0.769[a]	2.051[a]	0.549
SDS	10.3	0.563[b]	1.346[b]	0.438
Sonication	12.8	0.779[a]	1.969[a]	0.606
P	-	0.008	0.025	-
lsd	-	0.145	0.478	-

Reverse TRFs

Treatment	Richness (S)	Diversity (1-D)	Diversity (H)	Evenness (E)
SDW	10.8[ab]	0.687[a]	1.574[a]	0.451[a]
PPB	12.5[a]	0.730[a]	1.773[a]	0.496[a]
Tween	10.8[ab]	0.639[a]	1.481[a]	0.417[a]
SDS	8.0[b]	0.336[b]	0.833[b]	0.317[b]
Sonication	12.3[a]	0.709[a]	1.781[a]	0.512[a]
P	0.039	< 0.001	0.003	0.027
lsd	3.1	0.178	0.495	0.125

Similar results were observed by analysing OTUs of the reverse TRFs: the PCA separated populations removed from the leaf surface by buffer with surfactants from the ones isolated by the other treatments (PC2 = 11.3%) that included water, buffer and sonication (Figure 3.7). The microbial richness, diversity and evenness were also reduced by the addition of SDS to the extraction buffer (Table 3.5), implying a less rich population dominated by a few OTUs. Overall, the reverse

profiles had a much higher richness than that observed by the *in silico* estimations, but all ecological indices were lower than the ones calculated from the OTUs of forward TRFs. Future analyses will focus on the latter only.

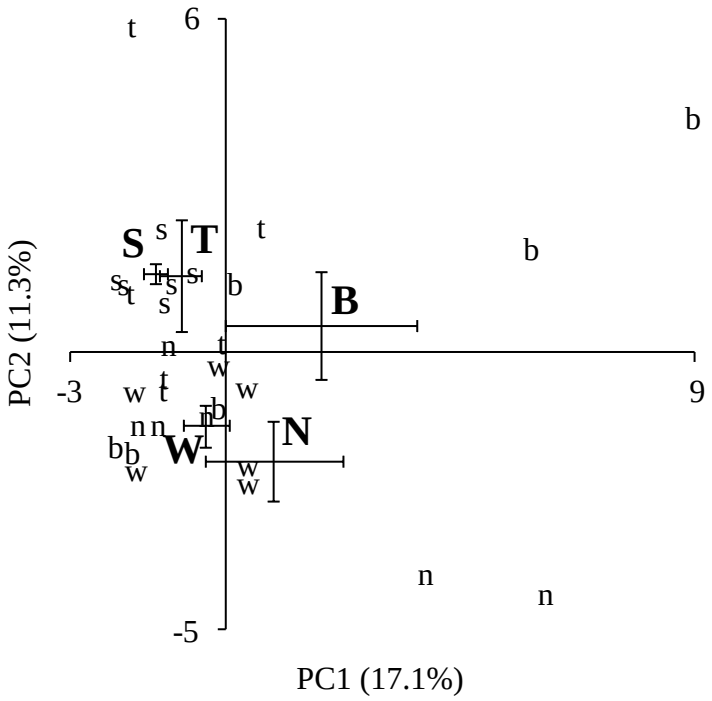

Figure 3.7: PCA (PC1 = 17.1% and PC2 = 11.3%) of epiphytic OTUs from reverse TRFs isolated using five techniques: washing with SDW (w), washing with PPB (b), washing with PPB and SDS (s), washing with PPB and Tween (t) and sonicating with PPB (n). Bold uppercase data with SEM error bars correspond to the average of each treatment (n = 6).

The endophytic communities had a different structure from the epiphytic ones. Both communities had a similar richness, but differed in their evenness and both diversity indices ($P < 0.001$, $P = 0.01$ and $P = 0.001$ respectively), suggesting that the OTUs relative abundance is more even across endophytes than epiphytes (Table 3.6). The number of leaves used for the extraction, the nature of extraction solution and the extraction method had no significant effect on the analysis of endophytic population (Annex Tables A.7 and A.8).

Table 3.6: Comparison of ecological indices (richness (S), Simpson Index (1-D), Shannon Index (H) and evenness (E)) between epiphytic and endophytic communities. Significant differences were identified in the Simpson Index (P = 0.01, lsd = 0.029), the Shannon Index (P = 0.001, lsd = 0.261) and the evenness (P < 0.001, lsd = 0.064).

Populations	Richness (S)	Diversity (1-D)	Diversity (H)	Evenness (E)
Endophytes	16.4	0.860	2.334	0.721
Epiphytes	13.1	0.822	1.887	0.551
P	-	0.010	0.001	<0.001
lsd	-	0.029	0.261	0.064

3.3.4 Comparison with RISA Profiles

Results obtained with the RISA profiling method (Annex Figure A.3) were compared to those obtained using T-RFLP and showed a lower degree of analysis. Monitoring the bacterial endophytic populations was biased due to the presence of a large and bright band around 250-300 bp, corresponding to the plant DNA. Even though the significant effect (P = 0.018) of treatments on the bacterial removal was similar to what was observed before (Table 3.5), epiphytic communities from the RISA profiles had a lower richness (Table 3.7) compared to the one analysed with T-RFLP (Table 3.5). Furthermore, the image analysis was fastidious compared to the automated genotyping method.

Table 3.7: Richness of epiphytic communities isolated with five methods and estimated using the RISA technique. The extraction solutions consisted of SDW, buffer with Tween (0.001%), buffer with SDS (0.1%) and buffer alone either combined with the washing procedure or sonication (P = 0.018, lsd = 1.8).

Treatments	SDW	Buffer	Tween	SDS	Sonication
Richness	7.7^{a}	7.3^{a}	6.7^{ab}	5.0^{b}	8.0^{a}

3.3.5 Identification of Bacterial and Fungal Isolates

A random selection of 51 bacteria was isolated from the previous estimation of the total bacterial population and identified by sequencing their 16S rDNA (Figure 3.8), which was phylogenetically compared with known related species (Annex Figures A.1 and A.2 and Table A.4 and A.5). The culturable bacterial population was largely dominated by bacteria belonging to the *Pseudomonas* genus and included *P. syringae* (41%), *P. fluorescens* (33%), *P. graminis* (8%) and *P. migulae* (4%). The third most common bacterium was identified as *Erwinia tasmaniensis* (12%). Both *Erwinia* and *Pseudomonas* bacteria belong to the *Gamma-proteobacteria* class. Another unidentified *Gamma-proteobacterium* was also isolated.

The theoretical TRF sizes of each identified bacterial species did not perfectly match the experimental validation, but were close (Table 3.8). The *Pseudomonas* spp. TRF size ranged from 203 to 205 bp and *E. tasmaniensis* emerged at 369 bp. The analysis of complex population increases the variability of OTUs length and the binning process (i.e. the alignment of all TRFs) may create a shift in the TRF size. The culturable *Pseudomonas* and *Erwinia* bacteria could have been binned in two OTUs each: 204 and 206 or 368 and 369. The four OTUs accounted for 11.6, 1.9, 9.0 and 1.5 percent of the whole population respectively (Annex Table A.6). However, other bacterial species can have similar TRF length, be included in the same OTU bin and participate to the OTU abundance.

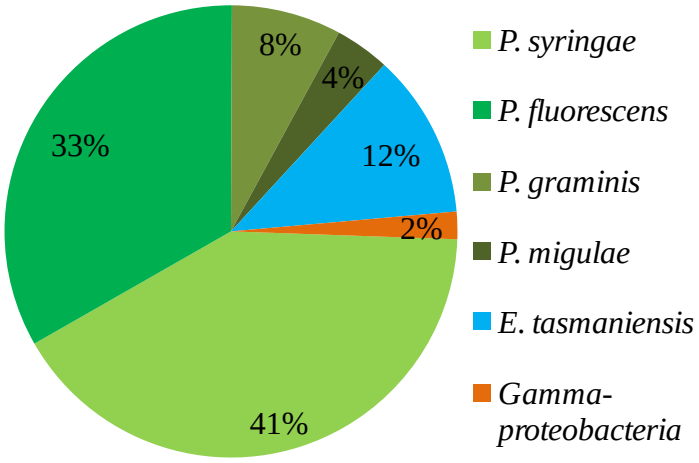

Figure 3.8: Proportions of culturable bacterial species in the epiphytic communities of field-grown barley. Bacteria were largely dominated by various *Pseudomonas* species and *Erwinia tasmaniensis*.

Table 3.8: *In silico* and experimental TRF sizes of the isolated culturable bacteria.

		TRF size (bp)			
		in silico		experimental	
Isolate	Identity	Forward	Reverse	Forward	Reverse
312G	*Pseudomonas syringae*	209	299	205	296
311A1	*Pseudomonas fluorescens*	157 - 207	301	204	296
311F	*Pseudomonas graminis*	207	301	204	299
312L	*Pseudomonas migulae*	206	299	203	298
312B	*Erwinia tasmaniensis*	371	164	369	164

A subset of seven filamentous fungi was selected based on their different morphology *in vitro* and identified by sequencing their 18S rDNA (Figure 3.9). No phylogenetic comparison with related fungal species was carried out. All fungi were ascomycota. Three out of seven have been identified only at the class or genus level, because there were multiple matching organisms with high similarity. The identified fungi included three *Dothideomycete* (*Boeremia exigua* and two *Dothideomycete* sp.), one *Sordariomycete* (*Arthrinium* sp.), one *Leotiomycete* (*Botryotinia*

fuckeliana), one *Eurotiomycete* (*Penicillium piceum*) and one mitosporic *Ascomycota* (*Humicola fuscoatra*).

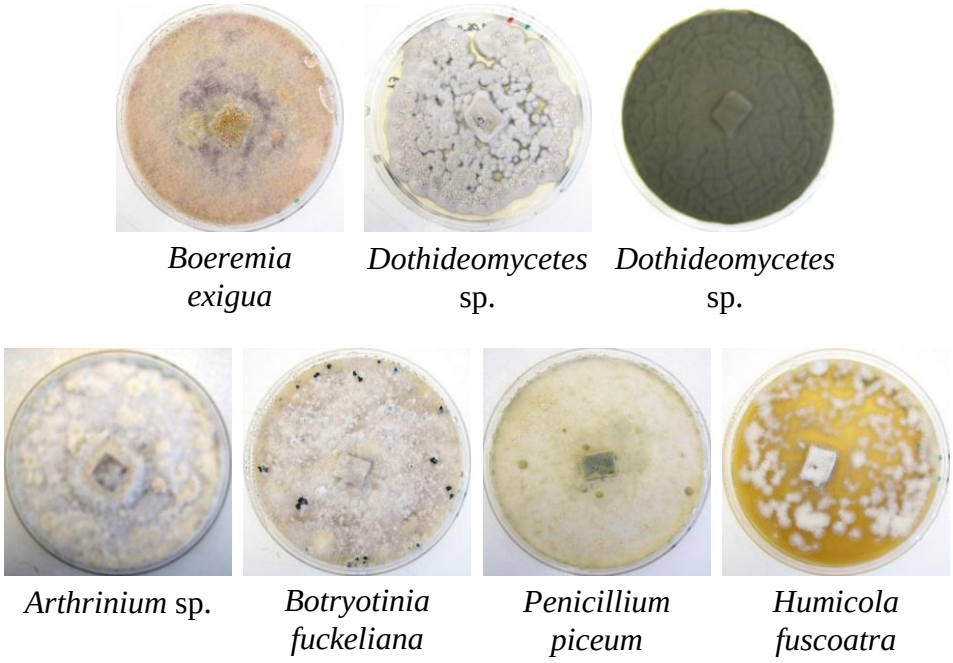

Figure 3.9: Identity of seven epiphytic culturable fungi, isolated based on different phenotypes.

3.4 DISCUSSION

The phyllosphere is a complex habitat hosting diverse microorganisms and its study requires thorough methodology. The T-RFLP technique combined with *Hha*I is a powerful tool to study the barley phyllosphere. Unlike the RISA method, it allows an estimation of the community structures (ecological indices), group individuals (PCA) and focus on particular OTUs of interest. However, the molecular profiling can be affected by many experimental factors. Here, the microbial removal method was shown to significantly modify the structure of the epiphytic populations. All treatments tested improved the removal of at least one kind of microorganism

compared to the buffer alone (Figure 3.2), suggesting that they were able to disrupt the bacterial biofilms and/or other microbial adhesion mechanisms differentially.

A biofilm is a complex structure, mostly formed of polysaccharides held tight by cations such as calcium (Ca^{2+}) (Sutherland, 2001; Morris and Monier, 2003). It provides many advantages such as an anchor to the leaf surface and protection against UV and osmotic shifts (Davey and O'Toole, 2000). Hence, bacteria trapped in biofilms may not be effectively removed from the leaf surface. Both EDTA and hypochlorite treatments promoted the removal of bacteria, but also filamentous fungi from the leaf surface. They were shown to cause partial removal of biofilm proteins in a bioreactor (Chen and Stewart, 2000): EDTA is supposed to disrupt the biofilm structure by chelating cations, such as Ca^{2+}, essential to biofilm integrity (Turakhia *et al.*, 1983), whereas hypochlorite causes biofilm protein removal by an unknown mechanism (Chen and Stewart, 2000).

Another way to promote microbial removal consists of gently disrupting the leaf surface. Surfactants improved the isolation of both bacteria and filamentous fungi, but Triton and SDS hindered yeast isolation. Anionic surfactants (SDS) were shown to solubilise the leaf epicuticular waxes (Tamura *et al.*, 2001), whereas nonionic surfactants (Tween and Triton) induced a reduction of the stiffness of plant wax surface (Grant *et al.*, 2008). However, using chemically similar surfactants (Tween and Triton) did not result in similar effects on the whole populations. The surfactant concentration also affected differentially the extraction. Nevertheless, the overall proportions of the bacterial populations were conserved, no matter what surfactant was used (Figure 3.6). Even though adding chemicals to the isolation buffers improved the global isolation of microorganisms, certain of them were preferentially isolated. Adding SDS and Tween resulted in a shift in the relative

abundance of a few OTUs, suggesting either an improved or impaired removal (Figure 3.6). Furthermore, the diversity of the epiphytic population significantly was reduced (Table 3.5), which means that the population is more dominated by a few microbes. As the aim of this study was to investigate the complex ecology of the phyllosphere, no extra chemicals were added to the buffer. The technique used also affected differentially the outcome of the isolation. Sonication was better than washing: as leaves had to be roughly chopped to fit in the flask for washing, epiphytic solutions were green, indicating the presence of chlorophyll and other plant debris. To avoid plant DNA to be detected on the T-RFLP profiles, the sonication was preferred to the washing method and no surfactant or other chemical was added to the extraction buffer.

The barley phyllosphere of Sumo harboured many microorganisms: mostly bacteria, then yeast and filamentous fungi, reaching over one million individuals per gram of fresh leaf weight (Figure 3.2). Bacteria are usually described as the most common organism on the leaf surface (Lindow and Brandl, 2003), and the fungal population, dominated by ascomycota, was much larger on older leaves (Thompson *et al.*, 1993; Jumpponen and Jones, 2009; Glushakova and Chernov, 2010), which was consistent with our findings (Figure 3.2 and 3.9). Overall, more bacteria were observed on barley leaves by Fountaine *et al.* (2009), but these leaves were much younger than the ones used in this study. This difference in the number of bacteria between young and old leaves was in contradiction with the findings of Thompson *et al.* (1993) on sugar beet, where it was shown that older leaves have more bacteria than young ones. However, these two analyses cannot be directly compared with each other as they are based on different cultivars and different locations. Significant differences in the number of culturable leaf-associated bacteria were observed on

various rice cultivars (de Costa *et al.*, 2006a). Even Fountaine *et al.* (2009) found significant differences between the two monitored cultivars: Optic and Cellar.

The *Pseudomonas* bacteria were the most common culturable bacteria, which was consistent with the literature (Rotem *et al.*, 1976; Ercolani, 1991; Thompson *et al.*, 1993). They have many different roles: *P. syringae* strains are well-known pathogens and ice nucleation-active bacteria (Hirano and Upper, 2000), whereas some *P. fluorescens* strains can be pathogenic (Cui *et al.*, 2005) or act as a BCA (Paulsen *et al.*, 2005). The other two *Pseudomonas* species have no known role: *P. graminis* was identified on grasses (Behrendt *et al.*, 1999) and *P. migulae* in water streams (Verhille *et al.*, 1999). *Erwinia tasmaniensis* is a potent BCA on apple trees against the fire blight pathogen, caused by the closely related bacterium *Erwinia amylovora* (Geider *et al.*, 2006). The roles of these culturable bacteria on barley leaves are unknown, but could potentially interact with disease causal agents.

The diffusates from a fluorescent *Pseudomonad* strain isolated from *R. commune* lesions were shown to induce total lysis of *R. commune* protoplasts, but lysis of spores was achieved from diffusates from *R. commune*-infected leaf lesions, suggesting complex interactions between microorganisms (Rotem *et al.*, 1976). Furthermore, culturable bacteria represent only a fraction of the whole leaf-associated population (Yang *et al.*, 2001). The ratio between culturable and unculturable bacteria (estimated using DGGE) from the rhizosphere has been shown to vary between 0.1 and 10% depending on the soil type (Torsvik *et al.*, 1998). Similarly, T-RFLP profiles revealed that the identified culturable bacteria represent only a fraction (up to 24%, Annex Table A.6) of the total community, which was of a similar order to what Wilson and Lindow (1992) observed with *P. syringae* on bean leaves.

The role and, most importantly, the identity of other unculturable bacteria of the Sumo leaves are still unknown. The OTUs corresponding to the culturable bacteria were easily found *in silico* and validated experimentally. The identification of other OTUs from databases, such as MiCA (Shyu *et al.*, 2007), is not possible. The most common OTU (62; Annex Table A.6) matched only seven different bacterial species, none of which were known plant-associated bacteri (data not shown). Furthermore, predicted and experimental TRF size did not match perfectly (Table 3.8). Using the MiCA database, the 61, 62 and 63 OTUs corresponded to over 80 different genera and more than 1,300 accession numbers. A clone library would be essential to identify the missing OTUs, but no interaction study could be undertaken without culturable isolates.

The roles of fungi also should not be ignored. As the plant ages they represent an increasing proportion of the whole microbial community (Thompson *et al.*, 1993; Jumpponen and Jones, 2009; Glushakova and Chernov, 2010). Many yeast, when inoculated on barley leaves before challenge with the causal agent of the barley leaf scald, induced a significant increase of visible symptoms (Fountaine *et al.*, 2009). Such knowledge could help to develop more integrated crop protection strategies, to not only kill causal agents, but also helper microorganisms (Newton and Toth, 1999; Newton *et al.*, 2010b).

CHAPTER 4: INTERACTIONS BETWEEN MICROORGANISMS

4.1 INTRODUCTION

The major diseases identified on cereals in Europe are caused by fungal pathogens, but bacteria are the most common microbial colonisers of the phyllosphere (Figure 3.2; Lindow and Brandl, 2003). Water and nutrient availabilities are considered as the main limiting factors for microbial growth on the leaf surface (Mercier and Lindow, 2000). Thus, bacteria have evolved various strategies to survive in this harsh environment. For example, the abilities of bacteria to be motile and produce biofilms were shown to give a fitness advantage to epiphytes (Haefele and Lindow, 1987; Yu *et al.*, 1999). As bacteria proliferate and colonise the leaf surface, the accumulation of signal molecules leads to the triggering of the formation of special structures via a mechanism sensing the population density called quorum sensing (QS). Many virulence factors are controlled by QS (Liu *et al.*, 2008) as well as some steps of the nodulation process of the beneficial *Rhizobia* bacteria (Gonzalez and Marketon, 2003).

Such fitness advantages facilitate the colonisation of plants and can also promote interactions with other microorganisms. The trophic interactions between microorganisms and the plant are very diverse and dynamic, depending on the plant or environmental conditions (Newton *et al.*, 2010a). Six epiphytic bacteria, including four *Pseudomonas* species and *Erwinia tasmaniensis* (Figure 3.8), were previously identified on Sumo leaves grown under field conditions at GS59 and reached a total of half a million individuals per gram of fresh weight (Figure 3.2). The different bacteria were shown to have various functions on other plants, such as being pathogenic (Hirano and Upper, 2000; Cui *et al.*, 2005) or acting as BCAs (Paulsen *et*

al., 2005; Geider *et al.*, 2006). However, the functions of these culturable bacteria on barley are not known.

Using a detached leaf assay, various bacteria have been identified as "helpers" of the causal agent of the wheat glume blotch pathogen *Phaeosphaeria nodorum* (Dewey *et al.*, 1999) i.e. promoting the development of symptoms (Newton and Toth, 1999). On field-grown barley and wheat crops, the presence of a bacterial potato pathogen has been correlated with significant changes in the amount of visible symptoms of fungal diseases (Newton *et al.*, 2004). The cereals were grown following the growth of a potato-infected crop. More powdery mildew (caused by *Blumeria graminis* f.sp. *tritici*) and septoria leaf blotch (caused by *Mycospherella graminicola*, formerly *Septoria tritici*) symptoms were observed on wheat, whereas less scald symptoms were observed on barley early in the season.

Pectobacterium atrosepticum, formerly known as *Erwinia carotovora* subsp. *atroseptica*, is a soil-borne potato pathogen and the causal agent of blackleg on stems and soft rot on tubers. As an opportunistic pathogen, it has been described as causing disease only on potato when host defences were impaired. Although *P. atrosepticum* has a limited host range, it has been shown to be able to colonise *Brassicaceae* roots (Perombelon and Hyman, 1989). Comparative genomics studies have revealed that the bacterium possesses a wide range of potential interaction mechanisms, including the presence of six types of secretion systems (from T1SS to T6SS), nitrogen fixation machineries, opine/rhizopine uptake and catabolism, antibiotic and toxin production (Toth *et al.*, 2006). Therefore, it has been hypothesised that the bacterium could have beneficial effects on *Brassicaceae* (Newton *et al.*, 2010a).

The presence of *P. atrosepticum* in the barley field was validated using molecular detection and its ability to colonise barley leaves was experimented *in*

planta. The biocontrol abilities of the *P. atrosepticum* and the previously isolated epiphytic bacteria were tested *in vitro* against *R. commune* and i*n planta* against *B. graminis* f.sp. *hordei*. Finally, the mechanisms used by those microbes to survive in the phyllosphere and interact with other microorganisms were assessed *in vitro* by measuring their motility (swimming and swarming), their biofilm production and by testing various mutants.

4.2 MATERIALS AND METHODS

4.2.1 Bacteria

Two isolates of each bacterial species identified as epiphytes of field-grown barley (Table 4.1 and Annex Figures A.1 and A.2) were used for further characterisation of potential interactions with barley disease causal agents. The *P. atrosepticum* 1043^{Str} WT strain and some of its pathogenicity-related mutants (Table 4.1) were kindly provided by Prof. I.K. Toth from the James Hutton Institute (Dundee, UK). The WT strain had a natural streptomycin resistance, whereas mutants were introgressed with a kanamycin resistance gene. The mutants were non-functional in certain elements of their pathogenicity machineries, including the QS (*expI*), various types of secretion systems (T2SS: *outD*, T3SS: *hrcC*, T4SS: *virB4*, T6SS: *ECA3444*) and different antimicrobial or toxic compounds: phenazine (*ehpB*), coronafacic acid (*cfa6*) and two potential toxins identified via comparative genomics studies (*ECA0482* and *ECA1488*). All mutants showed an impaired pathogenicity on potato (Bell *et al.*, 2004; Holeva *et al.*, 2004; Liu *et al.*, 2008; Toth, 2010). The growth and storage conditions as well as the inoculum preparation and application of all bacterial strains and mutants were carried out as previously described in Section 2.2.3.

The presence of *P. atrosepticum* in field samples was detected by PCR using species specific primers: ECA1f (5' CGG CAT CAT AAA AAC ACG 3') and ECA2r (5' GCA CAC TTC ATC CAG CGA 3') (Deboer and Ward, 1995). The PCR reaction (50 μL) was similar to the procedure for identification of epiphytes (Section 3.2.2) but the template consisted of 10 ng of leaf washing DNA as used for the T-RFLP optimisation (Section 3.3.3). The identity of the amplification products was validated by sequencing and comparison with entries on online databases as detailed in Section 3.2.2.

Table 4.1: List of epiphytic bacterial isolates as well as *Pectobacterium atrosepticum* and some of its pathogenicity-related mutants used for fitness and biocontrol characterisations.

Epiphytes from field-grown barley	312B	- *Erwinia tasmaniensis*
	331Q	- *Erwinia tasmaniensis*
	313H	- *Pseudomonas syringae*
	312G	- *Pseudomonas syringae*
	313P	- *Pseudomonas fluorescens*
	311A1	- *Pseudomonas fluorescens*
	311F	- *Pseudomonas graminis*
	312C	- *Pseudomonas graminis*
	312L	- *Pseudomonas migulae*
	313N	- *Pseudomonas migulae*
P. atrosepticum wild-type and pathogenicity mutants	1043^{Str}	- *Pectobacterium atrosepticum* (WT)
	expI	- quorum sensing (QS) mutant
	outD	- type 2 secretion system (T2SS) mutant
	hrcC	- type 3 secretion system (T3SS) mutant
	virB4	- type 4 secretion system (T4SS) mutant
	ECA3444	- type 6 secretion system (T6SS) mutant
	ehpB	- phenazine antibiotic mutant
	cfa6	- coronafacic acid mutant
	ECA0482	- possible toxin mutant 1
	ECA1488	- possible toxin mutant 2

4.2.2 *In vitro* Interactions between Microbes

4.2.2.1 Assessment of Bacterial Motility and Biofilm Formation

Biofilm formation was observed after an extended (two days) growth in liquid LB at 28°C (Figure 4.1 C). The swimming and swarming motility of the epiphytic bacteria (Figure 4.1 A and B) were tested using a 0.3% and 0.6% LB medium respectively (Appendix 10.2.3), adapted from Stavrinides *et al.* (2009). For the swimming test, the centre of the 0.3% plate was stabbed with a sterile toothpick dipped in a bacterial inoculum (OD_{600} = 0.3) and for the swarming test, a 5 µL droplet was point-inoculated in the middle of the 0.6% plate. Plates were then sealed with parafilm, inverted and incubated at 28°C for three days. The swimming motility was quantified as the diameter of bacterial growth, whereas the swarming motility and biofilm formation was qualitatively assessed (presence / absence).

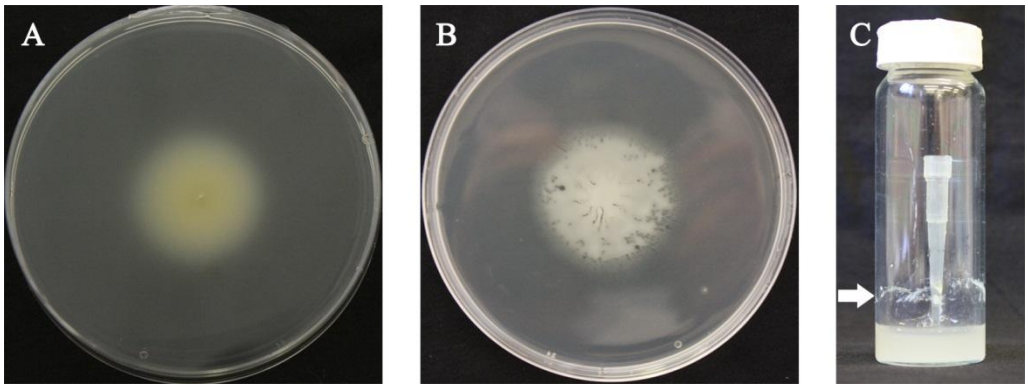

Figure 4.1: *In vitro* assessment of bacterial motility (A: swimming and B: swarming) and biofilm formation (C), indicated with a white arrow.

4.2.2.2 Dual-Inoculation Plate Assay

The dual-inoculation plate assay was adapted from Matarese *et al.* (2011). Five hyphal chunks of *R. commune* (diameter = 0.5 cm) were placed equidistantly on a CzV8CM plate (Figure 4.2 A). Four droplets of 5 µL of bacterial inoculum (10^5

cells/mL) or mock solution were point-inoculated equidistantly between all hyphal plugs (Figure 4.2 B). Plates were sealed with parafilm and incubated for two weeks at 28°C. Plates were then placed on a white box and photographed. Using the ImageJ software, pictures were converted to black and white with a constant threshold and the area of *R. commune* growth was determined.

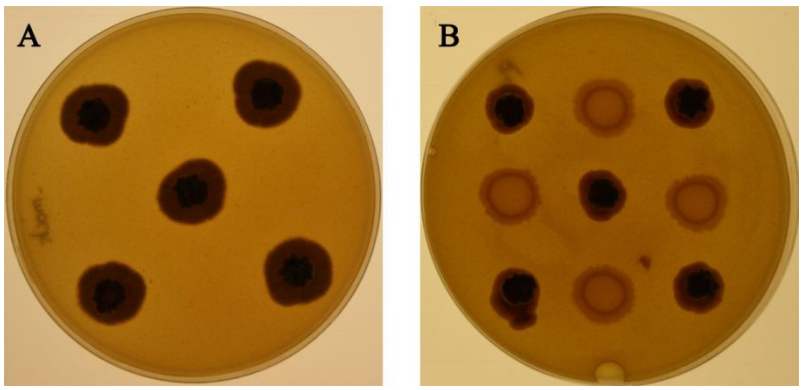

Figure 4.2: Dual-inoculation plate assay setup with five equidistantly-placed hyphal plugs of *R. commune* and four equidistantly-placed drops of mock solution (A) or bacterial inoculum of epiphytes (B). The picture was taken two weeks after inoculation.

4.2.2.3 Co-Culture in Liquid Medium

The effect of *P. atrosepticum* on *R. commune* spores was determined by growing them both in potato dextrose broth (PDB) growth medium (Appendix 10.2.2) favourable to the germination of the fungal spores. In a centrifuge tube, 2 x 10^5 fungal spores were transferred in 50 mL of PDB, as well as 1 mL of bacterial inoculum (10^5 cells/mL) or mock solution. The co-cultures were incubated at 18°C for four days (until the broth turned turbid) under gentle agitation. Each day, a 100 µL aliquot of each co-culture was plated in triplicate on CzV8CM medium with or without ampicillin and incubated at 18°C. A week later the number of rescued *R. commune* colonies was counted.

In order to observe direct interactions between *P. atrosepticum* and *R. commune*, a similar setup was carried out in a smaller volume (2 mL). A fraction was observed under the light microscope 30 min after incubation at room temperature under gentle agitation.

4.2.3 *In planta* Experiments

Optic seedlings were grown under normal glasshouse conditions and inoculated as described in Section 2.1.2. The survival of *P. atrosepticum* on barley leaves was estimated by counting the number of viable cfu per leaf fresh weight unit. Five leaves were collected at different time points over 12 days. They were weighed and ground using a mortar and pestle in 10 mL of PPB. Aliquots (100 µL) of 10-fold serial dilutions were plated on LB plates supplemented with streptomycin (Appendix 10.2). After two days of incubation at 28°C, the number of bacteria was counted. The effect of *P. atrosepticum* on fungal diseases was carried out using a detached leaf assay as described in Section 2.2.1 and 2.2.2.

4.3 RESULTS

4.3.1 Detection of *P. atrosepticum* in the Field

The pathogen *P. atrosepticum* is a culturable bacterium that has been isolated from the leaves of field-grown barley crops under high inoculum pressure (Newton *et al.*, 2004). Under normal field conditions and using a non-specific growth medium, *P. atrosepticum* was not isolated from Sumo leaves (Figure 3.8). However, using species specific primers, *P. atrosepticum* was detected in 67% of the samples used to optimise the T-RFLP procedure (Figure 4.3). The identity of the amplification products was validated via sequencing and comparing them to online

database accessions (data not shown). All sequences matched the genome of *P. atrosepticum* strain 1043 with a maximum identity of 99%.

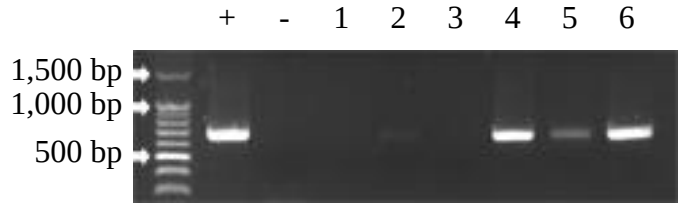

Figure 4.3: PCR detection of *P. atrosepticum* on Sumo leaves at GS59 from six biological replicates grown under field conditions.

4.3.2 Characterisation of Epiphytes

The motility and biofilm formation abilities of the epiphytic bacteria were assessed *in vitro* (Table 4.2). All bacteria were able to swim through the 0.3% agar, but the effectiveness varied based on the bacterial species: *E. tasmaniensis* and *P. migulae* were best able to swim furthest, whereas *P. syringae* swam the least. Only *P. syringae* was able to swarm and only *E. tasmaniensis* produced a biofilm *in vitro*. None of the tested epiphytes had all three abilities. However, *P. atrosepticum* was demonstrated to be able to swim, swarm and produce a biofilm (Toth, 2010).

Table 4.2: *In vitro* characterisation of motility (swimming and swarming) and biofilm formation of epiphytes isolated from leaves of field-grown barley. The swimming ability was quantified by measuring the diameter of the bacterial halo growth (± SEM), whereas the swarming motility and biofilm production was only qualitatively recorded (+: presence, -: absence). Experiments were carried out using two strains of each epiphyte in triplicate (n = 6).

Bacteria	Swimming (cm)	Swarming	Biofilm
Erwinia tasmaniensis	3.43 ± 0.63	-	+
Pseudomonas syringae	2.33 ± 0.31	+	-
Pseudomonas fluorescens	2.92 ± 0.44	-	-
Pseudomonas graminis	2.90 ± 0.40	-	-
Pseudomonas migulae	3.87 ± 1.08	-	-

4.3.3 *In vitro* Effects on Disease

The indirect effects of the five epiphytes and *P. atrosepticum* on the growth of *R. commune* were investigated *in vitro* using a dual-inoculation plate assay (Table 4.3). Compared to the control and mock-treated plates, all microbes induced a reduction of the fungal growth. Even though the growth medium was very rich (Appendix 10.2.1), the global reduction of the fungal growth could be due to an *in vitro* competition for nutrients. However, two bacteria, *P. syringae* and *P. atrosepticum*, had a significantly ($P < 0.001$) stronger effect on *R. commune* growth than the other bacteria had.

Table 4.3: *In vitro* effects of bacterial epiphytes, *P. atrosepticum* and related pathogenicity mutants (*Pba*) on the growth of *R. commune* using a dual-inoculation plate assay (± SD). The area of *R. commune* growth (cm^2) was recorded from three replicates ($P < 0.001$, lsd = 0.11).

Bacteria	*R. commune* growth area (cm^2)
Control	2.43 ± 0.17
Mock	2.18 ± 0.18
Pseudomonas fluorescens (311A1)	1.58 ± 0.23
Pseudomonas graminis (312C)	1.60 ± 0.19
Pseudomonas migulae (313N)	1.47 ± 0.11
Pseudomonas syringae (312G)	1.07 ± 0.16
Erwinia tasmaniensis (311Q)	1.53 ± 0.18
Pectobacterium atrosepticum	1.26 ± 0.09
Pba expI (quorum sensing)	1.81 ± 0.20
Pba outD (T2SS)	1.28 ± 0.08
Pba hrcC (T3SS)	1.30 ± 0.08
Pba virB4 (T4SS)	1.94 ± 0.32
Pba ECA3444 (T6SS)	1.29 ± 0.12
Pba cfa6 (coronafacic acid)	1.23 ± 0.14
Pba ehpF (phenazine)	1.22 ± 0.05
Pba ECA0482 (potential toxin 1)	1.14 ± 0.13
Pba ECA1488 (potential toxin 2)	1.24 ± 0.11

To gain a further understanding of the mechanisms involved in this reduction of *R. commune* growth, various pathogenicity mutants of *P. atrosepticum* (Table 4.1) were tested using the dual-inoculation plate assay (Table 4.3). All mutants except two had a similar strong negative effect on the growth of *R. commune* as the WT strain *in vitro*. A significant increase of the fungal growth ($P < 0.001$) was observed with two mutants, *expI* and *virB4*. The compromised effect of *P. atrosepticum* on *R. commune* growth resulted in the loss of functional quorum sensing (QS) and type IV secretion system (T4SS) machineries.

The direct interactions between *R. commune* spores and *P. atrosepticum* were investigated by co-cultivating both microorganisms together. The number of rescued viable *R. commune* fungal colonies was significantly reduced ($P = 0.02$) when in the presence of *P. atrosepticum* (Table 4.4). Adhesion between a bacterial cell and a fungal spore was also observed under the light microscope (Figure 4.4).

Table 4.4: Number of *R. commune* colonies (± SD) on various media with ampicillin or not, rescued from liquid cultures that contained *R. commune* spores alone or with *P. atrosepticum* ($P = 0.02$).

Microbes in liquid culture	*R. commune*	*R. commune*	*R. commune* + *P. atrosepticum*
Ampicillin	-	+	+
Number of *R. commune* colonies rescued	25.7 ± 18.5^{ab}	27.3 ± 6.8^{a}	14.0 ± 2.0^{b}

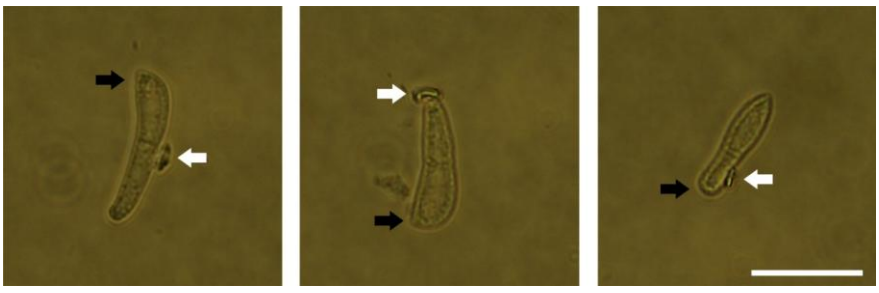

Figure 4.4: Adhesion of *P. atrospeticum* (white arrow) to *R. commune* spores (black arrow) *in vitro* after 30 min incubation (scale = 10 μm).

4.3.4 *In planta* Effects on Disease

Before investigating the effect of *P. atrosepticum* on the development of foliar diseases, its growth on Optic leaves was determined (Figure 4.5). The culturable bacterial population gradually increased with time (P = 0.007) and peaked at nearly 10^6 cfu/g_{FW}. Overall, the bacterium *P. atrosepticum* was able to actively colonise the barley phyllosphere.

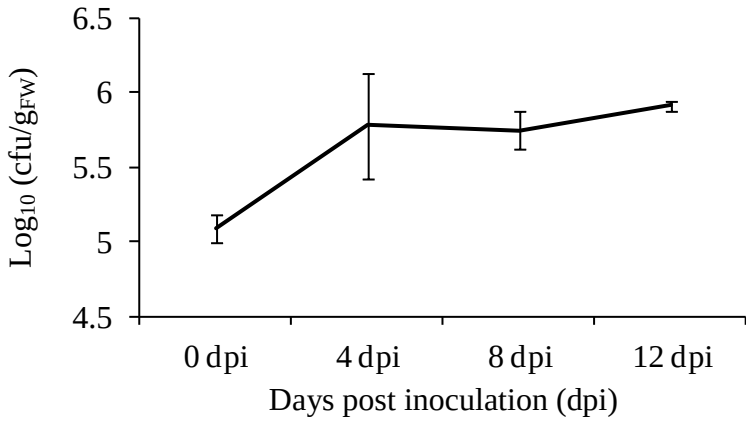

Figure 4.5: Growth dynamic of *P. atrosepticum*, expressed in logarithm-transformed cfu per gram of leaf fresh weight (g_{FW}), over 12 days post inoculation (dpi) on Optic leaves. Error bars represent SD (n = 4). The bacterial growth significantly increased with time (P = 0.007, lsd = 0.28).

The ability of *P. atrosepticum* to affect *R. commune* pathogenicity *in planta* was assessed on Optic using a detached leaf assay (Table 4.5), but no particular effect was recorded. The ability to affect disease resistance against powdery mildew was also investigated, as it had been shown that the presence of *P. atrosepticum* was correlated with an increase of powdery mildew symptoms on wheat (Newton *et al.*, 2004). On barley, symptoms appeared eight days after inoculation and gradually increased to reach a maximum a week later (Figure 4.6). A significant increase (P < 0.001) of the number of colonies per leaf area unit was observed on *P. atrosepticum*-

treated leaves. This observation was validated three times (Table 4.5) and an increase of the number of infected leaves was also observed.

Table 4.5: Effects of *P. atrosepticum* on *R. commune* and *B. graminis* infection *in planta* on Optic leaf segments at 14 days post inoculation. The disease severity was assessed as the proportion of infected leaves and the number or size of visible symptoms (± SD).

		Replicate	Control	+ *P. atrosepticum*
R. commune	Proportion of infected leaves		0.25	0.50
	Size of lesion per infected leaves		0.54 ± 0.08	0.54 ± 0.13
B. graminis	Proportion of infected leaves	1	0.83	1
		2	0.38	0.63
		3	0.48	0.57
	Number of symptoms per cm^2	1	1.65 ± 1.05	2.16 ± 0.81
		2	0.49 ± 0.34	0.90 ± 0.42
		3	0.75 ± 0.23	1.19 ± 0.79

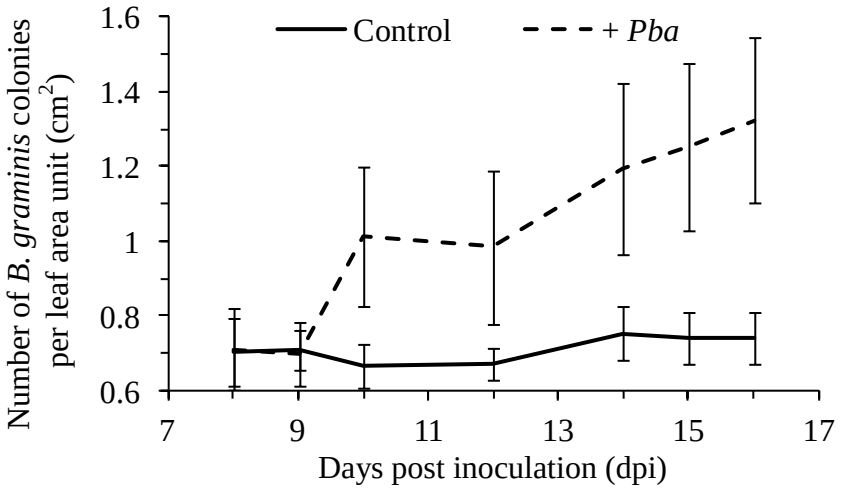

Figure 4.6: *In planta* effects of *P. atrosepticum* on *B. graminis* infection from 8 to 16 days post inoculation on Optic detached leaves (n = 21). The bacterium significantly altered the resistance to powdery mildew 14 days onwards after inoculation ($P < 0.001$, lsd = 0.40). Error bars represent SEM.

4.4 DISCUSSION

The presence of the bacterial potato pathogen, *P. atrosepticum* was previously shown to be correlated with significant changes in fungal disease resistance on field-grown wheat and barley (Newton *et al.,* 2004). Such results implied that the severity of disease causal agents could be significantly affected by the naturally occurring bacterial microflora, but whether *P. atrosepticum* was directly responsible for such changes still had to be determined. Two different *in vitro* assays showed that *P. atrosepticum* was able to reduce the growth of *R. commune* via direct and indirect interactions (Table 4.3 and 4.4). No contact between the bacteria and the hyphae occurred during the dual-inoculation plate assay, suggesting that diffusible compounds were responsible for the reduction of fungal growth. Similarly, cell-free diffusates from a leaf-associated *Pseudomonas* were demonstrated to enhance *R. commune* protoplast lysis (Rotem *et al.*, 1976). However, microscopic observations revealed that the bacterium could directly adhere to the fungal spores (Figure 4.4), hence multiple biocontrol mechanisms might be involved in the reduction of *R. commune* growth and spore germination.

An understanding in the mechanisms involved in *R. commune* biocontrol was sought using a selection of pathogenicity mutants of *P. atrosepticum*. As diffusible compounds were thought to be involved in this process, various mutants with non-functional antimicrobial or toxic compounds were tested *in vitro,* but none showed significant effects (Table 4.3). However, two other mutants, one with a non-functional QS machinery and the other with a non-functional T4SS, reduced *R. commune* growth less than the WT strain did. The QS process senses the bacterial density to trigger the expression of specific proteins and structures. The *P. atrosepticum* QS was demonstrated to alter the expression of a quarter of the

bacterial transcriptome *in vivo* (Liu *et al.*, 2008). The expression of two components of the T4SS was also significantly affected by a non-functional mutation of the bacterial QS 12 h after inoculation on potato tubers (Liu *et al.*, 2008).

A vast range of molecules are secreted or assimilated through the T4SS, including plasmid DNA and effector proteins (Ding *et al.*, 2003). The T4SS of *Agrobacterium tumefaciens* has been extensively described, as it is the secretion system that delivers the infectious transfer DNA (T-DNA) from the tumour-inducing plasmid to plant hosts (Christie, 2001). The T-DNA codes for proteins involved in the *de novo* biosynthesis of phytohormones and the opine carbohydrates that the bacteria feed on. The over-expression of phytohormones is tumorigenic, causing crown gall symptoms. The T4SS is not only used by bacteria to inject macromolecules in the host, but can secrete proteins directly in their environment as well: *Bordetella pertussis* excretes the pertussis toxin essential to its pathogenicity (Burns, 2003). The T4SS of *Pseudomonas aeruginosa* has also been described as involved in twitching motility (Mattick, 2002). Similarly to another secretion system (T3SS) that enables bacteria to interact with various plants and other eukaryotes (Preston, 2007), the T4SS was shown to interact with eukaryotes other than the compatible host (Bundock *et al.*, 1995). Hence, machineries labelled as "pathogenicity-related" enable the bacteria to interact with various organisms. However, even though the T4SS of *P. atrosepticum* may be involved in the control of *R. commune* growth *in vitro*, the actual active molecule is unknown.

In planta, the bacterium was shown to be able to colonise the barley phyllosphere actively (Figure 4.5), but induced resistance against *R. commune* on Optic leaves triggered by *P. atrosepticum* was not determined. Dewey *et al.* (1999) inoculated both bacteria and fungal spores at the same time in their detached leaf

assay. Here, leaves were sprayed with the bacteria and point-inoculated later with an *R. commune* spore suspension. The overall *P. atrosepticum* population on the leaf was high (Figure 4.5), but there may not have been enough clustered bacteria to trigger the interaction mechanisms and the leaf abrasion could have participated in the removal of bacteria from the inoculation site. As different barley cultivars were used in the field experiment, the Pipkin and Halcyon winter-types (Newton *et al.*, 2004); the *P. atrosepticum*-induced resistance to *R. commune* could also be affected by different barley genotypes. Conversely, a significant increase of the powdery mildew symptoms was recorded both here (Figure 4.6) and on wheat in the field (Newton *et al.*, 2004). Therefore, it seems that *P. atrosepticum* was at least partly responsible for the effect observed in the field.

Beside the potential involvement of the T4SS in the control of fungal diseases on barley leaves, other indirect mechanisms might be taking place *in planta* affecting positively and negatively disease establishment, but this would require further investigations. For example, the presence of *P. atrosepticum* in high number could affect the nutritional balance of microorganisms on the leaf surface, which was shown to affect *B. graminis* colony density on the barley leaf (Jensen and Munk, 1997). The plant itself could also be challenged by *P. atrosepticum* and would in return affect the colonisation of pathogens. The bacterium produces a toxin called coronafacic acid (Toth *et al.*, 2006). This compound is a structural analogue of jasmonic acid (JA), a plant defence phytohormone. It was hypothesised that the bacterial toxin inhibits salicylic acid (SA)-dependent defences, as this other defence-inducing phytohormone is antagonistic to JA (Glazebrook, 2005). This could explain the differential outcome on powdery mildew and scald symptoms caused by *P. atrosepticum* in the field, as JA-dependent defences are considered to target

necrotrophic pathogens, such as *R. commune*, whereas SA-induced defences are triggered following biotrophic pathogens infection, such as *B. graminis* (Glazebrook, 2005).

Even though *P. atrosepticum* gave interesting results to investigate interactions occurring with fungal diseases of the barley phyllosphere, this bacterium has no biocontrol interest. Furthermore, looking at one microorganism is too restrictive. Dewey *et al.* (1999) identified three different bacteria closely associated with *Mycospherella graminicola* spores and affecting positively the disease establishment on wheat leaves. The phyllosphere is a very rich habitat, colonised by multiple organisms, which are not culturable (Yang *et al.*, 2001). Using molecular techniques (T-RFLP), the bacterial richness of the barley phyllosphere was shown to be significantly higher than when using culturable methods (Table 3.6 and Figure 3.8). Multiple microorganisms could be required to produce a significant visible effect as well. A cell-free diffusate of a complex bacterial suspension isolated from *R. commune* leaf lesions induced the lysis of *R. commune* spores, whereas diffusates from a single isolated *Pseudomonas* species did not achieve such a result (Rotem *et al.*, 1976).

Out of the five epiphytes used in this study, only one (*P. syringae*) significantly reduced the growth of *R. commune* similar to that achieved by *P. atrosepticum in vitro* (Table 4.3). Some isolates of the bacterium *P. syringae* are also well-known phytopathogens (Hirano and Upper, 2000). *Xanthomonas maltophilia*, a weak dicotyledon pathogen, had the greatest effect in enhancing *M. graminicola* symptoms on wheat leaves (Dewey *et al.*, 1999). Overall, this suggests that pathogens possess multiple adaptive traits that would help them to survive on different incompatible hosts and interact with various organisms. Both *P.*

atrosepticum and *P. syringae* were motile (Table 4.2) and colonised the barley leaves (Figure 4.5) even though pathogenically it is a non-host. Both could also produce biofilms (Table 4.2). The formation of a *P. syringae* biofilm was not detected in the experimental system used, but other strains were shown to produce some thus providing a fitness advantage to their survival in the phyllosphere (Yu *et al.*, 1999). Finally, both bacteria have been recovered from many different habitats linked with the water cycle: ocean water, rainwater, aerosols and snow (Franc and Demott, 1998; Morris *et al.*, 2008), demonstrating great adaptable skills.

Overall, plant-associated bacteria demonstrated a considerable plasticity to adapt to very different environments and neighbouring microorganisms. Even though the two bacteria that demonstrated control of *R. commune* were phytopathogens, they still could be used to identify biocontrol mechanisms taking place in the phyllosphere. Furthermore, these results can be integrated in phytosanitory control strategies: for example, crop rotation from potato to barley crops should be reconsidered or treated appropriately, if high levels of *P. atrosepticum* were present in the potato crop.

CHAPTER 5: EFFECTS OF THE PLANT GENOTYPE AND AGRO-CHEMICAL TREATMENTS ON THE PHYLLOSPHERE MICRIOBIAL COMMUNITIES

5.1 INTRODUCTION

Plants grown in the field are under constant but varied biotic and abiotic stresses. The second most common cause of yield loss in barley originates from fungal diseases (Oerke and Dehne, 2004). To reduce the deleterious effects of diseases on yield, agro-chemical treatments are applied throughout the season. Fungicides are commonly used, but because of increasing EU restrictions (e.g. Regulation (EC) No. 1107/2009), alternative treatments and strategies are being sought, including the use of elicitors. Elicitors, generally, have no microbiocidal activity, but they stimulate a quick triggering of plant defences upon challenge by a pathogen (Walters *et al.*, 2008b).

Managing the plant-associated microbiome has emerged as a new potential integrated and sustainable strategy to fight diseases (Newton *et al.*, 2010b). Under controlled conditions, various factors have already been demonstrated to alter bacterial survival and colonisation of the phyllosphere. These include (i) environmental factors such as the relative humidity (O'Brien and Lindow, 1989), (ii) physiological factors, specific to particular plant species and the amount of sugar produced by the plant (O'Brien and Lindow, 1989; Mercier and Lindow, 2000), and (iii) microbial factors, including bacterial motility, ability to produce plant phytohormone and to aggregate (Haefele and Lindow, 1987; Brandl and Lindow, 1998; Monier and Lindow, 2005). However, in the field, greater variability in the microbial populations has been observed, which makes it more difficult to predict the

microbial behaviour. Furthermore, negative effects of fungicides were recorded on non-target bacterial communities (Walter *et al.*, 2007).

Here, the effects of different treatments on phyllobacterial communities were investigated on eight field-grown winter-type barley cultivars. The eight cultivars exhibited various degrees of tolerance towards *R. commune,* from resistant to susceptible and treatments included conventional fungicides as recommended by the SAC Technical Note TN611 (Oxley and Burnett, 2008) and an elicitor mixture that had previously shown effective protection in the field (Walters *et al.*, 2011). Interest primarily focused on endophytic communities as they can directly interact with the established fungal pathogens.

Over two years, leaf samples were collected from each treatment application, and interest focused on T2 samples. As the *R. commune* population changes over time (Abang *et al.*, 2006), interactions between the primary *R. commune* inoculum (before disease escape) and the leaf-associated bacteria is investigated here. Furthermore, at GS30, all plants have not initiated their sexual reproduction stage, but they have been exposed to foliar pathogens for long enough to allow treatments to have an effect on foliar diseases. The results on the composition of the endophytic bacterial population are presented here. Compared to epiphytes, microbes living in the leaf are less exposed to drastic environmental changes and are therefore theoretically more manageable in the optic of increased crop protection. Finally, the rapid sub-cuticular growth of *R. commune* could act as an escape strategy against epiphytic microbes with biocontrol activities (Newton *et al.*, 2004). Therefore, endophytes were preferred to epiphytes for this preliminary study.

Firstly, the main constituents of the endophytic bacterial communities at GS30+ were identified. The effects of various winter-type barley cultivars and

different agro-chemical treatments (i.e. conventional fungicides or an elicitor mixture) on the composition of the bacterial endophytes as well as on yield and disease severity were then assessed. Finally, it was sought whether or not a correlation between the structure of the bacterial community and the disease severity was observed.

5.2 MATERIALS AND METHODS

All procedures have previously been described in the Sections 2 and 3, and included plant growth conditions (Section 2.1.1), removal of microorganisms (Section 3.2.1), molecular profiling of bacterial communities and statistical analysis (Section 3.2.5).

5.3 RESULTS

5.3.1 The Endophytic Communities

A total of 57 OTUs were detected. The ten most common OTUs represented 76% of the total population (Figure 5.1). They were detected in over 80% of all populations, except the 63, 231 and 369 OTUs, which were present in 30 to 60% of the populations. The identity of only two OTUs (204 and 369) was known from previous results (Table 3.8) and represented *Pseudomonas* sp. and *Erwinia* and *Pectobacterium* sp. respectively.

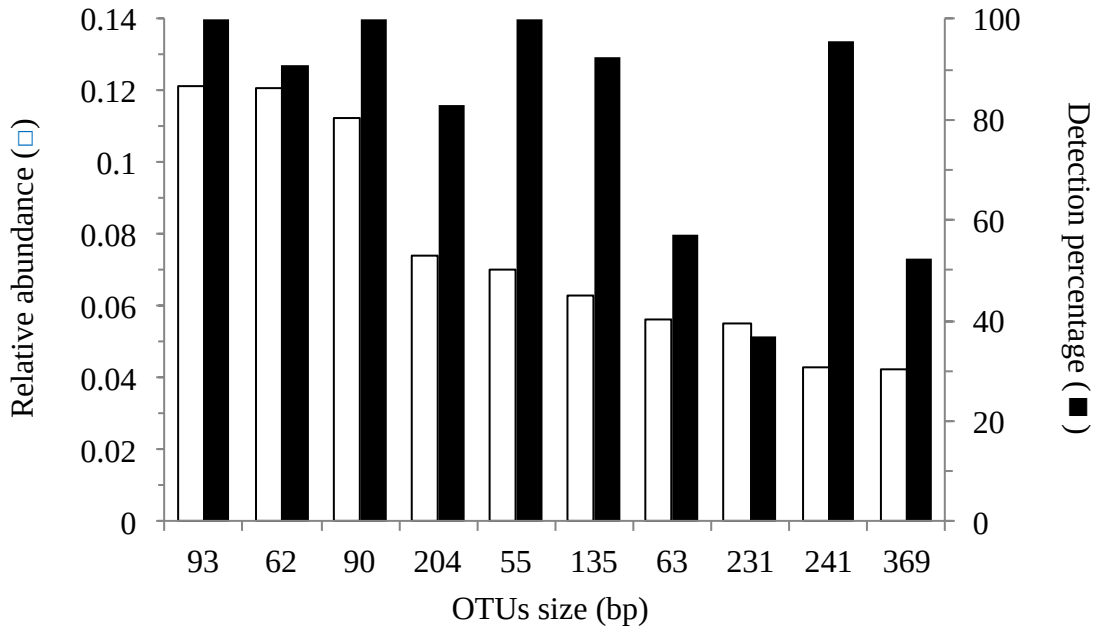

Figure 5.1: Relative abundance (white) and detection percentage (black) of the ten most common endophytic OTUs organised by decreasing relative abundance. They represent 76% of the total bacterial population.

The multivariate analysis of the endophytic bacterial populations from eight control and fungicide- or elicitor-treated barley cultivars grown in the field exhibited great variability (Figure 5.2). Samples did not cluster in clearly distinctive populations based on either the applied treatments or the cultivars. Overall, the average of all cultivars from control plots was separated from the average for the treated ones according to the first principal component (PC1 = 15.7%) (Figure 5.2 A). The first principal component participated little in a separation based on the cultivars, but the two extremes (Igri and Maris Otter) were well separated (Figure 5.2 B). The second principal component did not contribute towards explaining the sample diversity based on treatments or cultivars.

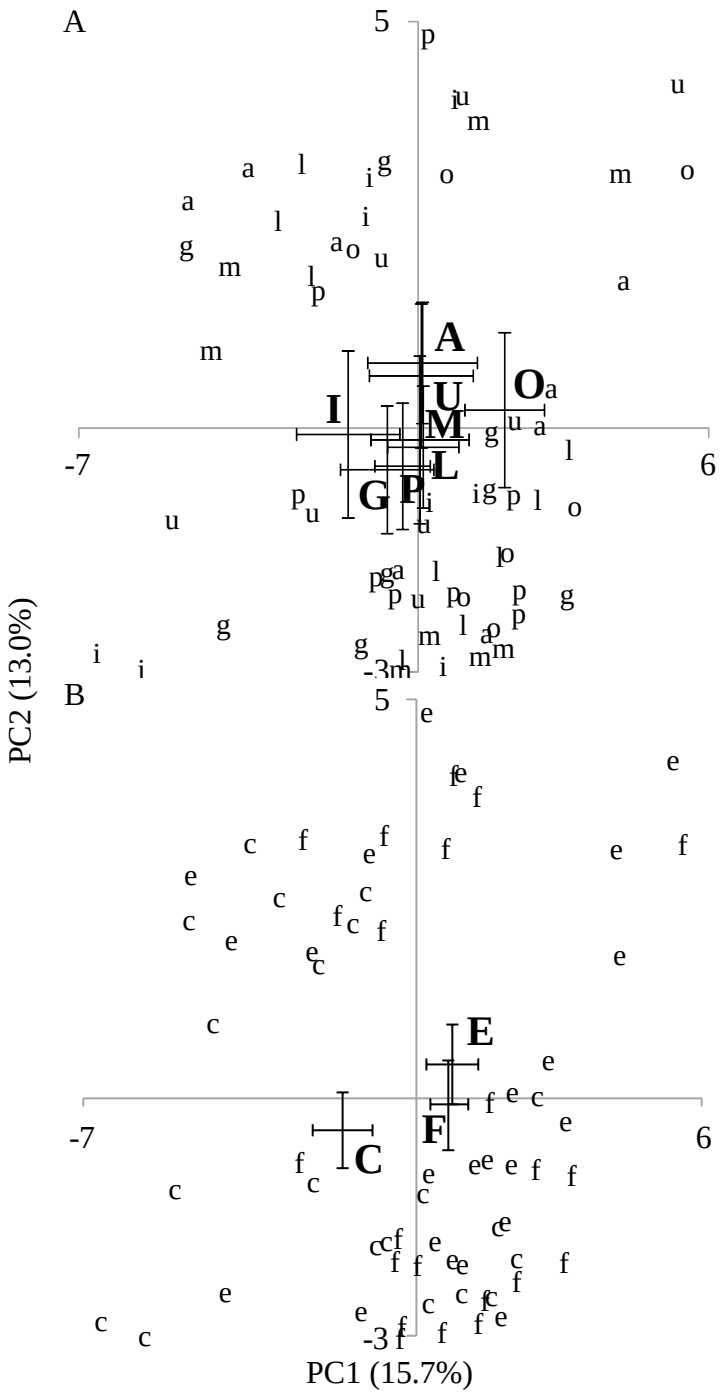

Figure 5.2: PCA (PC1 = 15.7% and PC2 = 13.0%) of endophytic communities at GS30+ from eight triplicate field-grown winter-type barley cultivars (Figure A): Flagon (g), Igri (i), Leonie (l), Manitou (M), Maris Otter (o), Pearl (p), Saffron (a) and Sumo (u), which were treated two weeks before sampling with conventional fungicides (f), an elicitor mixture (e) or nothing (c) (Figure B). Capital bold letters correspond to the average per treatments and the error bars represent the SEM.

The relative abundance of only six OTUs (135, 178, 204, 24, 271 and 294) out of the 57 detected was significantly affected by the cultivars they were associated with (Table 5.1). Three of them (135, 204 and 241) were among the most common endophytic OTUs (Figure 5.1). The relative abundance of four OTUs (135, 241, 271 and 294) was significantly different between the Igri and Maris Otter and represented 9 and 17% respectively of the total endophytic communities. They could explain the differences observed on Figure 5.2 B, as their loading vector values participated positively to the first principal component (Annex Table A.10). However, other OTUs were as much involved and did not significantly differ between cultivars. Two OTUs were affected by the treatment but accounted for less than 5% of the whole populations. The relative abundance of 11 OTUs was altered by the combined effect of treatment and cultivar and they represented nearly 65% of the bacterial endophytes. Most of these OTUs also participated positively in the first principal component (Annex Table A.10).

Table 5.1: Effects of the cultivar specificity on the relative abundance of certain OTUs. Three of them (†) were among the most common endophytic OTUs.

Cultivars	Relative abundance of selected OTUs						Sum
	135†	178	204†	241†	271	294	
Igri	0.047 [a]	0.001 [a]	0.066 [bd]	0.031 [a]	0.008 [ab]	0.006 [a]	0.158
Sumo	0.061 [ab]	0.001 [a]	0.038 [a]	0.046 [bc]	0.010 [ac]	0.013 [ab]	0.170
Flagon	0.042 [a]	0.003 [a]	0.082 [d]	0.037 [ab]	0.003 [a]	0.006 [a]	0.173
Manitou	0.056 [ab]	0.008 [ab]	0.058 [ad]	0.043 [bc]	0.010 [ac]	0.012 [ab]	0.188
Leonie	0.066 [ac]	0.006 [ab]	0.066 [bd]	0.042 [bc]	0.010 [ac]	0.019 [b]	0.210
Saffron	0.090 [c]	0.014 [b]	0.042 [ab]	0.045 [bc]	0.008 [ab]	0.015 [ab]	0.214
Pearl	0.078 [bc]	0.007 [ab]	0.070 [cd]	0.045 [bc]	0.013 [bc]	0.011 [ab]	0.223
Maris Otter	0.083 [bc]	0.005 [a]	0.049 [ac]	0.051 [c]	0.017 [c]	0.020 [b]	0.226
P	0.015	0.038	0.012	0.015	0.037	0.043	
lsd	0.029	0.008	0.024	0.010	0.007	0.010	

The bacterial diversity as shown using the Simpsons Index was significantly different between cultivars ($P < 0.001$) and also varied following treatment application ($P = 0.047$) (Table 5.2). Igri had the lowest diversity, whereas Leonie had the highest. Conversely to the elicitor application, treating plants with fungicides resulted in a significant increase of the bacterial diversity Statistical differences between phyllosphere communities of treated and control plants were observed using the evenness index (Table 5.3), but not the Shannon Index (Annex Table A.9).

Table 5.2: Simpson diversity Index of endophytic communities from field-grown barley cultivars treated with either conventional fungicides or an elicitor mixture or nothing. The diversity was significantly affected by the treatments (P = 0.047, lsd = 0.027)[†] and the cultivar (P < 0.001, lsd = 0.021)[‡]. The effect of the treatments on the bacterial diversity was also affected by the cultivars (P < 0.001, lsd = 0.040).

		Treatment			
		Control	Elicitor	Fungicide	Average[‡]
Cultivar	Igri	0.680	0.897	0.913	0.830[a]
	Flagon	0.859	0.863	0.897	0.873[b]
	Sumo	0.852	0.882	0.906	0.880[b]
	Saffron	0.896	0.878	0.894	0.889[bc]
	Maris Otter	0.901	0.886	0.885	0.891[bc]
	Pearl	0.898	0.906	0.877	0.893[bc]
	Manitou	0.906	0.869	0.907	0.894[bc]
	Leonie	0.899	0.901	0.902	0.901[c]
	Average[†]	0.861[a]	0.885[ab]	0.898[b]	

Table 5.3: Evenness of endophytic communities from field-grown barley cultivars treated with either conventional fungicides or an elicitor mixture or nothing. The diversity was significantly affected by the treatments (P = 0.008, lsd = 0.041)[†] and the cultivar (P = 0.004, lsd = 0.058)[‡]. The effect of the treatments on the bacterial diversity was also affected by the cultivars (P < 0.001, lsd = 0.098).

		Treatment			
		Control	Elicitor	Fungicide	Average[‡]
Cultivar	Igri	0.338	0.798	0.782	0.639[a]
	Sumo	0.466	0.803	0.729	0.666[ab]
	Saffron	0.724	0.669	0.714	0.702[bc]
	Flagon	0.770	0.635	0.757	0.721[bc]
	Manitou	0.765	0.696	0.753	0.738[c]
	Pearl	0.742	0.770	0.704	0.738[c]
	Leonie	0.715	0.719	0.791	0.742[c]
	Maris Otter	0.742	0.731	0.757	0.744[c]
	Average[†]	0.658[a]	0.728[b]	0.748[b]	

5.3.2 Plant Fitness

Over two years, leaf scald caused by *R. commune* was the most prevalent disease observed in the field (Table 5.4 and Annex Table A.13). Occasionally, powdery mildew symptoms were detected as well (data not shown). A significant difference in the percentage of leaf scald symptoms was identified between cultivars ($P < 0.001$). The most susceptible cultivar was Igri, whereas the most resistant was Leonie. Treatments also significantly altered the number of symptoms observed ($P = 0.03$). Fungicides considerably reduced the number of *R. commune* infections. The effect of elicitors depended on the cultivation year: in the 2009-2010 growing season, the symptom levels were similar to the untreated crops, whereas in 2010-2011 the protection was equivalent to the fungicide-induced protection at GS30+.

Table 5.4: Percentage of *R. commune* disease infection at GS30+ from eight cultivars from either untreated, elicitor- or fungicide-treated plots over two years (2009-2010 and 2010-2011). The disease percentage was significantly affected by the treatment ($P = 0.03$, lsd = 1.13)[†] and the cultivar ($P < 0.001$, lsd = 0.90)[‡]. Treatment effects significantly differed with year as well ($P = 0.01$, lsd = 1.15)□.

		R. commune disease percentage						
Treatments		Control		Elicitor		Fungicide		
	Year	09-10	10-11	09-10	10-11	09-10	10-11	Average[‡]
Cultivars	Igri	6.17	5.00	5.17	0.57	0.73	0.47	3.02^{a}
	Saffron	2.20	6.00	2.00	0.87	0.47	1.37	2.15^{ab}
	Sumo	4.23	1.40	3.40	1.10	0.03	2.07	2.04^{b}
	Maris Otter	2.33	2.33	2.70	0.33	0.13	0.10	1.32^{bc}
	Flagon	2.00	0.60	1.37	0.40	0.33	0.47	0.86^{cd}
	Manitou	1.53	0.33	1.10	0.40	0.20	1.10	0.78^{cd}
	Pearl	0.40	0.47	0.47	0.27	0.13	0.03	0.29^{d}
	Leonie	0.00	0.07	0.07	0.00	0.00	0.00	0.02^{d}
	Average□	2.36^{a}	2.02^{a}	2.03^{a}	0.49^{b}	0.25^{b}	0.70^{b}	
	Average[†]	2.19^{a}		1.26^{ab}		0.48^{b}		

Significant differences ($P < 0.001$) were also observed between years regarding yield, with 2009-2010 being the most productive year (Table 5.5 and Annex Table A.12). Cultivars as well as treatments significantly affected the harvest yield ($P = 0.005$ and $P < 0.001$ respectively). Igri exhibited the highest susceptibility to *R. commune* in the field and had the lowest yield. Leonie had the highest resistance to *R. commune*, but its yield was similar to Igri. Manitou had the highest yield. The application of fungicide resulted in an increase of yield (+11%), whereas elicitors did not improve the yield compared to the control crops.

Table 5.5: Yield (t/ha) from eight winter-type barley cultivars from either untreated or fungicide-treated or elicitor-treated plots over two years (2009-2010 and 2010-2011). The yield was significantly affected by the experimental year ($P < 0.001$ – not represented), the treatment ($P < 0.001$, lsd = 0.14)[†] and the cultivar ($P = 0.005$, lsd = 0.23)[‡].

		Yield (t/ha)						
Treatments		Control		Elicitor		Fungicide		
Year		09-10	10-11	09-10	10-11	09-10	10-11	Average[‡]
Cultivar	Igri	4.55	3.39	4.50	3.72	4.77	3.95	4.15[a]
	Leonie	4.62	3.31	4.72	3.53	4.99	3.84	4.17[a]
	Maris Otter	4.35	3.55	4.67	3.70	4.87	3.94	4.18[a]
	Sumo	4.39	3.65	4.64	3.69	5.03	4.21	4.27[ab]
	Saffron	4.75	3.54	4.86	3.42	5.38	4.11	4.34[ac]
	Flagon	5.10	3.42	4.68	3.75	5.35	4.17	4.41[bc]
	Pearl	4.95	4.11	4.46	3.74	5.13	4.32	4.45[bc]
	Manitou	5.34	3.43	5.26	3.63	5.50	4.04	4.54[c]
	Average	4.76	3.55	4.73	3.65	5.13	4.07	
	Average[†]	4.15[a]		4.19[a]		4.60[b]		

5.3.3 Relationship between Diversity and Susceptibility

In 2009-2010, a significant negative correlation (P = 0.03) was observed between the bacterial diversity and the percentage of leaf infection by *R. commune* observed in the field (Figure 5.3). The less diverse the bacterial communities were, the more leaf scald symptoms were observed. The Shannon Index was preferred to the Simpson's, because the latter varies only between 1 and 0 and the analysis resulted in a skewed distribution. However, more data are required to validate the correlation as more diversity values are needed from leaves with high infection.

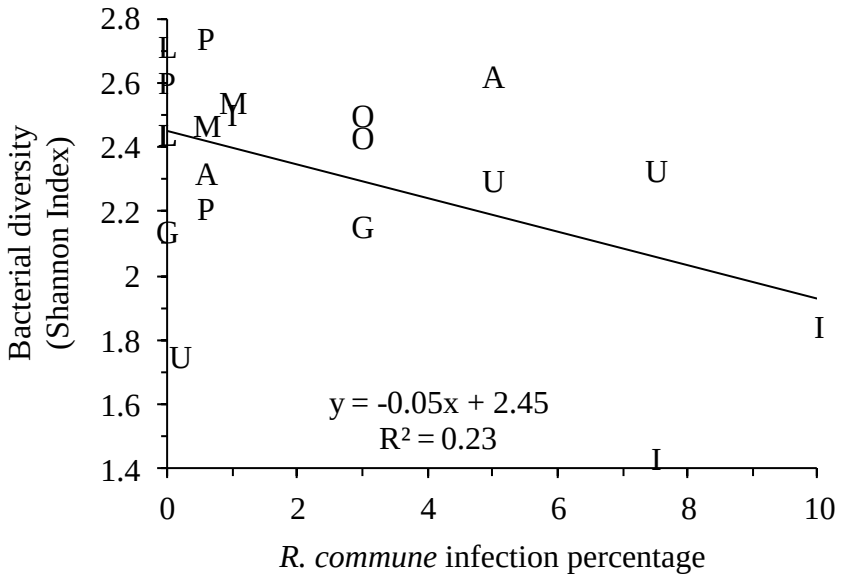

Figure 5.3: Relationship between the bacterial diversity and the visible amount of *R. commune* symptoms from eight non-treated cultivars: Flagon (G), Igri (I), Manitou (M) Maris Otter (O), Leonie (L), Pearl (P), Saffron (A) and Sumo (U). The regression was significant (P = 0.03).

5.4 DISCUSSION

Many plant, microbial and environmental factors have been identified as influencing the microbial phyllosphere under controlled conditions. However, in nature, microorganisms are exposed to all these factors and understanding the

fluctuations of the communities requires field experimentations. Lambais *et al.* (2006) demonstrated that various plant species from the Atlantic forest of Brazil harboured very different bacterial communities. Differences were also found in the field between various rice and maize varieties (de Costa *et al.*, 2006a; Balint-Kurti *et al.*, 2010). In barley leaves, the main endophytes were detected in all cultivars (Figure 5.1), but the relative abundance of some of them was significantly affected by the cultivar they were isolated from (Table 5.1). The overall bacterial diversity and evenness was also affected by the cultivars (Table 5.2 and 5.3).

The barley cultivars were originally selected because of their wide range of resistance against foliar fungal pathogens and a negative correlation between the bacterial diversity and the amount of visible leaf scald symptoms was identified. Using culturable techniques, de Costa *et al.* (2006a) identified significant differences in bacterial colonisation between various field-grown rice cultivars, which were found to be related to anatomical and physiological characteristics. The cultivars also had various degrees of resistance to rice blast (*Magnaporthe oryzae*) and bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*), but no correlation between the bacterial load and genotype resistance was identified. However, culturable microorganisms are only a minor part of the whole phyllosphere (Wilson and Lindow, 1992).

Using molecular techniques, Balint-Kurti *et al.* (2010) estimated the bacterial populations on a recombinant inbred maize population. Six chromosomal regions were correlated with the bacterial diversity. One of them exhibited a strong overlap with a locus controlling resistance to the Southern Leaf Blight. The most susceptible line had the highest diversity, whereas the most resistant had the lowest. An opposite trend was observed here on field-grown barley leaves (Figure 5.3). However, more experimental validations are required and whether it is a cause or consequence of the

presence of the disease still has to be determined. Some causal agents have been shown to induce contrasting changes in phyllosphere populations (Suda *et al.*, 2009; Zhou *et al.*, 2010) and results of Balint-Kurti *et al.* (2010) suggest that plants actively manipulate their phyllosphere ecology to improve their resistance against diseases.

Agro-chemical treatments affected the bacterial phyllosphere (Table 5.2) but fungicides alone led to a significant increase in bacterial diversity. Fungicide use disturbs the fungal ecology (Fokkema and Nooij, 1981; Bertelsen *et al.*, 2001; Walter *et al.*, 2007), but it also affects non-target microorganisms, such as bacteria. Application of the cypermethrin insecticide induced an increase in the bacterial population and a shift to Gram-negative bacteria on cucumber and pepper (Zhang *et al.*, 2008; Zhang *et al.*, 2009b). In a study on apple leaves, Walter *et al.*, (2007) showed that even though variation was observed depending on the fungicide type and application rate, repeated fungicide spraying led to an overall reduction of the microbial phyllosphere (bacteria, yeast and fungi).

Similarly, Fountaine *et al.* (2009) observed a significant reduction of culturable bacteria on field-grown barley leaves after fungicide treatment. Here, application of fungicides resulted in an increase in the bacterial diversity and evenness (Table 5.2 and 5.3), which is similar to the findings on field-grown wheat (Gu *et al.*, 2010). A higher diversity means that the bacterial population is more homogeneous and less dominated by a few species. Fountaine *et al.* (2009) cultivated bacteria on the KB medium, which is a good medium on which grow *Pseudomonas*. These bacteria were one of the most abundant and most detected bacteria in our study (203 OTU, Figure 5.1). Hence, their reduction due to fungicide applications would be predicted to lead to a rise in the bacterial diversity.

The application of an elicitor mixture also resulted in an increased bacterial evenness similar to that observed in fungicide treated plants (Table 5.3). The elicitor mixture was composed of Bion (a SA analogue), cis-jasmonate and beta-aminobutyric acid (BABA, a gamma-aminobutyric acid (GABA) related chemical). Individually, each eliciting compound was shown to affect differentially the phyllosphere ecology. A significant reduction in the endophytic populations was observed on *A. thaliana* after Bion application, whereas the epiphytic diversity increased after cisJA treatment (Kniskern *et al.*, 2007; Traw *et al.*, 2007). A positive correlation between the activity level of the glutamate decarboxylase enzyme, which is involved in GABA synthesis, and the epiphytic bacterial diversity was observed on field-grown maize leaves (Balint-Kurti *et al.*, 2010).

The elicitor mixture tested was previously shown to increase resistance to *R. commune* and *B. graminis* on field-grown spring barley, but this also resulted in a reduced resistance to *Ramularia collo-cygni* (Walters *et al.*, 2011). Molecular analyses revealed that the elicitor mixture stimulated the SA-dependent defences (i.e. the PR1 gene expression) and down-regulated genes involved in JA-dependent transduction signal (LOX2 gene). Hence, Bion seems to be mostly active in the mixture, but the opposite effect is observed on bacterial diversity (Table 5.2 and 5.3).

Even though elicitors modify the phyllosphere in a similar manner to fungicides, they provided only partial resistance to *R. commune* compared to the fungicide-treated plants (Table 5.4). Furthermore, yield was not increased on elicitor treated crops (Walters *et al.*, 2011), whereas it was on fungicide-treated ones (Table 5.5). The improved fitness resulting from the fungicide application was correlated with a rise in the green leaf area duration (Bertelsen *et al.*, 2001). Saprophytic fungi are suspected to increase leaf senescence and broad spectrum fungicides would limit

their negative action. Elicitors generally have no direct effect on microorganisms and only stimulate the plant defences. Saprophytes should therefore not be affected by elicitor application. Many types of yeast isolated from field-grown barley leaves have led to a significantly increased susceptibility to *R. commune in planta* under controlled conditions (Fountaine *et al.*, 2009). Hence, even though elicitors provide some protection against diseases, they may not be able to promote plant tolerance to other indirectly detrimental microorganisms.

Elicitors increased the bacterial diversity and evenness to a similar level to that on barley cultivars resistant to *R. commune* and the fungicide-treated ones. Whether or not a diverse endophytic bacterial community participates directly or indirectly to the resistance to *R. commune* in the field still has to be determined. The absence of yield gain from the application of elicitors and the partial protection to foliar diseases observed in this study does not support the argument for a ban of fungicides, but elicitors still represent a potential sustainable tool for crop protection.

CHAPTER 6: EFFECTS OF THE LEAF SURFACE ON THE PHYLLOSPHERE MICROBIAL COMMUNTIES

6.1 INTRODUCTION

The leaf surface is actively colonised by a vast diversity of microorganisms (Andrews and Harris, 2000). Previous results have shown that colonisation of the phyllosphere is affected by crop protection treatments and differed between cultivars. Many physiological factors controlled by the plant genotype can affect the microbial colonisation in the phyllosphere. One of them, the epicuticular wax layer, is the first physico-chemical barrier that microorganisms encounter on the leaf surface.

Epicuticular waxes comprise the outer-most layer of the cuticle and represent a key interface between the plant and the environment (Juniper and Jeffree, 1983). The leaf surface is only interrupted by a few specific structures, most commonly stomata and trichomes. Stomata are gas-exchange structures and trichomes, hair-like structure, have multiple purposes, from secretion to protection. Others structures such as hydrathodes, involved in the water exchange with the environment, or papillae of unknown functions, are less common. The waxes form a hydrophobic layer on the leaf surface and are composed of many diverse compounds, including long-chain hydrocarbons, alkyl esters, primary alcohols and fatty acids (Shepherd and Griffiths, 2006). The biosynthesis of these compounds has been extensively reviewed, but their delivery mechanisms still remain unclear (Kunst and Samuels, 2003). The composition of the epicuticular waxes can differ based on, for example, the plant genotype, the time of year, or the tissue it covers (Tulloch, 1973; Tsuba *et al.*, 2002). The wax chemistry affects its structure as well (Shepherd and Griffiths, 2006) and results in crystals of various shapes (Barthlott *et al.*, 1998). For example,

von Wettstein-Knowles (1974) identified that β-diketones and hydroxyl-β-diketones were associated with the formation of tube-shaped waxes on barley leaf surfaces.

The development of epicuticular waxes was essential to the colonisation of land by plants and its primary role is thought to be to reduce the uncontrolled loss of water (Edwards *et al.*, 1996). They also inhibit losses of other solutes and gases. However, the waxes are not totally impermeable and some compounds can travel through the cuticle. This is of great importance for agro-chemical treatments, such as fertilisers and pesticides, which can be manufactured to disperse and be active even within the plant (Nawrath, 2006). The cuticular wax also has photo-protective properties, increasing the reflectance, hence reducing photo-damage and temperature increase (Shepherd and Griffiths, 2006).

As previously stated, the cuticle is the first innate barrier against microorganisms including pathogens. Bacteria penetrate the plant via natural openings (stomata, hydrathodes) and wounds. Fungi possess various mechanisms to penetrate the cuticle, via the production of cell wall-degrading enzymes (e.g. cutinase) and the formation of special structures (appressoria) to directly pierce through the cuticle and the epidermal cells by increasing turgor pressure, via accumulation of compounds such as glycerol (Tucker and Talbot, 2001).

With so many diverse important physiological roles, modifying the epicuticular wax composition could result in severely deleterious phenotypes. However, the following chapter investigate how the epicuticular wax layer affects the plant fitness and the leaf-associated bacterial populations of field-grown barley. The wax layer of existing glossy mutants was characterised by determining protuberances density and wax structure and density using environmental scanning electronic microscopy (ESEM), as well as the weight and composition of the wax

layer. Field experiments were carried out to estimate the effect of glossy mutations on yield and resistance to foliar disease. Finally, T-RFLP measurements enabled to identify epiphytic constituents and how they are affected by wax mutations

6.2 MATERIALS AND METHODS

6.2.1 Plant Genotypes

Six glossy mutants derived from two spring-type barley WT (Bowman and Optic) were selected. The mutants were identified by their specific leaf phenotype, with a shiny glossy and lighter green appearance (Figure 6.1). The two Bowman glossy mutants (2015 and 2019) originated from other barley cultivars, which mutation was backcrossed in the Bowman line. The mutations were both located on chromosome 4HL at the glf1.a and glf3.d alleles respectively (Table 6.1) (Druka *et al.*, 2011). Conversely, the four Optic mutants (06-33, 37-54, 48-82 and 149-16) were generated following chemical mutagenesis of seeds using ethyl-methane sulphonate. They were selected based on their phenotype (Caldwell *et al.*, 2004), but no genetic characterisation has been undertaken on these mutants.

Table 6.1: Genetic characterisation of the Bowman glossy mutants 2015 and 2019 adapted from Druka *et al.* (2011).

Mutants	Line ID	Backcross (BC)	Mutant group	Locus or allele name	Chromosome	Donor
2015	BW385	Bowman (BC=<7)	Eceriferum	Glossy leaf 1 (glf1.a)	4HL	Himalaya
2019	BW386	Bowman (BC=<7)	Eceriferum	Glossy leaf 3 (glf3.d)	4HL	Gateway

Figure 6.1: Leaf appearance of barley Bowman and Optic WTs and two representatively different wax mutants: 2019 and 06-33. The mutants appear glossy with a lighter green colour.

For the microscopic observations of the leaf surface and the chemical analysis of the wax composition, the eight varieties were grown under glasshouse conditions as described in Section 2.1.2. A selection of six was grown in field: the 2015 and 2019 Bowman mutants, the 37-54 and 48-82 Optic glossy mutants and their respective WTs. Disease symptoms of scald and powdery mildew were recorded towards the end of the growing season (GS59+) before the inflorescence emergence (Section 2.1.1.3). Top leaves were also sampled for T-RFLP analysis of the epiphytic bacterial populations (Section 3.2.5).

6.2.2 Environmental Scanning Electron Microscopy

The leaf surfaces of four week-old barley seedlings grown under glasshouse conditions were imaged using environmental scanning electron microscopy (ESEM) with the Philips XL30 ESEM FEG microscope. The leaf segments were prepared according to conventional procedures and sputter-coated with 12 nm thick gold-

palladium in a Cressington 208 sputter coater (Cressington Scientific Instruments Ltd., UK).

The adaxial side of the middle section of the second leaf was compared between the six glossy mutants and their WT cultivars. Two different leaves were imaged twice at three magnifications: x 50, x 2,000 and x 10,000. The lowest magnification allowed the number of trichomes and stomata per leaf area unit within the image field (2.64 mm^2) to be determined. The intermediate and high magnification images were used to estimate the distribution and the structure of epicuticular waxes respectively. The waxes structure was identified using the classification of Barthlott *et al.* (1998).

6.2.3 Wax Analysis

The area of five four week-old leaves grown under glasshouse conditions was determined using the ImageJ software as described in Section 2.2.2. Each measurement was done in triplicate. The leaves were held with forceps and immersed in 600 mL of dichloromethane (Fisher Scientific, UK) for one minute to solubilise the epicuticular waxes from the leaf surface. The solution was filtered through a paper filter (Whatman Grade 1) and then concentrated by evaporating the solvent under gentle heat and a flow of nitrogen gas. The solution was filtered again through a paper filter and transferred to a clean vial. After evaporating the remaining solvent using nitrogen gas, the crystallised waxes were weighed and stored at -20°C until further analysis.

The profiling of the epicuticular wax composition and the identification of its chemical constituents was carried out using gas chromatography and mass spectrometry (GC-MS). Briefly, a fixed amount of waxes (0.5 mg) was mixed with 20 µg of methyl nonadecanoate, used as internal standard. The mixture was dried in a

vacuum centrifuge and later derivatised for 1 h at 50°C in 100 µL of a 1:1 mixture of BSTFA (Fisher Scientific, UK) and anhydrous pyridine (Sigma-Aldrich, DE). The automated analysis of the samples was carried out by a Trace GC Ultra GC-MS apparatus (Fisher Scientific, UK). Data were finally analysed with the Xcalibur software (Fisher Scientific, UK).

6.3 RESULTS

6.3.1 Microscopic Observations

The ESEM observations of the leaf surface of barley WT and their related mutants revealed major changes due to the mutation to a glossy phenotype. Significant differences were observed in the number of certain leaf protuberances as well as the epicuticular wax densities and structures.

6.3.1.1 The Leaf Protuberances

The leaf surface was not a smooth and flat surface, but was undulating (grooves and ridges) matching the shape of the underlying epithelial cells (Figure 6.2). Various plant structures participated to this uneven topography. Both stomata (GC) and trichomes were organised in a parallel succession emerging between the underlying epithelial cells (Figure 6.2 A), whereas small cuticular papillae (CP) were visible on top of the epithelial cells in a non-distinctive pattern (Figure 6.2 B). Three kinds of trichomes were observed: long hair (LH), medium hooked trichomes (HT) and short trichomes (ST) (Figure 6.2 A). The presence of cuticular papillae could not be quantified, as they could not be seen when epicuticular waxes covered the leaf surface (Figure 6.2 A). Occasionally, microorganisms could be seen attached to the leaf surface with a biofilm (BB) (Figure 6.2 C) or growing across the leaf surface (Figure 6.2 D).

No difference in the number of stomata was found between the cultivars (Table 6.2). However, significant differences in the total number of trichomes ($P < 0.001$) were observed between cultivars (Table 6.2): Optic had fewer trichomes than Bowman. Overall, some glossy mutants (the 37-54 and 48-82 Optic mutants and the 2015 and 2019 Bowman mutants) had fewer trichomes compared to their WTs, but this trend was not significant.

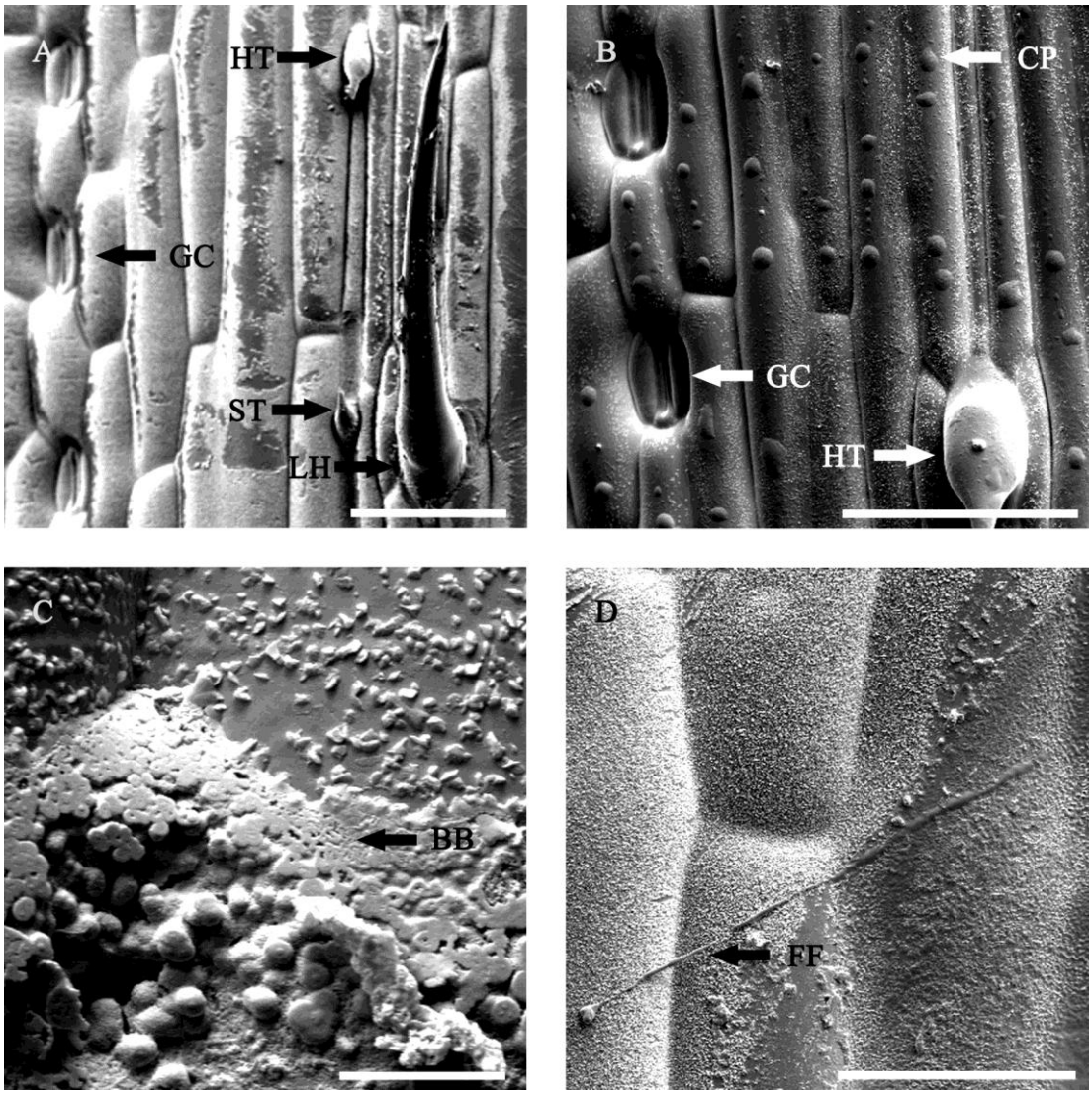

Figure 6.2: ESEM images of structures identified on the leaf surface. The plant protuberances included stomata surrounded by two guard cells (GC), long hairs (LH), hooked trichomes (HT), short trichomes (ST) and small cuticular papillae (CP) (Figures A and B, scale = 100 μm). Bacteria and/or yeast could be found aggregated in biofilm (BB, Figure C, scale = 5 μm), whereas filamentous fungi (FF) could be seen growing across the leaf surface (Figure C, scale = 50 μm).

Table 6.2: Number of stomata and trichomes per leaf unit area (mm^2) of two barley cultivars and six glossy mutants. Only the number of trichomes was significantly different between the varieties tested ($P < 0.001$, lsd = 2.34).

	Number of structures per mm^2	
Cultivars	Stomata	Trichomes
Bowman	29.41	9.17^{a}
2015	30.54	7.09^{ac}
2019	35.37	7.94^{ab}
Optic	34.14	5.58^{ce}
06-33	29.98	6.05^{bd}
37-54	26.00	3.78^{de}
48-82	32.34	3.69^{e}
149-16	30.54	7.85^{ac}

6.3.1.2 The Epicuticular Waxes

The rest of the surface was covered with flaky crystalloids, which corresponded to the epicuticular waxes (Figure 6.3). The two WT cultivars both had an extensive layer of epicuticular waxes (Figure 6.3 A and D). A similar density was observed on the other glossy mutants except for two: 2019 Bowman and 06-33 Optic (Figure 6.3 C and E). Because of this reduced presence of waxes, small cuticular papillae were made more easily observable (Figure 6.2 B).

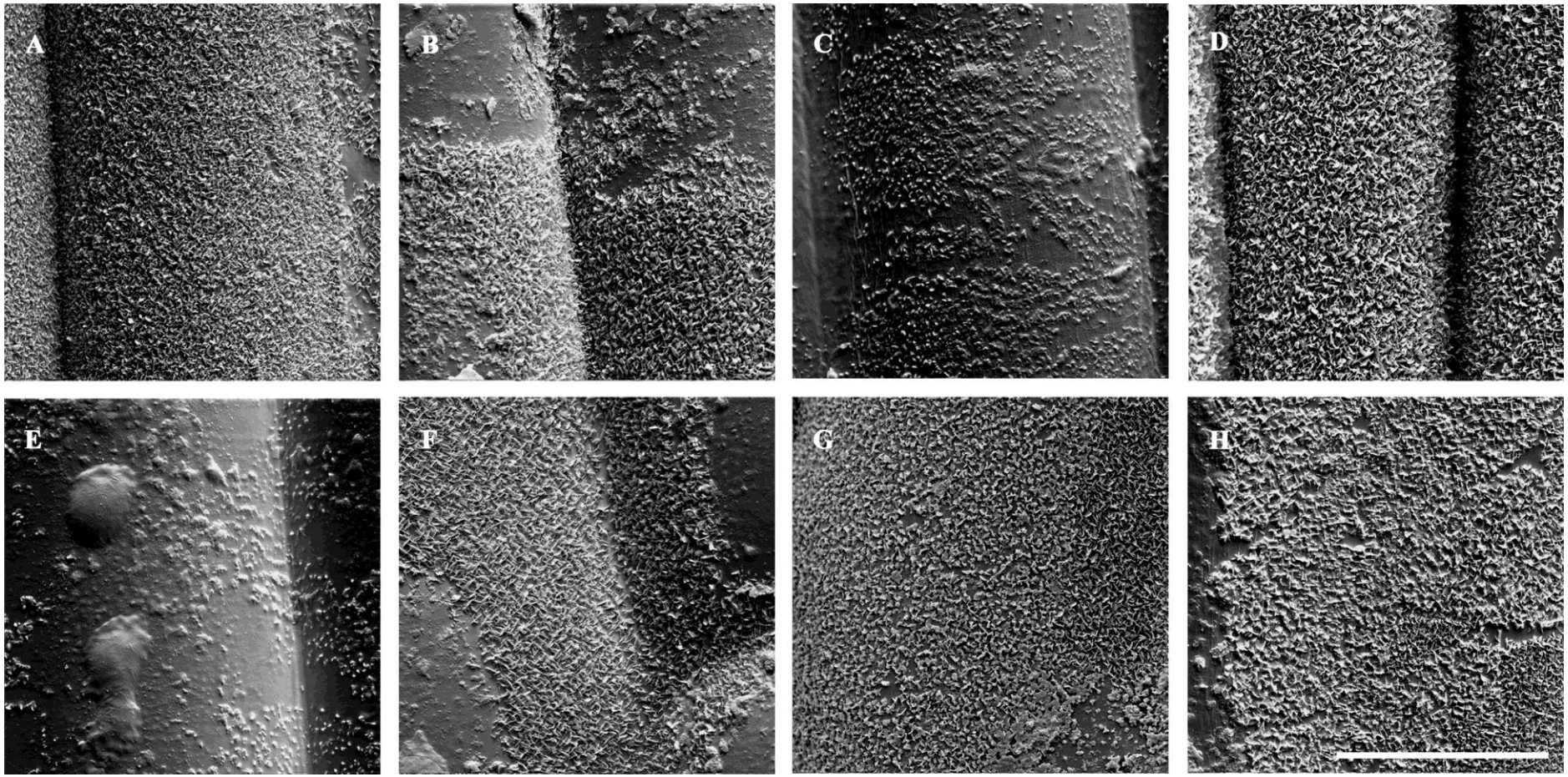

Figure 6.3: Representative ESEM images at a 2,000 magnification (scale = 10 μm) of the leaf surface of two barley cultivars and their associated glossy mutants: Bowman (A), 2015 (B), 2019 (C), Optic (D), 06-33 (E), 37-54 (F), 48-82 (G) and 149-16 (H).

Wax crystals had a flat shape with sharp irregular edges, called platelets (Figure 6.4). They were all interlocked without any particular orientation. No obvious differences in the wax shape, density or orientation could be identified between the two barley WTs, Optic and Bowman. However, ESEM images revealed a strong modification of the wax structure of some mutants compared to their respective WT. The two glossy mutants 2019 Bowman and 06-33 Optic, which previously showed a reduced wax density (Figure 6.3), also had a severely altered wax structure. The irregular platelets were replaced by small lumps of irregular shape unevenly distributed across the leaf surface. At the intermediate magnification, the surface of the 48-82 Optic mutant showed a similar coverage of its surface with waxes compared to the WT (Figure 6.3 D and G), but at a higher magnification the wax crystals did not look as fully emerged (Figure 6.4 G). Other glossy mutants looked similar to their respective WT.

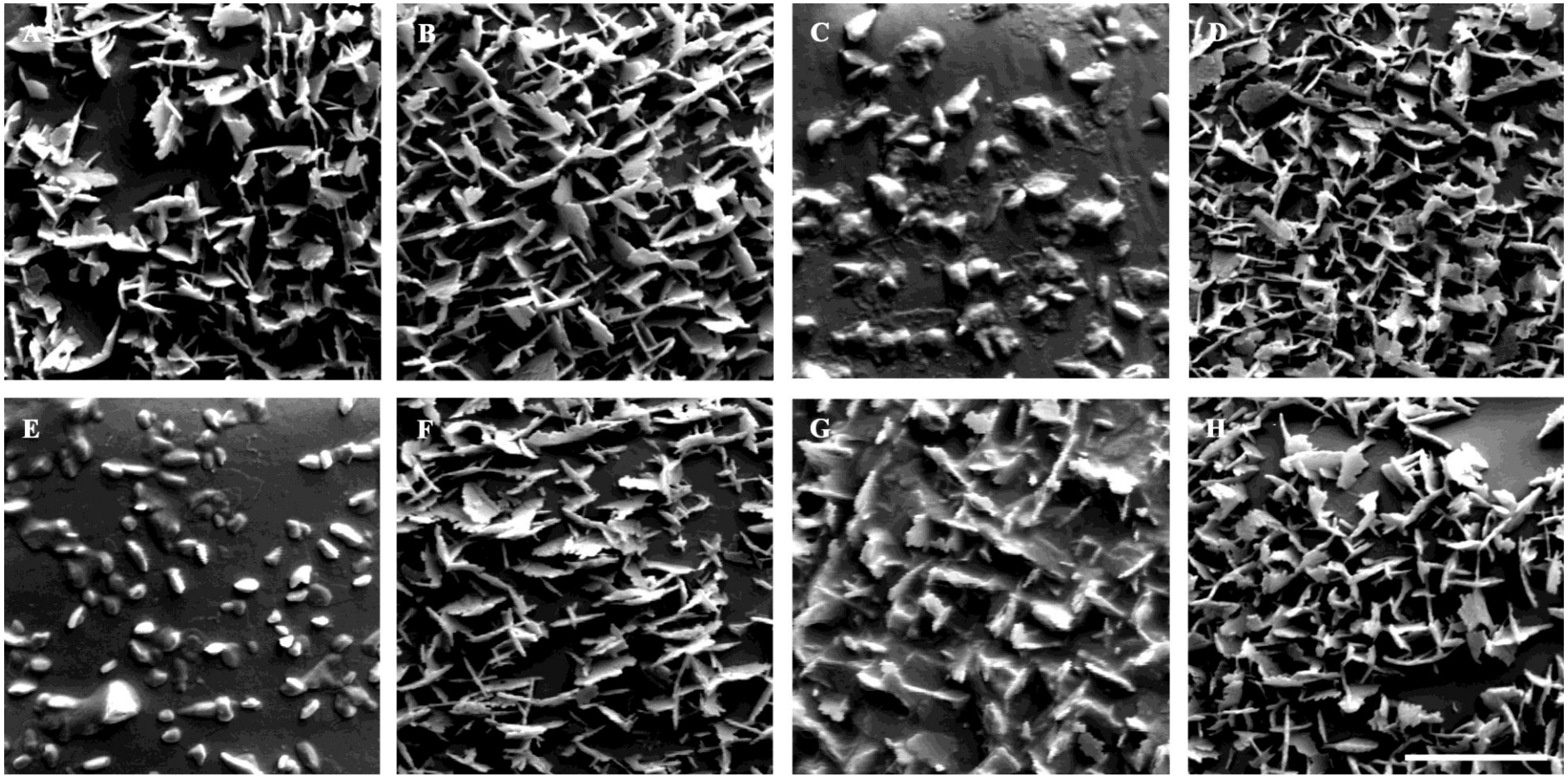

Figure 6.4: Representative ESEM images at a 10,000 magnification (scale = 2 μm) of the leaf surface of two barley cultivars and their associated glossy mutants: Bowman (A), 2015 (B), 2019 (C), Optic (D), 06-33 (E), 37-54 (F), 48-82 (G) and 149-16 (H).

6.3.2 Wax Chemistry

Epicuticular waxes were extracted from the whole leaf, from both abaxial and adaxial surfaces. No significant differences in the weight of wax per leaf area unit could be identified between the two WTs. Similarly, most of the glossy mutants showed the same level of wax. However, the three glossy mutants, which were shown previously to differ in wax density and structure, had a significantly different amount of wax ($P < 0.001$) compared to their respective WTs (Figure 6.5). The reduced wax density observed on ESEM pictures for the 2019 Bowman and 06-33 Optic glossy mutants (Figure 6.3 C and E) was correlated with a significant reduction in the amount of wax removed from their leaf surfaces compared to their respective WTs. The 48-82 Optic mutant, which showed merged wax crystals (Figure 6.4 G), had a significantly higher amount of wax than the Optic WT.

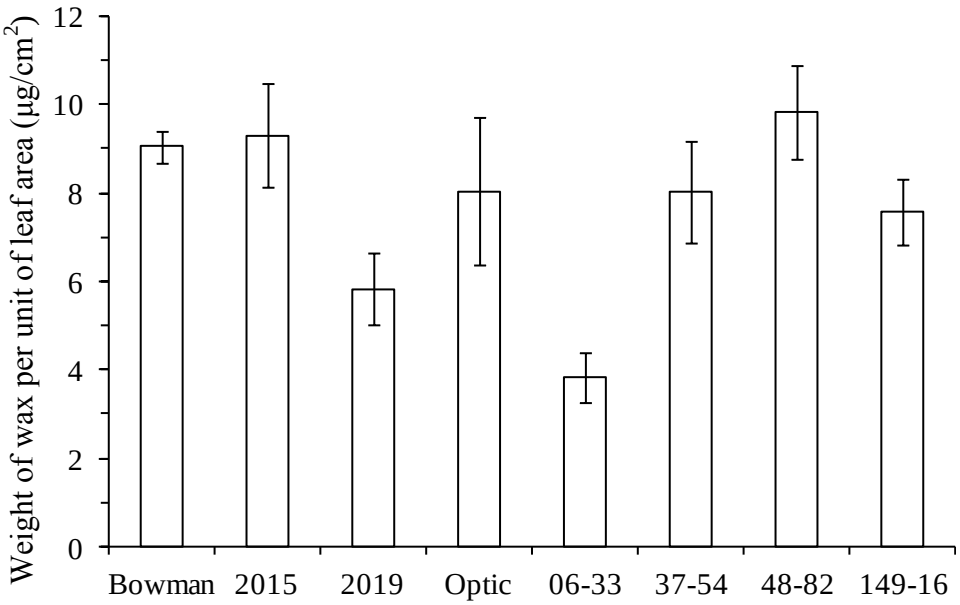

Figure 6.5: Weight of epicuticular wax per unit of leaf area (µg/cm^2) of various barley WTs and wax mutants ($P < 0.001$ lsd = 1.75). Error bars represent the SD.

The epicuticular waxes were composed of five major constituents: primary alcohols, long chain acids, esters, alkanes and another unidentified chemical with a benzyl group. The GC-MS analysis has not been entirely completed, but clear differences could be seen on the chromatograms (Figure 6.6). Only one representative chromatogram of each glossy mutant with significantly fewer waxes (2019 and 06-33) and their respective associated WTs are shown here.

Overall, all WT profiles were dominated by a single eluate (11). Its relative abundance was much higher than the other constituents and made the comparison difficult. Hence, profiles focused on eluates of lower abundance. Considerable differences could be identified between the two WTs with Bowman being dominated by a couple of eluates (eluates 6 and 11), whereas the main constituents of Optic were more homogeneously represented (eluates 6, 10, 11, 12 and 14). These dominating constituents were also part of the main constituent of the glossy mutant epicuticular waxes. However, the relative abundance of others constituents (eluates 5, 7, 8, 9, 13 and 14) was greater in glossy mutants. Finally, new constituents could be detected in the glossy mutants (eluates from 15 to 18) and they were identified as esters (Figure 6.6).

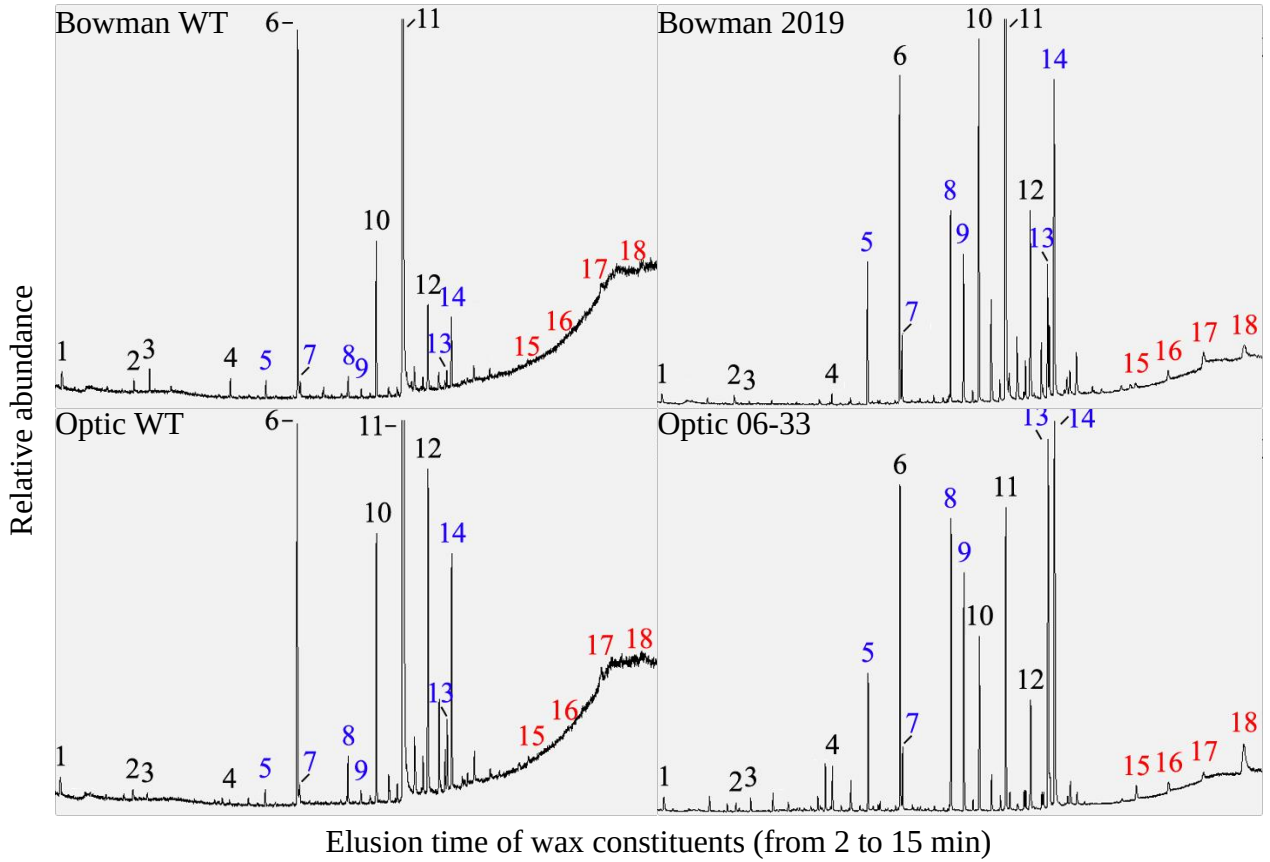

Figure 6.6: GC-MS chromatograms of two barley WTs (Bowman and Optic) and two related glossy mutants (2019 and 06-33). The chromatograms focused between the 2 and 15 min elution time frame and the peak height represent relative abundance. A selection of 18 eluates was labelled using three colours: black (similarly present in all samples), blue (reduced in glossy mutants) and red (present only in mutants).

6.3.3 Effects on Diseases, Yield and the Bacterial Phyllosphere in the Field

In the field, the glossy mutants (2015, 2019, 37-54 and 48-82) exhibited alterations in both yield and resistance to leaf-associated fungal diseases compared to their respective WTs (Table 6.3). Even though the fluctuations were similar from one year to another, variation occurred between the yield and disease infection measurements. Yield could not be assessed for the Bowman mutants in the first year as they exhibited a very low germination rate and thus a low plant density in the plots. Overall, the glossy mutants had a significant lower yield, with up to 27% reduction for 2015 Bowman in 2010. The 2019 Bowman mutant had a higher yield than its WT both years, but without statistical significance. The glossy mutants showed a significant increased susceptibility to leaf scald disease, caused by *R. commune*. It also showed a contrasting significantly increased resistance to powdery mildew caused by *B. graminis* f.sp. *hordei* observed in the field.

Table 6.3: Yield (t/ha) and diseases infection percentage at GS59+ of leaf scald and powdery mildew caused by *R. commune* (*Rc*) and *B. graminis* f.sp. *hordei* (*Bgh*) respectively, over three years. Plots were grown in the field in triplicate for three years (nd: not determined).

		Cultivars						
		Bowman	2015	2019	Optic	37-54	48-82	P
2009	Yield (t/ha)	3.2^{a}	nd	nd	5.5^{a}	4.5^{b}	4.0^{b}	<0.01
	Rc disease %	0.10	nd	nd	1.67	2.33	3.00	
	Bgh disease %	0.10^{b}	nd	nd	3.00^{a}	0.55^{b}	0.60^{b}	0.05
2010	Yield (t/ha)	4.8^{ab}	3.6^{c}	5.4^{a}	7.1^{ab}	6.3^{bc}	6.1^{bc}	0.01
	Rc disease %	0	0	0.73^{c}	5.33^{bc}	20.0^{ab}	28.3^{a}	0.01
	Bgh disease %	15.0^{ab}	25.0^{a}	8.33^{b}	0	0	0	0.02
2011	Yield (t/ha)	4.0^{a}	nd	4.6^{a}	5.8^{a}	nd	4.7^{b}	0.02
	Rc disease %	0	nd	0	0	nd	0	
	Bgh disease %	6.67^{a}	nd	0.37^{c}	3.67^{ab}	nd	3.00^{b}	<0.01

Changes in the epiphytic bacterial populations were also observed on the field-grown leaves of two glossy mutants (2019 and 48-82) and their WTs (Bowman and Optic) in 2010. The PCA (Figure 6.7) showed great variation between the epiphytic communities, but overall the communities of the glossy mutants leaves were separated from the WTs according to the first principal component (PC1 = 56.9%). The cultivar also influenced the separation of the communities by the third principal component (PC3 = 10.9%).

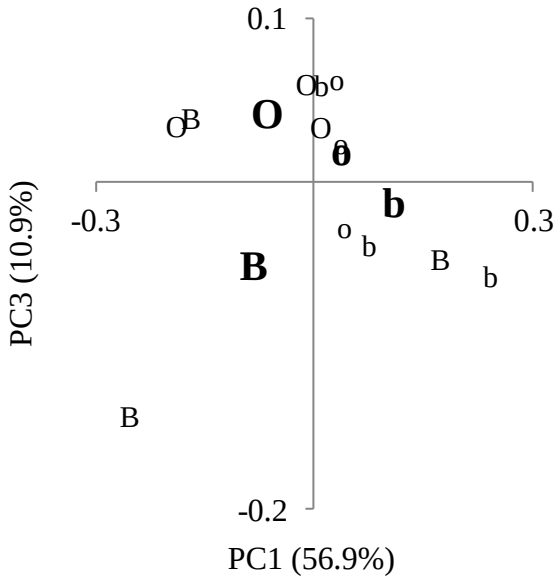

Figure 6.7: PCA of epiphytic communities from two barley glossy mutants (b: 2019 and o: 48-82) and their related WTs (B: Bowman and O: Optic). Bold letters represent the average of three.

The relative abundance of four of the main OTUs involved in the separation due to the first principal component (197, 203, 406 and 436; Table 6.4) differed and fluctuated similarly between glossy mutants and their WTs (Figure 6.8). Three of them (197, 203 and 436) were reduced in the epiphytic communities of the glossy mutants, whereas the fourth (406) was increased. However, there was no significant effect of the leaf wax mutation on the relative abundance of these OTUs as

individuals. As a group, the relative abundance of the 436, 203 and 197 OTUs was significantly (P = 0.04) reduced by approximately 40% in the glossy mutants compared to the WTs (Figure 6.8).

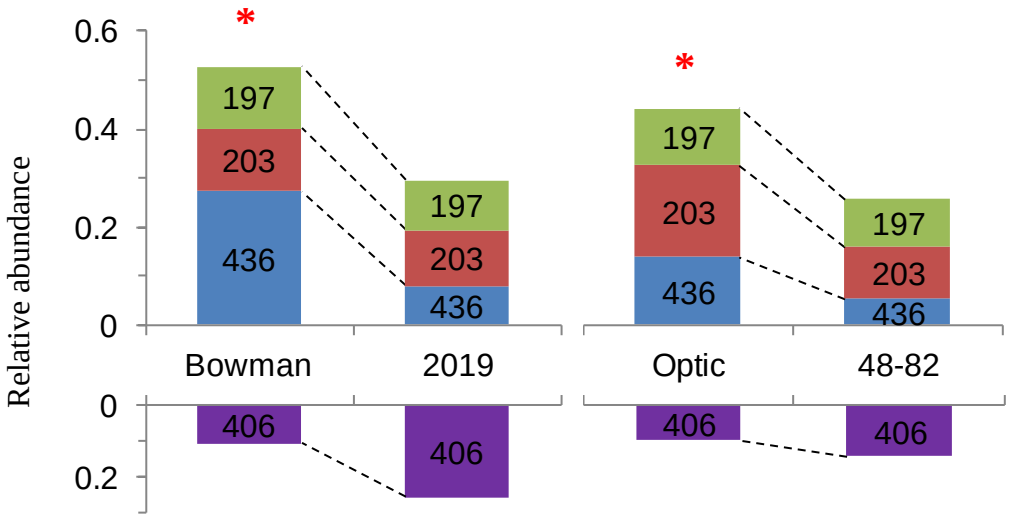

Figure 6.8: Relative abundance of the main OTUs participating in the first principal component of the PCA that separated the WTs (Bowman and Optic) from the glossy mutants (2019 and 48-82). As a group, the relative abundance of the 197, 203 and 436 OTUs was significantly reduced on leaves of glossy mutants (P = 0.04, lsd = 0.205).

Table 6.4: OTUs' loading vector values associated with the first principal component of PCA (PC1) analysis of the effect of the epicuticular wax mutation on epiphytic communities of field-grown barley leaves.

OTU	Loading Vector	OTU	Loading Vector	OTU	Loading Vector
436	-0.522	292	-0.014	53	0.005
203	-0.135	233	-0.010	62	0.018
197	-0.103	364	-0.008	300	0.020
230	-0.089	90	-0.002	193	0.027
341	-0.059	295	-0.001	348	0.034
77	-0.026	158	0.000	410	0.038
57	-0.026	82	0.002	368	0.043
109	-0.020	86	0.004	406	0.824

6.4 DISCUSSION

Overall, mutations of the leaf surface properties to a glossy phenotype resulted in a reduction of the number of trichomes (Table 6.2), modifications to the amount, density and structure of epicuticular waxes (Figure 6.3, 6.4 and 6.5) and their major chemical constituents (Figure 6.6). Platelets are known as the most common crystalloids on leaf surfaces (Barthlott *et al.*, 1998) and their alteration leads to considerable modification of microbial colonisation and pathogen resistance on the leaves as well as the plant fitness in the field.

The colonisation of the leaf surface by epiphytes is greatly dependant on the amount of available free sugars (Mercier and Lindow, 2000). Various carbohydrates, including glucose, fructose and sucrose originating from photosynthesis, leak through the leaf surface by passive diffusion. The composition and the amount of the sugars vary notably with plant species (Tukey, 1970). A positive and proportional relationship has been established between the amount of sugars on the leaf surface of various plant species and the number of epiphytic bacteria (Mercier and Lindow, 2000). The major component affecting the diffusion of sugars is the waxy plant cuticle (van der Wal and Leveau, 2011). Leaf wettability, i.e. its ability to retain water, also influences the distribution of sugars. Sugars can accumulate considerably in water droplets on the leaf surface where bacteria are often found (van der Wal and Leveau, 2011). The wettability is directly related to the composition of the epicuticular waxes and thus, they control directly sugar diffusion and indirectly sugar retention, which is essential for microbial colonisation.

Under controlled conditions, mutations of the maize epicuticular wax layer resulted in an overall increase in the inoculated epiphytic bacterial populations (Marcell and Beattie, 2002). However, *Pantoea agglomerans* exhibited opposite

dynamics between different glossy mutants. Under field conditions, the epiphytic populations on barley leaves differed greatly between the 48-82 Optic and 2019 Bowman glossy mutants and their respective WTs (Figure 6.7). If the communities of all bacterial species were showing similar increases, the relative abundance would not change, but the relative abundance of major OTUs of the epiphytic populations was greatly affected on glossy mutants. Hence, bacterial populations responded differently to the 48-82 Optic and 2019 Bowman glossy mutants, which showed very different leaf wax alterations of their leaf surface.

On one hand, the 2019 Bowman wax mutant had a significantly reduced amount of wax (Figure 6.5), thus a reduced density (Figure 6.3 C). Wax crystalloids also had a severed shape (Figure 6.4 C). A reduction in the wax density was hypothesised to contribute to the water capillary movements on the maize leaf surface (Marcell and Beattie, 2002). A low wax density would result in higher water movements and more water trapped around the crystals. This could promote a more extensive bacterial colonisation of the leaf surface by providing larger amounts of water and carbohydrates (Marcell and Beattie, 2002).

On the other hand, the 48-82 Optic glossy mutant had significantly more waxes compared to its WT (Figure 6.5). The wax density was similar to the WT, although the crystalloids structure was different. The number of trichomes present on the leaf surface was also reduced on the glossy leaves (Table 6.2). A positive correlation was found between the number of trichomes and the epiphytic bacterial populations on rice leaves (de Costa *et al.,* 2006a). Hooked trichomes contribute to the leaf roughness (Sato and Tadeka, 1993), which affects water drop retention (Brewer *et al.*, 1991): the more trichomes, the more water drops are retained. Thus, the water availability on the 48-82 Optic leaves should be reduced and the bacterial

growth impaired. The absence of trichomes on *Arabidopsis thaliana* leaves did not significantly alter the bacterial epiphytic communities (Reisberg *et al.*, 2012). However, changes in the relative abundance of the main epiphytic OTUs of field-grown barley leaves were similar to the ones observed with the Bowman varieties (Figure 6.8). Hence, it is possible that mechanisms, other than those affecting the water availability on the leaf surface, and that are common in the Bowman and Optic glossy mutants, are involved in the control of the epiphytic populations.

Even though the analysis of the composition of wax constituents is not yet complete, considerable differences were observed between glossy mutants and WTs (Figure 6.6). The epicuticular waxes do not only affect microbial colonisation by altering the availability of water and nutrients, but also certain of its chemical compounds can directly interfere with microbial life cycles. The removal of epicuticular waxes resulted in an increased germination rate and infection structure development of powdery mildew causal agents on *Lolium* spp. and cereal leaves (Carver and Thomas, 1990; Carver *et al.*, 1990). On *Lolium* spp. leaves, the abaxial surface exhibited little to no powdery mildew symptoms compared to the adaxial side. Under the microscope, both surfaces were covered with epicuticular waxes of different shape. As the chemical composition affects the wax structure, it was hypothesised that certain chemicals would impair powdery mildew infection (Carver *et al.*, 1990).

Pre-penetration of the fungal pathogen *B. graminis* f.sp. *hordei* was promoted *in vitro* by the most common aldehyde of barley leaves, the C-26 n-hexacosane (Tsuba *et al.*, 2002). This chemical and several other very-long-chain aldehydes affected the pre-penetration in a dose- and chain-length dependent manner *in vitro* (Hansjakob *et al.*, 2010). Recently, it was demonstrated *in vivo* that *B. graminis*

could not induce its pre-penetration processes on leaves lacking very-long-chain aldehydes, but the processes were restored by applying n-hexacosane to the leaves before infection (Hansjakob *et al.*, 2011). Similar results were observed on the leaves of a *Medicago truncatula* wax mutant, where the pre-penetration inhibition of different host and non-host pathogens was correlated with a significant reduction in a C-30 primary alcohol (Uppalapati *et al.*, 2012). Other factors such as the surface hydrophobicity, which is directly dependent on the chemical composition of waxes, participate in the promotion of pre-penetration of *B. graminis* (Zabka *et al.*, 2008).

Alterations in the integrity of the epicuticular wax layer could increase the leaching of molecules from the interior of the leaf to the surface. Photosynthetic sugars including, glucose, fructose and sucrose are the main carbohydrates diffusing through the cuticle (Tukey, 1970). However, other metabolic by-products and secondary metabolites can manipulate leaf-associated microorganisms. For instance, methanol is a volatile side-product of cell-wall biosynthesis and it can be metabolised by *Methylobacterium* bacteria, which are a common phyllosphere coloniser (Lindow and Brandl, 2003; Delmotte *et al.*, 2009). Recently, a positive correlation between the concentration of carotenoids and glucosinolates in the leaf with the composition of the bacterial communities was identified (Ruppel *et al.*, 2008). Whether these compounds are intentionally released to alter the phyllobacteria is unknown, but modifications of the leaf surface properties should affect the diffusion of these microbial nutrients and modify the microbial balance.

The presence of disease causal agents was demonstrated to challenge the innate leaf-associated bacterial populations as well (Suda *et al.*, 2009; Zhou *et al.*, 2010). The changes in the relative abundance of the main OTUs was similar between Optic and Bowman glossy mutants (Figure 6.8), but the barley cultivars were

differentially affected by diseases in the field (Table 6.3). The Bowman varieties were mostly infected by *B. graminis* and the Optic ones by *R. commune*. Thus the disease causal agents should have a minor role here in the control of the bacterial phyllosphere, but the epiphytes could have an effect on disease establishment. The 203 OTU may partly represent *Pseudomonas* species (Table 3.8). The most common of them, *P. syringae*, showed biocontrol activity against *R. commune in vitro* in previous experiments (Table 4.3). The reduction of the 203 OTU relative abundance may contribute to the increased *R. commune* susceptibility of glossy mutants.

The evolutional development of the epicuticular waxes was a crucial step in the land colonisation by plants (Edwards *et al.*, 1996) and they are at the interface between plants and their environment (Barthlott *et al.*, 1998). Overall, many factors that are dependent on the epicuticular wax composition may participate in the manipulation of the bacterial communities in the barley phyllosphere. Manipulating the composition of the leaf waxes is complex and can have lethal consequences for the plant. The glf1 glossy mutant of the Bonus cultivar is highly sterile, semi-dwarf, relatively weak and late in heading (Anon., 1993). Glossy mutants in the field also experienced a yield reduction (Table 6.3). Interestingly, however, the 2019 Bowman mutant exhibited an increased resistance against *B. graminis* as well as a 5 to 10% increase in yield (Table 6.3). Whether this is directly related to a reduced amount of n-hexacosane in the composition of the epicuticular waxes still has to be determined, but it could be a valuable and potentially sustainable trait to select for in the breeding process.

CHAPTER 7: SYSTEMIC EFFECTS OF A ROOT-ASSOCIATED MICROORGANISM ON THE PHYLLOSPHERE MICROBIAL COMMUNTIES

7.1 INTRODUCTION

Crop protection can be achieved in the phyllosphere by directly applying a BCA under certain conditions. Various bacterial and fungal BCAs are commercially available in Europe (Nicot *et al.*, 2011). Combining BCAs can synergistically improve crop protection and reduce the variability of its effectiveness (Guetsky *et al.*, 2001). However, BCAs are usually developed as prophylactic methods (i.e. application before the causal agent's establishment). Many other factors such as temperature, relative humidity and spray-timing can also interfere with the efficacy of biocontrol measures. Furthermore, BCAs may have to be applied frequently to sustain the adequate levels of protection (Al Mazra'awi *et al.*, 2006). Overall, even though foliar BCAs are an interesting integrated crop protection strategy, they still present great variability in the field, which limits their usage (Kiss, 2003).

Using root-associated BCAs could reduce certain inconveniences affiliated with the foliar ones. The rhizosphere is a habitat where environmental conditions are less harsh than the phyllosphere, with more stable water levels and nutrient availability (Andrews and Harris, 2000). The rhizosphere contains a great number of microbes (Roesch *et al.*, 2007) able to interact with crops. Some of them can form tight interactions with the plant resulting in a vast range of beneficial effects. The arbuscular mycorrhizal fungi (AMF) are well-known beneficial endomycorrhiza that form tree-like structures within the root cells to interact with the plant. The main function of the AMF is to improve the phosphorus uptake from the soil, promoting plant growth and soil fitness (Parniske, 2008). It has also been shown to promote

local resistance to soil-borne pathogens (Azcon-Aguilar and Barea, 1996; Liu *et al.*, 2007) and systemic resistance to foliar bacterial pathogens (Liu *et al.*, 2007). Whether the systemic effect is directly due to the AMF or indirectly by improved plant fitness is not known (Parniske, 2008). Furthermore, little is known on the systemic effect of AMF on phyllosphere microbial communities. Even though the AMF symbioses represent most of the plant-fungus mutualistic interactions (Parniske, 2008), some plants, including barley and wheat, are "non-responsive" to AMF (Grace *et al.*, 2009), which means that the AMF colonisation on these plants results in negligible or negative growth response.

Piriformospora indica is a soil fungus that can improve plant growth and fitness (Waller *et al.*, 2005). This endomycorrhizal fungus belongs to the *Sebacinales* order (*Basidiomycota*) (Selosse *et al.*, 2009) and was originally isolated in the Indian Thar desert in 1997 (Verma *et al.*, 1998). It is able to colonise the roots of a large range of higher plants including *Arabidopsis thaliana*, legumes, (e.g. pea and soybean) and cereals such as barley, maize and rice (Pham *et al.*, 2004). Plant colonisation is restricted to the root cortex, but the beneficial effects of the fungus are both local and systemic. Waller *et al.* (2005) showed that this endophyte was able to (i) enhance plant growth, (ii) increase its yield, (iii) promote its tolerance to moderate salt stress, (iv) induce local resistance against root pathogens (i.e. *Fusarium culmorum* and *Cochliobolus sativus*), and (v) induce systemic resistance against the foliar pathogen *B. graminis* f.sp. *hordei* on barley. Such diverse beneficial effects on potentially so many economically important crops make *P. indica* a very interesting microorganism for biotechnological applications (Oelmuller *et al.*, 2009).

The systemic effects of *P. indica* on microorganisms were investigated on a selection of three commercially used barley cultivars. Primary interests focused on

the fresh weight increase and the changes in disease severity (including *B. graminis* and *R. commune*, causal agents of powdery mildew and leaf scald respectively) due to *P. indica* colonisation of the roots of the different cultivars. Finally, the systemic effect of *P. indica* colonisation on the leaf-associated communities was determined using T-RFLP.

7.2 MATERIALS AND METHODS

7.2.1 Fungus and Plant Growth Conditions

7.2.1.1 Fungal Growth Conditions and Inoculum Preparation

The *Piriformospora indica* isolate was kindly provided by Karl Heinz-Kogel's group from the Justus Liebig Universitaet (Giessen, Germany). The fungus was cultured on the same growth medium as *R. commune* (CzV8CM) but at room temperature for three weeks. Preparation of spore suspensions was adapted from the *R. commune* inoculum preparation method (Section 2.2.1), except that spores were washed off the plate by rubbing a glass rod on the plate with SDW. The inoculum was diluted at a 5.0 x 10^5 spores/mL concentration with a 0.1% (v/v) Tween 80 solution.

7.2.1.2 Plant Growth Conditions and Sampling

The effect of the endophyte on barley was assessed on three different cultivars: Optic, Ingrid and Bowman. Seeds were germinated as described in Section 2.1.2 and subsequently inoculated either with the *P. indica* inoculum or a Tween mock solution (0.1% v/v) for 2 h under gentle agitation.

Inoculated seedlings were placed in SCRI compost and grown under normal glasshouse conditions (Section 2.1.2). Once the third leaf had emerged (two to three weeks-old seedlings), the entire shoot was cut off and weighed. Ten segments of the

second leaf were used for the detached leaf assay (Section 2.1.2) to determine the effect of *P. indica* on the resistance against scald (Section 2.2.1) and powdery mildew (Section 2.2.2). Ten other uncut second leaves and the washed root systems were stored in the freezer at -20°C for T-RFLP analysis (Section 3.2.5) and molecular quantification of the fungi in the roots respectively. Experiments were replicated twice.

7.2.2 Molecular Quantification of the Fungal Endophyte

Roots were ground into a fine powder using liquid nitrogen, a mortar and a pestle. The DNA was extracted from 0.2 g of ground roots based on a CTAB-buffer procedure (Section 3.2.2). However, because roots were not entirely clean from soil residues, DNA samples contained PCR inhibitors, mainly humic acid. Hence, the DNA samples had to be cleaned by filtration through a Micro Bio-Spin column (Bio-Rad Laboratories, UK) containing PVPP (Sigma-Aldrich, UK). Briefly, 900 µL of a 10% (w/v) PVPP solution was added to a sterile column and centrifuged twice at 11,000 g for one minute to remove any liquid in excess. The DNA suspension was then gently pipetted on top of the PVPP and centrifuged once at 11,000 g for one minute. The purified eluate was transferred to a new sterile 1.5 mL microcentrifuge tube and DNA quantified with a spectrophotometer.

The quantification of the fungal endophyte was adapted from Deshmuck *et al.* (2006) and used quantitative PCR (qPCR) to relate the amount of fungal genomic DNA to the amount of barley root genomic DNA. The fungus was quantified using its translational elongation factor *EF-1α* gene (*PiTef*) and barley with two different reference genes: the ubiquitin (*HvUb*) and the 18S rRNA genes (*Hv18S*) (Table 7.1). The qPCR was performed in a 25 µL volume containing 1X of LightCycler® 480 SYBR Green I Master (Roche, DE), 0.35 µM of each primer, 10 ng of genomic DNA

and PCR-grade water. After an initial step at 95°C for 7 min, 40 cycles (94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec) were performed using a thermocycler fitted with the Chromo-4 Real-Time PCR Detector (Bio-Rad Laboratories, UK). A single fluorescent reading was obtained at the end of the 72°C step of each cycle. At the end of the 40 cycles, a melting curve control (from 60°C to 94°C with an increment of 0.5°C every 30 sec) was used to check for the absence of false positive amplicons.

Table 7.1: List of primers and their sequences used for qPCR.

Genes		Sequence (5' => 3')
PiTef	Forward	ACC GTC TTG GGG TTG TAT CC
	Reverse	TCG TCG CTG TCA ACA AGA TG
HvUb	Forward	CAG TAG TGG CGG TCG AAG TG
	Reverse	ACC CTC GCC GAC TAC AAC AT
Hv18S	Forward	GCG AGC ACC GAC CTA CTC
	Reverse	GGA CCG GAA TCC TAT GAT GTT

Data were analysed at a fixed threshold of 0.04 with the Opticon Monitor software version 3.1.32, supplied with the Chromo-4 PCR Detector. Each sample was run in triplicate and the Cycle threshold (Ct) averaged. The Ct value corresponds to the cycle number, at which the amplification curve starts its linear exponential phase. The relative DNA amount of *P. indica* was calculated as described by Wulf *et al.* (2003): $2^{-\Delta Ct}$, where: ΔCt = Ct (*PiTef*) – Ct (barley reference gene);.

7.3 RESULTS

7.3.1 Effects of *P. indica* on Barley Cultivars

7.3.1.1 Increase in the Shoot Fresh Weight

The effect of *P. indica* on the barley shoot fresh weight was monitored on three different spring barley cultivars: Bowman, Ingrid and Optic, using 20 replicates

of each (Table 7.2). There was a significant difference in shoot weight between the cultivars ($P < 0.001$) and an overall significant increase of the shoot fresh weight ($P < 0.001$). No interaction between the cultivar and treatment was found and all *P. indica*-infected plants had a similar shoot weight increase: +20% for Ingrid, +19% for Bowman and +16% for Optic.

Table 7.2: Shoot fresh weight (g) of three different barley cultivars (Bowman, Ingrid and Optic) either infected or not with *P. indica*. The averaged weight values (n = 20) were significantly affected by both the cultivar used ($P < 0.001$, lsd = 0.12) and the treatment applied ($P < 0.001$, lsd = 0.10).

	Shoot Fresh Weight (g)			
Treatment	Bowman	Ingrid	Optic	Average
Control	1.12	1.30	1.00	1.14^{a}
+ *P.indica*	1.34	1.57	1.17	1.36^{b}
Average	1.23^{b}	1.44^{c}	1.09^{a}	

7.3.1.2 Enhanced Plant Resistance against Powdery Mildew

All barley cultivars were susceptible to *B. graminis* and developed symptoms within six days after inoculation (Figure 7.1). The number of symptoms tended not to increase any further after 12 days. However, the maximum number of colonies observed was significantly different between the varieties ($P < 0.001$). Hence, the three barley varieties had significantly different susceptibilities to powdery mildew, with Ingrid being the most susceptible and Optic the most tolerant of the three.

When seedlings were initially infected with the fungal endophyte *P. indica*, a significant reduction in the number of pustules ($P = 0.05$) was observed only on Ingrid (Table 7.3). The apparent reduction of symptoms on the Optic leaf segments observed in both experiments was not significant. Bowman showed fewer symptoms

when infected with *P. indica* on the first occasion and more on the second (data not shown). Overall, the fungus *P. indica* induced a reduction in *B. graminis* symptoms on the cultivars tested but effectively only on Ingrid.

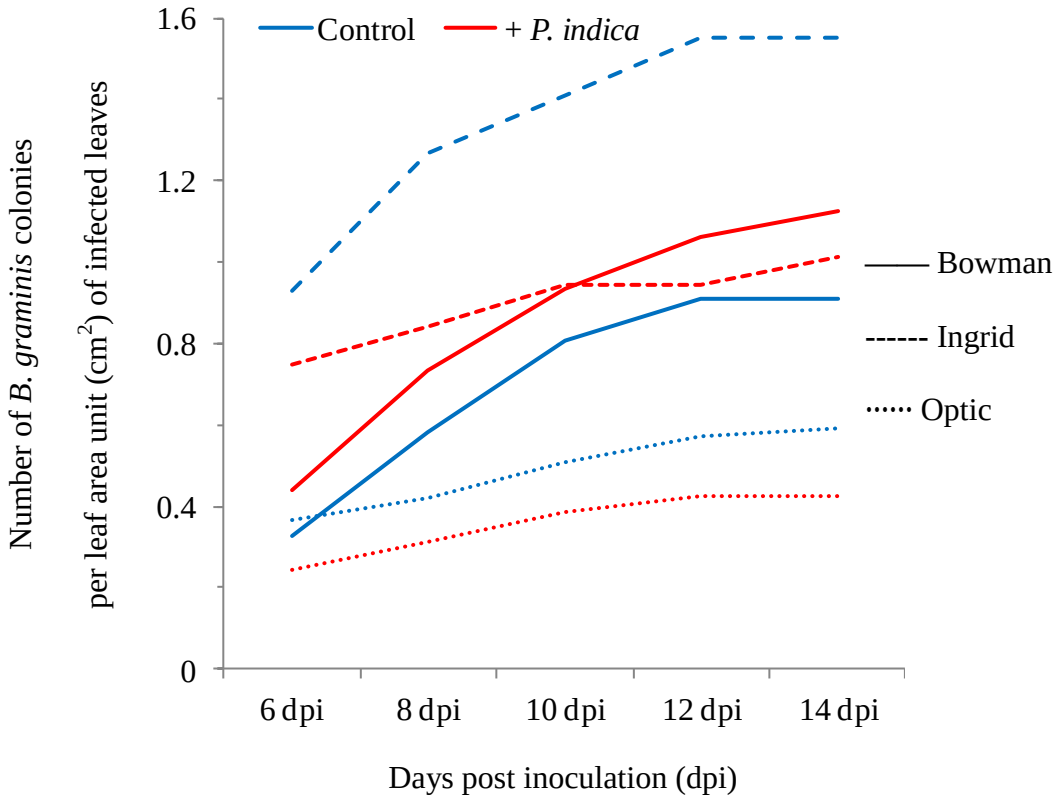

Figure 7.1: Number of *B. graminis* pustules per leaf area unit (cm^2) over time on three barley cultivars: Bowman (plain), Ingrid (dash) and Optic (dot). Seedlings were initially infected with *P. indica* (red) or a mock solution (blue). Values represent the average of two replicated experiments (n = 20) monitored over 14 days post inoculation.

Table 7.3: Average number of *B. graminis* colonies per leaf area unit (cm^2) from leaf segments of three barley varieties (Bowman, Ingrid and Optic) initially infected or not with *P. indica* (P = 0.05, lsd = 0.392).

	Treatment	
Cultivar	Control	+ *P. indica*
Bowman	0.728^{ab}	0.875^{b}
Ingrid	1.453^{c}	0.914^{b}
Optic	0.469^{a}	0.358^{a}

7.3.1.3 Modification of the Plant Resistance against Scald

Pre-inoculating seedlings with *P. indica* resulted in a transient reduction of the number of scald symptoms on leaf segments of Optic (Figure 7.2). The lesion size increased with time (P = 0.005) and was similar between both treated leaf segments (Table 7.4). However, the appearance time of the necrotic spots was different between the leaves of *P. indica*-infected seedlings and the control ones (Figure 7.3). Even though many *R. commune* infected leaf area of the *P. indica*-infected plants appeared water-soaked (Figure 7.2), the overall number of necrotic lesions was greatly reduced. However, a few days later (21 dpi) more lesions appeared and most of the leaf segments were as infected as the control (Figure 7.3). The *P. indica*-infected seedlings did not increase the final resistance to *R. commune* but slowed down the emergence of scald symptoms in leaves. Ingrid showed little infection to *R. commune* and no significant effect of *P. indica* on scald resistance was observed (data not shown).

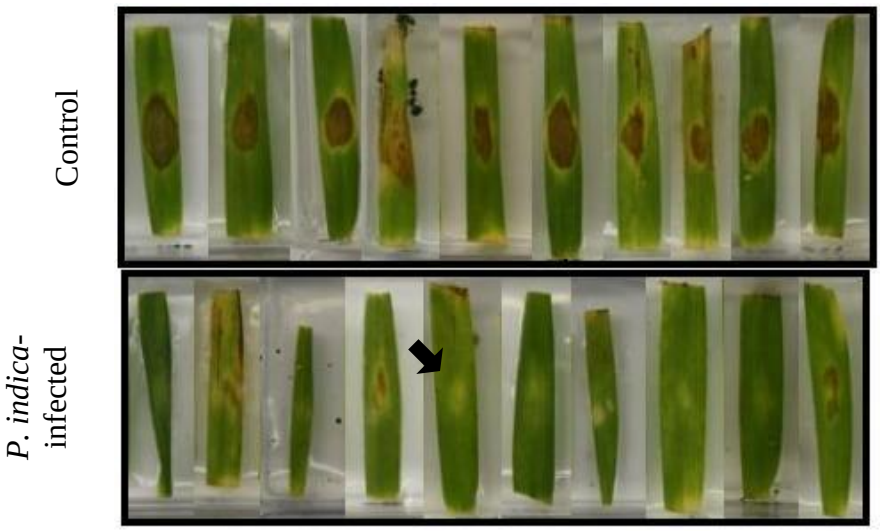

Figure 7.2: Pictures of scald symptoms on leaves of *P. indica*-infected (bottom) or control-treated (top) plants at 17 days post inoculation. Before emergence of necrotic lesions (white arrow), water-soaked infected areas are visible (black arrow).

Table 7.4: Average size of *R. commune* lesions (cm) on leaves of control and *P. indica*-infected Optic seedlings (n = 10) from 14 to 21 days post inoculation (dpi). The lesion sizes increased with time (P = 0.005, lsd = 0.054), but no significant differences between treatments was found.

R. commune lesion size (cm)	Days post inoculation (dpi)					
	14 dpi	15 dpi	17 dpi	18 dpi	21 dpi	Average
Control	0.966	0.921	1.000	1.061	1.081	1.006
+ *P. indica*	0.902	0.902	0.918	0.990	0.997	0.942
Average	0.934[a]	0.911[a]	0.959[a]	1.026[b]	1.039[b]	

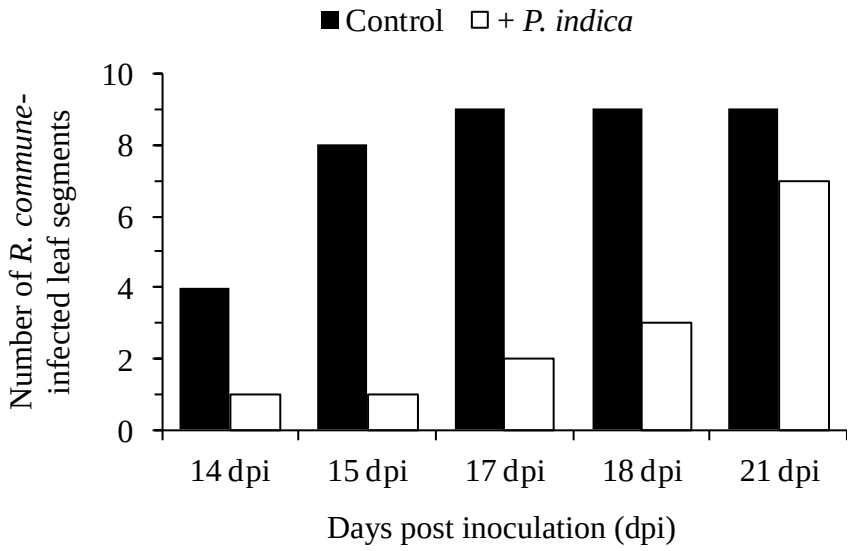

Figure 7.3: Appearance of necrotic spots from 14 to 21 days post inoculation (dpi) caused by *R. commune* on Optic leaf segments of seedlings infected with *P. indica* (white) or not (black). A significant reduction in the number of infected leaves was observed (Chi-square test, P = 0.001).

7.3.2 Quantification of *P. indica* in the Roots

The amount of *P. indica* was quantified from a fixed quantity of DNA via qPCR using the *PiTef* gene, normalised with the two barley reference genes. The choice of the best suited reference gene was validated by comparing the distribution of Ct values between each cultivar (Figure 7.4). The *Hv18S* gene was significantly variable between cultivars (P < 0.001), whereas the *HvUb* gene showed better

stability. Hence, the latter was chosen as the reference gene for qPCR quantification of *P. indica* in the three cultivars tested.

The fungus was able to colonise the roots of all spring barley cultivars. However, there were differences in the relative amount of endophytic colonization between the three varieties tested (Figure 7.5). The relative abundance of *P. indica* in the infected Ingrid roots was similar to that in Bowman roots, whereas it was significantly lower in the *P. indica*-infected Optic roots (P = 0.023).

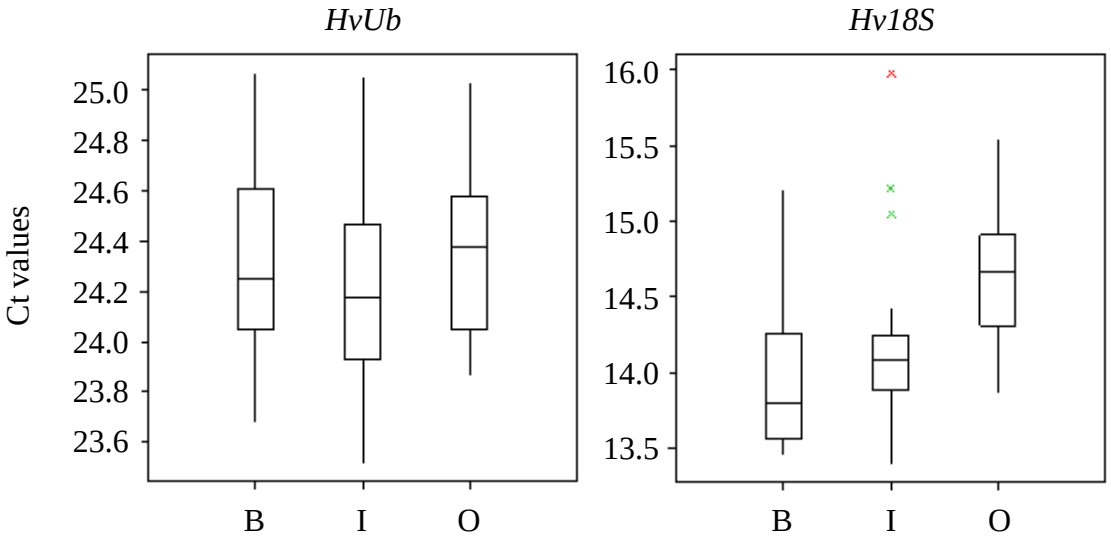

Figure 7.4: Boxplots of the Ct values of two barley reference genes (ubiquitin *HvUb* gene, and 18S rRNA *Hv18S* gene) used for the qPCR quantification of *P. indica* from three barley cultivars: Bowman (B), Ingrid (I) and Optic (O). Only Ct values of *Hv18S* were significantly cultivar-dependent (P < 0.001, lsd = 0.23, n = 10).

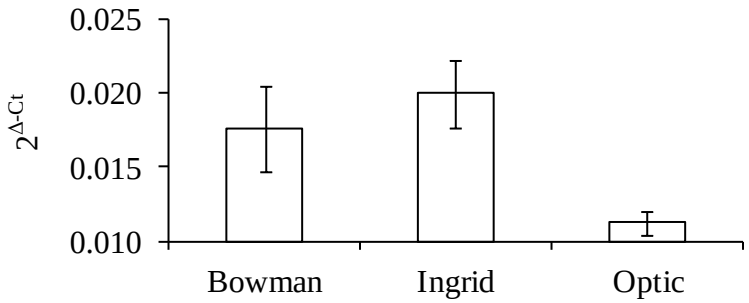

Figure 7.5: Relative quantification of *P. indica* from infected roots of three barley cultivars: Bowman, Ingrid and Optic; using *PiTef* as marker and *HvUb* as reference genes. Error bars represent the SEM (n = 10). Fewer endophytes were detected in the roots of Optic (P = 0.024, lsd = 0.006).

7.3.3 Systemic Effects of *P. indica* on the Phyllosphere Ecology

Using the T-RFLP method, the effect of *P. indica* on the phyllosphere structure was investigated. Five leaves of Tween mock-treated or *P. indica*-infected seedlings were sampled twice over two replicated experiments. A PCA analysis of epiphytic OTUs of the forward TRFs (Figure 7.6) showed that the first principal component (PC1 = 30.6%) separated the populations from seedlings mock-treated and infected with *P. indica*. Hence, this suggested that the leaf-associated epiphytic populations between mock and *P. indica*-treated seedlings mostly by the relative abundance of certain OTUs.

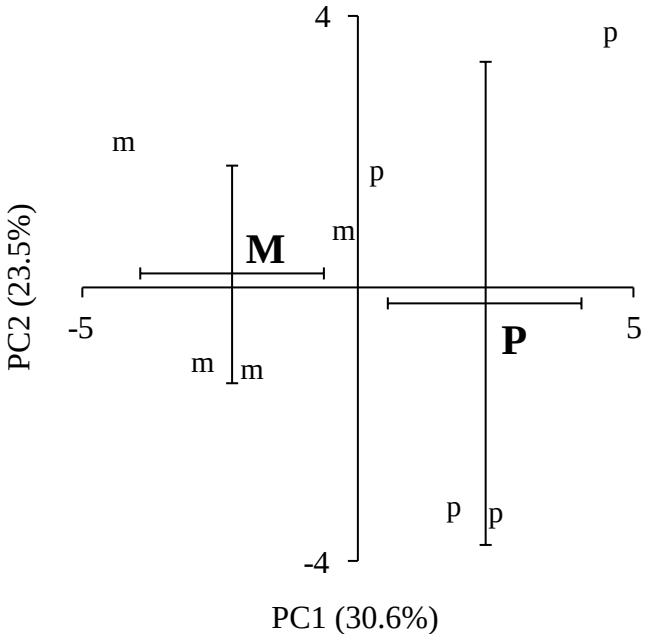

Figure 7.6: PCA of epiphytic populations from glasshouse-grown Ingrid leaves, which seedlings were infected with *P. indica* (p) or mock-treated (m). Populations labelled in bold represent the average of four replicates with SD error bars.

The OTUs involved the most in the separation between the two bacterial populations were identified based on their associated loading vector values during the PCA analysis (Table 7.5). On the one hand, the 61, 77, 83, 209 and 370 OTUs participated the most in the differentiation of the bacterial populations of the mock-

treated seedlings compared to the *P. indica*-infected ones (Figure 7.7). Individually, their relative proportion was not significantly different between the treatments. Overall, they were significantly more common (P = 0.004) and represented 30% of the total population, whereas they reached 20% in *P. indica*-infected plants. On the other hand, the 63, 73, 90 228 and 340 OTUs were more common on the leaf surface of *P. indica*-inoculated plants than the mock-treated ones (Figure 7.7). Conversely to the other five OTUs, they represented 30% of the bacterial community on leaves of plant infected with *P. indica*, whereas they attained 20% on non-infected crops. Similarly, no significant difference was identified in the relative abundance between each OTU alone, but as a group the difference between control *P. indica*-infected plants was significant (P = 0.041).

Table 7.5: OTUs' loading vector values associated with the first principal component of PCA (PC1) analysis of the effect of the root endophyte *Piriformospora indica* on epiphytic communities of glasshouse-grown barley leaves.

OTU	Loading Vector	OTU	Loading Vector	OTU	Loading Vector
370	-0.268	58	-0.095	175	0.155
209	-0.255	291	-0.090	52	0.160
61	-0.245	357	-0.090	137	0.229
83	-0.227	348	-0.050	73	0.236
77	-0.210	159	0.014	63	0.257
222	-0.200	206	0.118	340	0.262
201	-0.145	138	0.125	90	0.291
53	-0.140	279	0.135	228	0.322
237	-0.114	86	0.151		
196	-0.105	79	0.152		

The overall structure of the phyllosphere of control and *P. indica*-infected plants was estimated using ecological indices, but no significant difference in ecological indices (richness, diversities and evenness) of epiphytic populations and endophytic communities was found (Annex Table A.11).

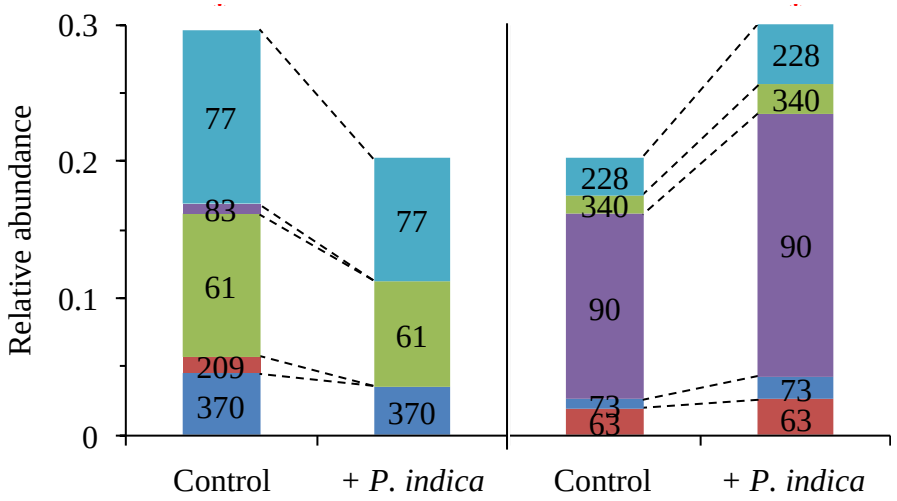

Figure 7.7: Relative abundance of the main differential leaf-associated OTUs between mock- and *P. indica*-treated seedlings. Individually, no OTU was significantly affected by the treatment. However, as a group the five most involved OTUs in the control leaves were significantly (P = 0.004) more common than in the *P. indica*-treated ones. Conversely, as a group the five most involved OTUs on the leaves of the *P. indica*-infected plants were significantly (P = 0.041) more common than in the mock-treated ones.

7.4 DISCUSSION

The endophytic fungus *P. indica* offers several potential improvements to plant health and crop protection from biotic and abiotic stresses. It promotes the growth of various plants and crops (Pham *et al.*, 2004). On barley, beneficial effects of the interaction with *P. indica* were observed on various cultivars: Ingrid (Waller *et al.*, 2005; Baltruschat *et al.*, 2008), Annabell (Waller *et al.*, 2005), California Mariout (Baltruschat *et al.*, 2008) and Golden Promise (Deshmukh *et al.*, 2006). Similarly, an increase in the shoot biomass of two other barley varieties (Bowman and Optic) was observed here (Table 7.2).

The root endophyte was shown to induce broad spectrum protection in the roots against the necrotroph *Fusarium culmorum* and the hemibiotroph *Cochliobolus sativus* (Waller *et al.*, 2005). It was also demonstrated to induce an increase in the resistance against the biotroph *B. graminis* on Ingrid, as previously described by Waller *et al.* (2005), but not significantly on Optic and Bowman (Table 7.3). For the first time, *P. indica* was shown to temporarily slow down symptom appearance of the fungus *R. commune* on Optic leaf segments, but not on Ingrid. Hence, *P. indica* has the ability to promote resistance against a wide range of pathogens.

However, the effect of *P. indica* on disease resistance seemed to be cultivar-dependent. The colonisation of the roots by *P. indica* was different between the various cultivars (Figure 7.4). Ingrid had the highest amount of *P. indica* and the best increase in shoot biomass and protection. Different *Sebacinales* fungi (the same order as *P. indica*) affected differentially the growth and resistance to *B. graminis* on barley (Deshmukh *et al.*, 2006). They also affected differently the expression of defence genes (Waller *et al.*, 2008). Conversely, if different *Sebacinales* isolates could interact differentially on one barley cultivar, one isolate should have been able to colonise differentially various cultivars and thereafter effects would differ too.

Although the plant and fungus genotypes appear to be important factors determining the success of the beneficial interaction with barley, the actual mechanisms of the systemic defence induction are still unknown. Leaves of *P. indica*-infected seedlings were shown to have neither a higher expression of defence genes (coding for an SA-responsive protein BCI-1, a JA-induced protein JIP-23 and the pathogenicity-related protein PR5), nor a faster induction of defence genes upon challenge with *B. graminis* (Waller *et al.*, 2005). Overall, a very limited number of genes, whose expressions were modified during the *P. indica* infection, were

identified either in roots or leaves (Waller *et al.*, 2008), which is consistent with what had already been observed during AMF colonization (Hause and Fester, 2005). However, other metabolic factors are changed systemically by *P. indica*. Salt tolerance in *P. indica*-infected barley was related to a strong increase in the anti-oxidant pool in the roots as well as in the leaves (Waller *et al.*, 2005; Baltruschat *et al.*, 2008). A priming of pH alkalinisation was also observed upon *B. graminis* infection (Felle *et al.*, 2009). Hence, this suggests a new systemic defence induction pathway yet to be characterised.

The systemic modifications of the phyllosphere composition could also participate in resistance to pathogens. Plant pathogens were shown to modify the phyllosphere bacterial populations (Suda *et al.*, 2009; Zhou *et al.*, 2010). Whether it is a cause or a consequence is still unknown. However, it would be interesting to monitor the changes in the phyllosphere of a *P. indica*-infected plant upon challenge with a foliar pathogen. Balint-Kurti *et al.* (2010) identified genetic markers in maize participating in the control of the bacterial phyllosphere diversity. They hypothesised that the plant could lower its bacterial diversity allowing certain helper bacteria to better control the southern leaf blight disease. Hence, a different bacterial population could be another factor participating in the increased resistance to powdery mildew or scald on barley, to identify how resilient the *P. indica*-induced phyllosphere communities are. Interestingly, the relative abundance of the 370 OTU was reduced in the leaves of the *P. indica*-infected plants (Figure 7.7). This OTU can comprise the bacterium *Pectobacterium atrosepticum*, which has previously shown to increase the powdery mildew symptoms *in planta* (Figure 4.6).

However, the fungus may not be the origin of beneficial interaction. Recently, different bacterial species have been detected, isolated and identified as

closely associated with several fungi of the *Sebacinales* order (Sharma *et al.*, 2008). For example, the *Rhizobium radiobacter* strain PABac-DSM was shown to be intimately associated with *P. indica* spores and hyphae. Despite extensive attempts to eradicate the bacterium, axenic cultures of pure *P. indica* have not yet been obtained. Most intriguingly, PABac-DSM induced growth promotion and systemic resistance against powdery mildew in barley seedlings comparable with the *P. indica*-induced phenotype. The bacteria *R. radiobacter* (also known as *Agrobacterium tumefaciens*) is a well-known soil-borne pathogen infecting dicotyledons and leading to the crown gall disease. However, the bacterium PABac-DSM lacks the virulence genes (Sharma *et al.*, 2008). Other *Rhizobium* strains are known as biocontrol agents (Pu and Goodman, 1993; Humphry *et al.*, 2007). Similarly, the AMF possess bacterial endosymbionts (Bianciotto *et al.*, 2000) that did not only affect the AMF activity (Xavier and Germida, 2003; Bharadwaj *et al.*, 2008), but could also affect directly plant pathogens and the whole plant (Bharadwaj *et al.*, 2008).

Overall, because of improved biomass and yield and increased resistance to pathogens and tolerance to salt-stress by *P. indica* and other related fungi (Waller *et al.*, 2005; Deshmukh *et al.*, 2006), as well as because of the changes in microbial populations in the phyllosphere, the *Sebacinales* present considerable interests for biotechnological approaches (Oelmuller *et al.*, 2009). However, previous findings by Serfling *et al.* (2007) indicated that *P. indica* is not well suited for use in the field in Northern Europe. The identification the plant and fungal genetic markers involved in the interaction specificity would greatly improve the colonisation of the plant by the fungus. Similar strategies are already being patented between other fungal endophytes and grasses (e.g. US Patent 6815591).

CHAPTER 8: DISCUSSION

Worldwide, about 50% of barley production has been estimated to be lost due to pests and diseases (Oerke and Dehne, 2004). Crop protection is achieved through multiple strategies, but there is increasing pressure to develop new methods that are more ecologically-benign, sustainable and integrated with food production requirements. Managing the phyllosphere is emerging as a potential way to reduce yield loss due to pathogens, by promoting microbial communities that are beneficial to crops and detrimental to disease causal agents. This work focused on the bacterial phyllosphere of barley, a crop of high economic value in Scotland, and identified how some of its constituents could affect diseases and how it was affected by various factors in the field. Finally, the potential to manipulate the phyllosphere will be discussed.

8.1 THE PHYLLOSPHERE

The phyllosphere of various plants, including *Arabidopsis thaliana*, trees and crops (Ercolani, 1991; Kniskern *et al.*, 2007; Gu *et al.*, 2010), has been studied previously, but the barley phyllosphere has not been extensively assessed as yet. A great number of different microbial species live on leaves. Bacteria have been described as the most common (Lindow and Brandl, 2003), but fungi (yeast and filamentous fungi) also represent a considerable part of the leaf microflora, especially on old leaves (Thompson *et al.*, 1993). Late in the growing season, the isolation and quantification of culturable microorganisms on field-grown barley leaves revealed that bacteria and yeast were the most common microorganisms (nearly 10^6 cfu/g_{FW} together) and filamentous fungi were present at a lower level (Chapter 3). The culturable bacterial community was largely dominated by

Pseudomonas species and one kind of *Erwinia* species, as was observed on other plants (Ercolani, 1991; Thompson *et al.*, 1993; Legard *et al.*, 1994).

However, culturable microorganisms are only a fraction of the whole microbial population. Using a molecular fingerprinting DNA technique, namely T-RFLP, the barley phyllosphere was estimated to comprise on average 13 and 16 OTUs living as epiphytes and endophytes respectively (Chapter 3). Many were present in both populations, suggesting an extensive colonisation of the phyllosphere by bacteria. The *Pseudomonas* genus and *Erwinia* were detected as two OTUs that represented up to 24% of the bacterial community (Chapter 3). Thus, the barley phyllosphere is a rich environment comprising multiple bacterial genera and species.

Living in the phyllosphere requires many adaptive traits in the microorganisms. Water and nutrients are considered as the two main limiting growth factors on the leaf surface (Andrews and Harris, 2000; Mercier and Lindow, 2000). Therefore, microbes have evolved mechanisms to accumulate effectively, incorporate and attract water and nutrients. All isolated bacteria from field-grown barley leaves exhibited swimming motility *in vitro* and some other species were able to swarm and develop biofilms (Chapter 4). Another bacterium, the potato pathogen *Pectobacterium atrosepticum,* the presence of which on barley was previously observed (Newton *et al.*, 2004) and validated here using molecular detection (Chapter 4), was also able to actively colonise the barley phyllosphere (Chapter 4) and demonstrated motility and biofilm formation (Toth, 2010).

Overall, out of all leaf-associated microbes, only a few species become pathogenic. Some of the isolated bacteria from the barley phyllosphere, including *Pseudomonas syringae, Pseudomonas fluorescens* and *Pectobacterium atrosepticum*, are well-known phytopathogens but not on barley (Hirano and Upper, 2000;

Perombelon, 2002; Cui *et al.*, 2005). The interactions between plants and microorganisms can vary a lot from beneficial/symbiotic to parasitic/pathogenic and a similar microbe can have contrasting effects on different plants (Newton *et al.*, 2010a). The ability of these microorganisms to survive and multiply in non-host environments, such as non-host plants, the soil or the water cycle (Perombelon and Hyman, 1989; Franc and Demott, 1998; Morris *et al.*, 2008), was demonstrated to be linked to the development of their pathogenicity (Morris *et al.*, 2010).

8.2 MICROBIAL INTERACTIONS IN THE PHYLLOSPHERE

The scope of *in planta* interactions between microorganisms and disease causal agents ranges from antagonistic (i.e. BCA) to synergistic (i.e. "helper"). The same microbe can have contrasting effects on disease development. The opportunistic potato pathogen, *Pectobacterium atrosepticum*, was previously correlated with a reduction of *R. commune* development on field-grown barley, and an increase of *Septoria tritici* and *Blumeria graminis* f.sp. *tritici* infection on field-grown wheat (Newton *et al.*, 2004). This bacterium as well as another one isolated from the barley phyllosphere, *Pseudomonas syringae*, showed biocontrol properties against *R. commune in vitro* (Chapter 4). These results could not be confirmed *in planta*, probably because of the experimental procedures that did not permit enough bacteria to interact with the fungal spores. However, *P. atrosepticum* had demonstrated "helper" activity by significantly promoting the development of *B. graminis* f.sp. *hordei* on barley leaves, corroborating the observations made on wheat by Newton *et al.* (2004).

The mechanism involved in the biocontrol of *P. atrosepticum* against *R. commune* has been sought using a selection of pathogenicity mutants (Chapter 4), in

which non-functional mutations significantly reduced the pathogenicity of the bacteria on potato (Bell *et al.*, 2004; Holeva *et al.*, 2004; Liu *et al.*, 2008; Toth, 2010). Observations implied that it was not a single mechanism that was involved in the biocontrol process, but rather multiple ones. The dual-inoculation assay suggested that a diffusible compound participated in the reduction of the growth of *R. commune in vitro*. The bacterium *P. atrosepticum* can produce various antibiotics and toxins. One of them, phenazine, was previously demonstrated to reduce the development of cankers on cypress (Raio *et al.*, 2011), but no mutants impaired in toxin biosynthesis have had a significantly different effect on *R. commune* growth *in vitro*, compared to the effect of the WT isolate (Chapter 4). Therefore, this would suggest the presence of another, but as yet unidentified, diffusible compound.

The absence of a functional type IV secretion system (T4SS) significantly reduced *in vitro* the biocontrol effectiveness of *P. atrosepticum* against *R. commune* growth (Chapter 4). This system is involved in the secretion of large molecules (proteins and DNA) in the environment or directly into eukaryotes (Bundock *et al.*, 1995; Christie, 2001). The T4SS of *Agrobacterium tumefaciens* is probably the best known and described system and is used by the bacteria to inject an infectious plasmid in the plant leading to crown gall disease (Christie, 2001). In *P. atrosepticum*, the T4SS participates in its pathogenicity (Bell *et al.*, 2004), but its secretome is unknown. The role of the T4SS of *P. atrosepticum* on *R. commune* biocontrol was detected using a dual-inoculation assay, implying that the mutant could not secrete the active compound responsible for the hindered fungal growth. However, direct contact between the bacterium and the fungal spore was observed (Chapter 4) and the T4SS could be involved in other biocontrol mechanisms.

In conclusion, different bacteria demonstrated biocontrol activities against *R. commune* and multiple mechanisms were involved in this process. However, contrasting effects could be observed on other diseases (Chapter 4; Newton *et al.*, 2004). Using a single microorganism as a BCA may not confer broad resistance, and introducing multiple BCAs at the same time has had distinct results, from synergistic to antagonistic (Denoth *et al.*, 2002). Hence, rather than introducing one or multiple non-endemic microbes in an established community, manipulating the plant innate microflora and/or its microenvironment could be a more sustainable solution. The effects of current crop protection practices on the plant-associated microorganisms have to be fully assessed, to identify mean to manipulate or control changes in the phyllosphere.

8.3 FACTORS AFFECTING THE PHYLLOSPHERE STRUCTURE

The first physico-chemical barrier that microorganisms encounter on the leaf surface is the epicuticular wax layer (Juniper and Jeffree, 1983). Glossy mutants, i.e. with altered epicuticular wax layers, have shown significantly reduced yield and contrasting disease susceptibility: the resistance degrees to *R. commune* and *B. graminis* were hindered and improved respectively (Chapter 6). The chemical composition was shown to affect the early infection steps of *B. graminis* (Carver and Thomas, 1990; Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2011), but the GC-MS analysis has not been completed and the changes in susceptibility could not be related to changes in the wax chemical composition. However, the phyllosphere composition could also have participated in the altered susceptibility. Even though the overall structure was not affected, a shift in the main OTUs occurred (Chapter 6). One of the reduced ones (203 OTU) on glossy leaves corresponded to the

Pseudomonas clade (Chapter 3), which has shown biocontrol activity against *R. commune* (Chapter 4).

The composition of the phyllosphere was significantly different between cultivars. Eight barley cultivars with a wide range of susceptibility to leaf scald and powdery mildew were grown under field conditions. A negative correlation was observed between the bacterial richness and the amount of scald symptoms observed, the most damaging disease present in the field at the time (Chapter 5). A contrasting correlation was observed on maize, where varieties genetically identified as susceptible to Southern Leaf Blight had a high bacterial diversity (Balint-Kurti *et al.*, 2010). Whether or not the observed correlation is linked with the resistance genes present in each barley cultivars or due to the presence of *R. commune* on the leaves is unknown, but such traits could potentially add a valuable extra protection.

If the crop genotype can influence the phyllosphere populations, the interaction with microorganisms of interest could also be manipulated and enhanced. Various root-associated microbes, including fungi and bacteria, have been shown to promote crop nutrition and protection (Waller *et al.*, 2005; Castellanos-Morales *et al.*, 2011; Henkes *et al.*, 2011; Fröhlich *et al.*, 2012). One of them, *Piriformospora indica,* which belongs to the *Sebacinales* order, has been shown to increase the plant yield and fresh weight, promote resistance against *B. graminis* and slow down *R. commune* progress (Chapter 7; Waller *et al.*, 2005). The relative abundance of the main OTUs in the phyllosphere of *P. indica*-infected plants was also significantly altered. The 370 OTU comprised of *P. atrosepticum*, which can actively colonise the phyllosphere and promote *B. graminis* infection (Chapter 4). Its relative abundance was reduced in *P. indica*-infected barley leaves. The mechanisms involved in the

systemic induced resistance are unknown, but a modification of the phyllosphere structure could participate in the reduced susceptibility.

Crop protection in the field is commonly achieved by using resistant cultivars and applying agro-chemical treatments. The crop genotype has been shown to manipulate the bacterial leaf-associated communities (Chapter 5). Here, the effect of conventional fungicides was compared to the effect of an elicitor mixture on non-target microorganisms. Both applications could modify the structure of the non-target microorganisms in the phyllosphere (Chapter 5). The elicitor mixture, developed by Walters *et al.* (2011), was composed of three analogues of phytohormones (SA, JA and GABA), that showed an enhanced protection against leaf scald and powdery mildew on barley. The fungicide induced an increase of the bacterial diversity to a similar level to the diversity observed on elicitor-treated leaves and resistant barley cultivars (Chapter 5). Similar results were also observed on field-grown wheat (Gu *et al.*, 2010). Whether or not a high diversity participated in the disease resistance is not clear, but the fungicide application achieved a better protection than the elicitor mixture.

8.4 SUCCESSFULLY MANAGING THE PHYLLOSPHERE

Even though interests in the phyllosphere are growing, successful manipulation of the leaf-associated communities is far from being achieved at the moment. Probably the only example of effective, sustainable and applied microflora modification is the treatment against recurrent antibiotic-associated diarrhoea in the human gut, caused by *Clostridium difficile*. Infection with *C. difficile* results in extensive colonisation of the intestines by the bacterium and significant changes in the microflora composition (Grehan *et al.*, 2010; Khoruts *et al.*, 2010). To cure the

patient, the gut microflora has to be entirely wiped out with high levels of antibiotic and a faecal transplant from a healthy related donor is carried out. The faecal transplant replaces the "diseased" bacterial population with a "healthy" one and the disease recedes and finally disappears (Khoruts *et al.*, 2010). The establishment of the healthy populations is also durable (Grehan *et al.*, 2010). Such replacement of microflora in plants is not possible, but other potential strategies have emerged to manipulate it.

The microbial colonisation of the phyllosphere is very dynamic (Kinkel, 1997; Beattie and Lindow, 1999) and changes over time (Redford and Fierer, 2009). The main bacteria composing the barley phyllosphere were present in all barley cultivars, but their relative abundance differed (Chapter 5). The Theory of Island Biogeography, describing the microbial colonisation of the leaf surface (Andrews and Harris, 2000), implies that the first microorganisms that meet all required conditions to grow and duplicate, are likely to be the most successful colonisers. However, various microbes may have redundant roles. Burke *et al.* (2011) determined that even though the bacterial communities associated with the green macroalga *Ulva australis* were very variable, their overall expressed functional genes were very similar. On various leaves, proteomic analyses revealed that microbial enzymes involved in the carbon and nitrogen assimilation were highly abundant across bacterial species and other abundant proteins of unknown function were also consistently detected in various genera (Delmotte *et al.*, 2009). Even though the roles, functions and effects of all microorganisms in the phyllosphere are beginning to be understood, the communities were shown to be affected by various anatomical, physiological and genetic characteristics of the plant.

Indirect manipulation the microbial colonisation by modification of the leaf surface properties should be wisely considered. The epicuticular waxes, the interface between the plant and the environment, fulfil numerous essential roles to plants in controlling water loss and photodamage (Shepherd and Griffiths, 2006). Even though the modification in abundance of certain OTUs could help the protection against foliar diseases, the glossy phenotype still resulted in a detrimental yield loss (Chapter 6). Hence, rather than disrupting the integrity of the epicuticular wax layer, an alternative solution would be to select in new cultivars for lower amounts of waxy chemicals known to trigger germination of *B. graminis* spores (Hansjakob *et al.*, 2010). It would be necessary though to investigate the effect of the absence of one wax constituent on other disease causal agents and how those pathogens are also affected by wax chemical components. Other anatomical factors, such as the number and kind of trichomes, can also modify some properties of the leaf surface, including its roughness and wettability (Brewer *et al.*, 1991; Sato and Tadeka, 1993). This would influence the phyllosphere colonisation (Chapter 5; de Costa *et al.*, 2006a), but the trichome density is also significantly affected by environmental factors (Perez-Estrada *et al.*, 2000). Overall, modifying the leaf surface would affect the initial colonisation step of new arriving microorganisms in the phyllosphere, but a detrimental microbe could still manage to infect the plant.

However, there is growing evidence that the phyllosphere is directly controlled by the plant genotype. Observations on barley and maize have been contradictory (Chapter 5; Balint-Kurti *et al.*, 2010), but epiphytes and endophytes were already shown not to respond similarly to treatments (Kniskern *et al.*, 2007). A genetic control of the phyllosphere implies that traits could be identified, selected and bred into cultivars of interest. The selection of such traits has to be carefully

chosen though. Maize breeding programme has naturally selected lines with higher levels of toxins that are ineffective against *Fusarium* pathogens (Saunders and Kohn, 2009). These toxins may give a colonisation advantage to *Fusarium* pathogens by limiting the growth of other fungal competitions.

However, the genetic control of the plant-microbe interaction is of particular interest for known beneficial microorganisms. The most described root-associated fungal symbiont, AMF, poorly interacts with barley roots (Grace *et al.*, 2009), but *P. indica* colonisation results in multiple beneficial improvements (Chapter 7; Waller *et al.*, 2005). The colonisation of barley roots by *P. indica* was demonstrated for the first time to be cultivar-dependent (Chapter 7). Although the endophyte was shown not to be effective in the field in Europe (Serfling *et al.*, 2007), numerous other fungal species of the same *Sebacinales* order have demonstrated beneficial effects on barley (Deshmukh *et al.*, 2006; Waller *et al.*, 2008). The identification of the traits involved in the control of the interaction between *P. indica* and barley roots is very valuable. Various plant genes have already been identified as involved in the colonisation of root symbionts (Parniske, 2008), but their role in *P. indica* colonisation has not been assessed yet. The breeding of the meadow fescue, *Festuca pratensis*, with a particular strain of symbiotic endophyte, *Neotyphodium siegelii*, has already been patented: US Patent 6815591, 6111170 and 6072107. Similar symbiont-host breeding could be achieved between other crops of high economic interest and other symbionts, such as the AMF. The early plant colonisation of the symbiont could also be achieved by coating the seeds with the endophyte of interest, but this technique still requires optimisation (Walsh *et al.*, 2001).

The multiple and various interactions between microorganisms and with the plant make it impossible to control it entirely. A balance that compromises reduced

susceptibility to diseases and improved plant fitness has to be reached and coupled with other crop protection methods (agro-chemical compounds and phytosanitary practices) to limit selection pressure on pathogens and improve the sustainability of crop protection strategies. However, the agro-chemical treatments would have to alter as little as possible the established microbial community. The phyllosphere bacterial communities are already greatly influenced by the applied fertilisers. The symbiosis with AMF was shown to be repressed by the plant when phosphorus was abundant (Nagy *et al.*, 2009) and many bacteria were demonstrated to be able to assimilate nitrogen (Delmotte *et al.*, 2009).

Both fungicide and elicitor applications increased the bacterial diversity (Chapter 5), but fungicides have a much more negative effect than elicitors on the whole phyllosphere. Repeated applications of fungicide disturb the whole plant-associated fungal ecology and reduce non-target bacteria up to 10,000 fold (Chapter 5; Walter *et al.*, 2007). Elicitors though did not affect the bacterial load associated with the leaves (Kniskern *et al.*, 2007). Thus it was hypothesised that elicitor would not affect the association with endophytic symbionts (Newton *et al.*, 2010b). The foliar application of an SA analogue was shown to affect the root colonisation of an AMF fungus on soybean, but only transiently (de Roman *et al.*, 2011). Hence, a combination of plant-symbiont breeding with adapted elicitor applications could achieve similar protection and yield gain to that obtained with conventional fungicide treatments, but more studies are needed to confirm whether this strategy is economic, long-lasting, efficient compared to current crop protection practices..

Finally, this work focused on bacteria living in and on leaves alone, because they were described as the most prevalent kind of microorganisms in the phyllosphere (Lindow and Brandl, 2003). However, other microorganisms need to be

further studied. The culturable analysis of the phyllosphere composition revealed that there were nearly as many yeast as bacteria (Chapter 3). Most of the isolated yeast from field-grown barley leaves improved *R. commune* infection when co-inoculated under controlled conditions (Fountaine *et al.*, 2009). Saprohytic fungi were also shown to increase the leaf senescence on wheat (Bertelsen *et al.*, 2001). Hence, the fungal phyllosphere and its modification by treatment application will have to be further investigated in the future. Human pathogens will have to be closely monitored as well (Brandl, 2006). The presence of enteric bacteria was detected in the experimental barley field (Holden, 2010). Managing the phyllosphere by limiting microbiocidal treatments and promoting microbial interactions may create opportunities for the survival and spread of such organisms on the crop and the harvest and will have to be fully assessed.

In conclusion, the phyllosphere is a complex and dynamic habitat that requires further studies. The full understanding of the interactions occurring between disease causal agents and their surrounding microflora will facilitate the development of more integrated and sustainable crop protection methods in the future.

8.5 CONCLUSIONS

- The phyllosphere of field-grown barley plants was identified as a rich habitat composed of multiple microorganisms;
- The dominating culturable bacteria were from the *Pseudomonas* genus and the most common one, *P. syringae*, demonstrated biocontrol activities against *R. commune in vitro*;
- Another bacterium, *Pectobacterium atrosepticum*, an opportunistic potato pathogen, could actively colonise the barley phyllosphere, inhibit the *in vitro* growth of *R. commune* and promote powdery mildew infection;
- The biocontrol mechanisms of *P. atrosepticum* against *R. commune* involved a secretion system (the T4SS) and diffusible compounds that were none of the toxins tested (phenazine, coronafacic acid and two other potential toxins);
- A root-associated endophyte, *Piriformospora indica*, known to increase plant fitness and resistance to powdery mildew, was demonstrated for the first time to temporarily impair *R. commune* colonisation;
- The endophytic colonisation was dependent on the barley genotype, which means that the symbiosis could be improve by selecting promoting traits;
- In the field, the percentage of scald symptoms observed on different cultivars was negatively correlated with the endophytic bacterial diversity;
- Mutations affecting the leaf surface significantly reduced yield and modified the phyllosphere, but differentially altered resistance to different pathogens;
- Both fungicides and elicitors promoted bacterial diversity, which could have helped to control scald disease and fungicides achieved better protection against *R. commune* than elicitors.

CHAPTER 9: REFERENCES

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CHAPTER 10: APPENDIX

10.1 FIELD AND GLASSHOUSE EXPERIMENTS

10.1.1 Field Plans

10.1.1.1 Lab Field 2010-2011

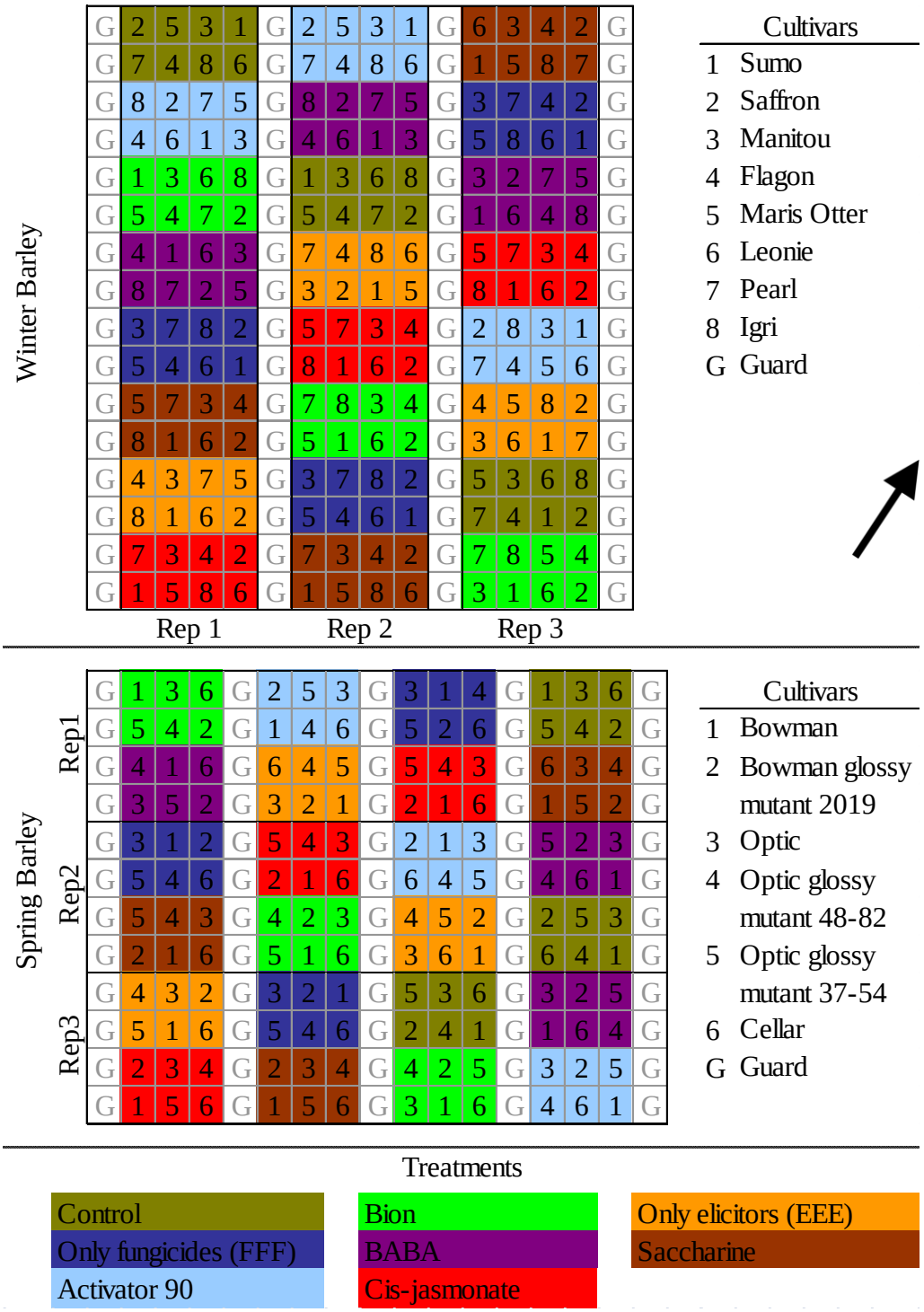

Figure 10.1 (previous page): Field plans of the Lab Field in 2010-2011 of both winter and spring barley including eight and six cultivars respectively according to a triplicate split-plot design covering a selection of eight different treatments. Arrow points towards north.

10.1.1.2 Lab Field 2009-2010

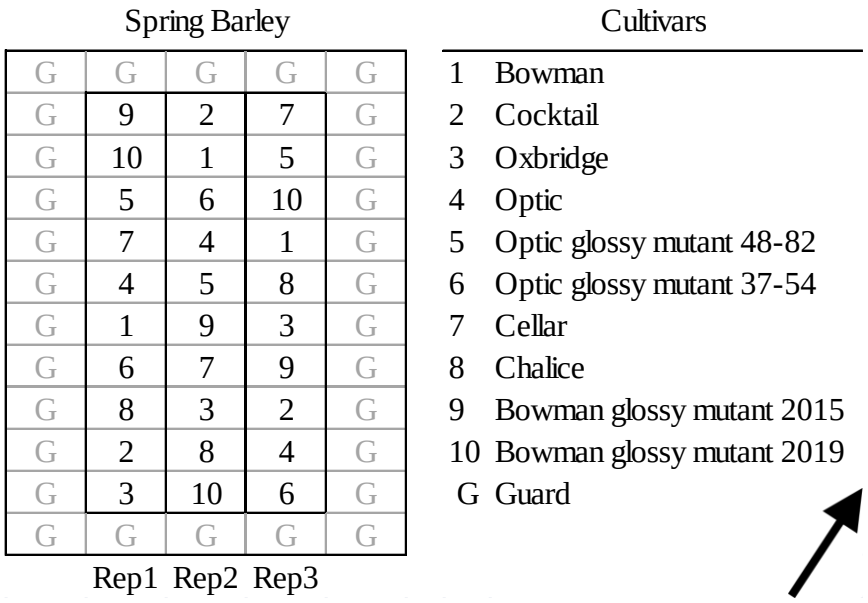

Figure 10.2: Field plan of the Lab Field in 2009-2010 including 10 cultivars of spring barley. Arrow points towards north.

Figure 10.3: Field plan of the Lab Field in 2009-2010 including 10 treatments on eight cultivars according to a triplicate split-plot design for winter barley. Arrow points towards north.

10.1.1.3 Lab Field 2008-2009

Winter-Sown Cultures

G	8	11	11	G
G	13	4	7	G
G	14	6	10	G
G	15	16	2	G
G	6	12	5	G
G	9	5	15	G
G	16	14	9	G
G	4	7	13	G
G	3	1	1	G
G	10	9	14	G
G	2	2	8	G
G	1	8	6	G
G	11	15	4	G
G	12	13	12	G
G	5	10	16	G
G	7	3	3	G

Rep1 Rep2 Rep3

Cultivars

1 Bowman
2 Coctail
3 Oxbridge
4 Optic
5 Optic glossy mutant 48-82
6 Optic glossy mutant 37-54
7 Cellar
8 Chalice
9 Sumo
10 Saffron
11 Manitou
12 Flagon
13 M.Otter
14 Leonie
15 Pearl
16 Igri
G Guard

Spring Barley

Rep1	G	2	5	G
	G	7	3	G
	G	1	6	G
	G	4	9	G
	G	10	8	G
Rep2	G	9	10	G
	G	3	5	G
	G	2	7	G
	G	1	6	G
	G	8	4	G
Rep3	G	9	7	G
	G	10	2	G
	G	3	8	G
	G	5	4	G
	G	6	1	G
	G	G	G	G

Cultivars

1 Bowman
2 Coctail
3 Oxbridge
4 Optic
5 Optic glossy mutant 48-82
6 Optic glossy mutant 37-54
7 Cellar
8 Chalice
9 Bowman glossy mutant 2015
10 Bowman glossy mutant 2019
G Guard

Figure 10.4: Field plans of the Lab Field in 2008-2009 including 16 winter-sown barley cultivars (eight WB and eight SB) as well as 10 SB cultivars according to a triplicate split-plot design. Arrow points towards north.

10.1.1.4 Rhynchosporium *Nursery 2010-2011*

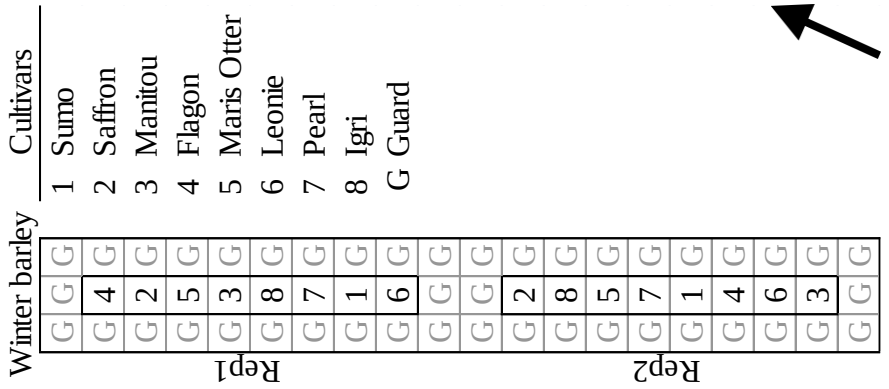

Figure 10.5: Field plan of winter barley in the *Rhynchosporium* nursery (H14) in 2010-2011 including eight cultivars in duplicate. Arrow points towards north.

10.1.1.5 Rhynchosporium *Nursery 2009-2010*

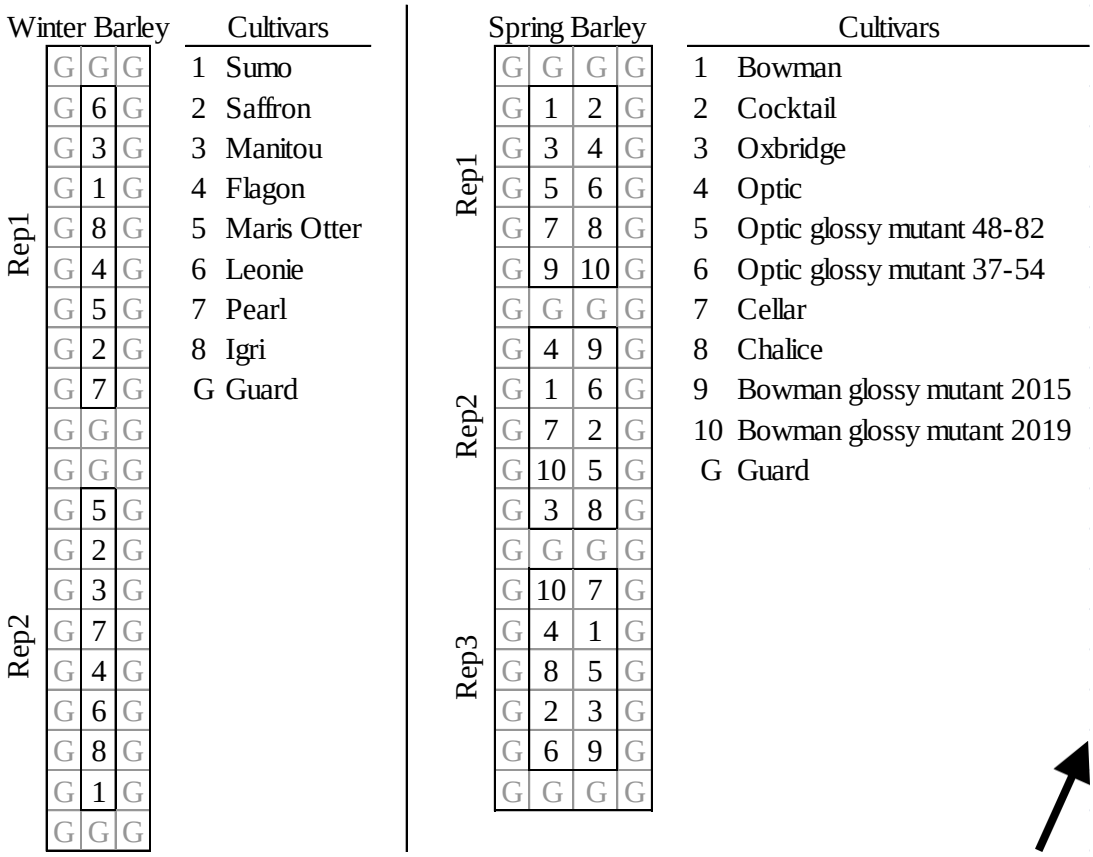

Figure 10.6: Field plans of winter and spring barley in the *Rhynchosporium* nursery (H14) in 2009-2010 including eight and 10 cultivars respectively in duplicate for WB and triplicate for SB. Arrow points towards north.

10.1.1.6 Rhynchosporium *Nursery 2008-2009*

Winter-Sown Barley

G	G	G	G	G
G	G	G	G	G
G	2	15	11	G
G	7	8	16	G
G	6	3	13	G
G	8	13	4	G
G	12	9	9	G
G	14	7	10	G
G	1	6	1	G
G	15	10	12	G
G	5	11	8	G
G	11	2	15	G
G	13	4	2	G
G	10	12	6	G
G	4	14	7	G
G	9	5	3	G
G	16	1	14	G
G	3	16	5	G
G	G	G	G	G
G	G	G	G	G

Rep1 Rep2 Rep3

Cultivars

1	Bowman
2	Coctail
3	Oxbridge
4	Optic
5	Optic glossy mutant 48-82
6	Optic glossy mutant 37-54
7	Cellar
8	Chalice
9	Sumo
10	Saffron
11	Manitou
12	Flagon
13	Maris Otter
14	Leonie
15	Pearl
16	Igri
G	Guard

Figure 10.7: Field plan of the *Rhynchosporium* nursery in 2008-2009 including 16 winter-sown barley cultivars (eight WB and eight SB) according to a triplicate split-plot design. Arrow points towards north.

10.1.2 Field Treatments

Table 10.1: List of fertiliser and herbicide treatments applied in the fields on WB and SB. Treatment time is only indicative, as application can be changed according to the weather conditions and / or the development on the crop.

	Treatment	Commodity	Month	Rate/ha
Winter Barley	N-P-K fertiliser	0-20-30	September	0.3 t
	Herbicide	Trump	October	5 L
	Foliar Mn spray	Manganese	November	3 L
	1st N top-dress	30-0-0-(7.5 SO_3)	February	0.17 t
	2nd N top-dress	30-0-0-(7.5 SO_3)	March	0.23 t
	Herbicide	Traton SX	March	45 g
	Herbicide	Duplosan	March	1 L
	Foliar Mn spray	Manganese	April	3 L
Spring Barley	N-P-K fertiliser	22-4-14-(7.5 SO_3)	March	0.35 t
	Herbicide	Stomp 400	March	5 L
	N top-dress	22-4-14-(7.5 SO_3)	April	0.15 t
	Foliar Mn spray	Manganese	May	3 L
	Herbicide	Traton SX	May	45 g
	Herbicide	Duplosan	May	1 L

10.1.3 SCRI Compost

Peat	1,200 L
Sand	400 L
Perlite	100 L
Dolomite limestone	2.5 kg
Ground limestone	2.5 kg

Synchrostart fast release fertiliser (12 N, 14 P, 24 K, 3 MgO) and micronutrients (B, Cu, Fe, Mn, Mo, Zn) and containing Intercept insecticide

1.5 kg

10.2 MICROBES GROWTH MEDIUM

10.2.1 CzV8CM Medium

Oxoid Czapek-Dox	56 g
Agar	10 g
V8 juice	200 mL
Calcium carbonate	4 g
Complement supplement	50 mL

Make up to 1 L with distilled water and autoclave.

The complete supplement contains the following chemicals:

Casein hydrolysate	20 g
Myc. Peptone	20 g
Yeast extract	20 g
Adenine [dissolved in 100 mL 1 N HCl]	3 g
Biotin	0.02 g
Pyridoxine	0.02 g
Thiamine HCl	0.02 g
Para-aminobenzoic acid	0.02 g
Nicotinic acid	0.02 g

Make up to 1 L with distilled water, adjust to pH 7.0 with concentrated NaOH and autoclave.

10.2.2 Potato Dextrose Broth (PDB)

Potato dextrose broth	26.5 g

Make up to 1 L and autoclave.

10.2.3 Luria-Bertani (LB) Broth and Agar

Select peptone	10 g
Select yeast	5 g
NaCl	10 g
Select agar (only for LB Agar)	15 g (1.5% for normal bacterial growth)
	6 g (0.6% for swarming motility test)
	3g (0.3% for swimming motility test)

Make up to 1 L, adjust to pH 7.5 with concentrated NaOH and autoclave.

10.2.4 Nutrient Agar (NA)

Oxoid Nutrient Broth	13 g
Agar	15 g

Make up to 1 L and autoclave.

10.2.5 Antibiotics

After solubilisation, the antibiotic solutions were filter-sterilised via a 0.2 μm membrane.

10.2.5.1 Streptomycin

Stock solution preparation:	1.0 g in 5 mL SDW	(200 mg/mL)
In growth medium:	0.5 mL in 1 L medium	(100 μg/mL)

10.2.5.2 Ampicillin

Stock solution preparation:	1.0 g in 5 mL SDW	(200 mg/mL)
In growth medium:	0.5 mL in 1 L medium	(100 μg/mL)

10.2.5.3 Kanamycin

Stock solution preparation:	1.0 g in 10 mL SDW	(100 mg/mL)
In growth medium:	0.5 mL in 1 L medium	(50 μg/mL)

10.3 MOLECULAR BIOLOGY

10.3.1 Stock Solutions

Before usage, all solutions were sterilised by autoclaving them (high pressure steam at 121°C for 30 min).

NaCl (5 M)	29.22 g	in 100 mL
EDTA (ethylenediaminetetraacetic acid) (0.5 M, pH 8.0)		
	14.61 g	in 75 mL

Increase the pH to 8.0 by adding NaOH pellets to dissolve completely the EDTA and make up to 100 mL.

Tris HCl (1 M, pH 8.0)	12.11 g	in 100 mL

Lower the pH to 8.0 by adding HCl.

K_2HPO_4 (1 M)	174.2 g	in 1 L
KH_2PO_4 (1 M)	136.1 g	in 1 L

10.3.2 Potassium Phosphate Buffer (PPB)

K_2HPO_4 (1 M)	61.5 mL
KH_2PO_4 (1 M)	38.5 mL

Make up to 1 L and autoclave. The PPB should have a pH of 7.0.

10.3.3 CetylTrimethylammonium Bromide (CTAB)-Based DNA Extraction Buffer

CTAB	4 g
NaCl (5 M)	56 mL
EDTA (0.5 M, pH 8.0)	8 mL
Tris HCl (1 M, pH 8.0)	20 mL
PVP (polyvinylpyrrolidone)	2 g

Make up to 200 mL and autoclave.

10.3.4 TEN Buffer

Tris HCl (1 M, pH 8.0)	50 mL
EDTA (0.5 M, pH 8.0)	50 mL
NaCl	14.1 g

Make up to 500 mL with distilled water and autoclave.

10.3.5 Phenanthroline-Based DNA Extraction Buffer

The buffer has to be freshly made for DNA extraction; hence quantities will depend on number of samples. Preparation for 10 samples is used here as an example. At all times the solution has to stay under the fume cabinet as phenanthroline and β-mercapto-ethanol are extremely poisonous.

TEN buffer (Appendix 10.3.4)	5 mL	
Phenanthroline	5 mg	0.1% (w/v)

Place on heated stirrer for 15 min until phenanthroline is dissolved.

β-mercaptoethanol	5 µL	0.1% (v/v)

PVP	0.1 g	2% (w/v)

Place on heated stirrer until totally dissolved.

10.3.6 Tris-Borate-Ethylene Diamine Tetraacetic (TBE) Buffer (10X)

Tris	108.0 g
Orthoboric acid	55.0 g
EDTA	9.3 g

Make up to 1 L with distilled water and autoclave

All electrophoresis gels were stained with ethidium bromide (0.5 μg/mL final concentration) and observed under a transilluminator (UVItec, UK).

ANNEX

A.1: DATABASE OF PHYLLOSPHERE MICROORGANISMS

A.1.1 Bacteria Identified in the Phyllosphere

Table A.1: (previous page) List of leaf-associated bacteria identified in the literature with their 16S rRNA gene. The bacteria, identified in a selection of articles numbered from 1 to 7 (see Table A.3), were organised by phylum, class, genus and species. As many complete 16S rRNA gene sequences as possible were gathered from this selection and accession numbers are listed on the right hand side.

Phylum / *Class* / *Genus* / *Species*	References						
	1	2	3	4	5	6	7
Actinobacteria							
Actinobacteria							
Aeromicrobium				1			
Agreia							
A. pratensis			1				
Agromyces							
A. salentinus			1				
Arthrobacter			1	1			
A. aureus		1					
A. crystallopoietes		1					
A. globiformis	1	1					
A. mysorens		1					
A. oxydans		1					
A. protophormiae		1					
A. ureafaciens		1					
A. viscosus		1					
Cellulomonas				1			
C. flavigena	1						
Cellulosimicrobium				1			
C. cellulans		1					
Clavibacter	1			1			
C. michiganensis		1	1				
Corynebacterium							
C. bovis		1					
Curtobacterium				1			
C. fangii			1				
C. flaccumfaciens		1	1				
C. herbarum			1				
C. plantarum	1						

Phylum / *Class* / *Genus* / *Species*	References						
	1	2	3	4	5	6	7
Actinobacteria							
Actinobacteria							
Friedmanniella				1			
Frigoribacterium			1	1			
Geodermatophilus				1			
Janibacter				1			
Kineococcus			1				
Kineosporia				1			
Knoellia				1			
Kocuria				1			
K. kristinae		1					
K. rosea		1					
Leifsonia				1			
Microbacterium				1			
M. barkeri		1					
M. esteraromaticum		1					
M. lacticum		1					
M. liquefaciens		1					
M. saperdae		1					
Micrococcus	1						
M. luteus	1	1					
M. lylae		1					
Mycobacterium				1			
Nakamurella				1			
Nocardia				1			
Nocardioides				1			
Oerskovia							
O. turbata		1					
Pseudoclavibacter							
P. helvolus			1				
Rathayibacter				1			
Rhodococcus				1			
R. corynebacterioides			1				
R. erythropolis			1				
R. rhodochrous		1					
Sinomonas							
S. atrocyanea		1					
Streptomyces							
S. chartreusis			1				
Terracoccus				1			

Phylum / Class / Genus / Species	References						
	1	2	3	4	5	6	7
Bacteroidetes						1	
Flavobacteria						1	
Chryseobacterium				1			
C. balustinum		1					
C. indologenes		1					
Flavobacterium			1	1			
F. johnsoniae		1					
Sphingobacteria							
Sphingobacterium							
S. multivorum		1					
Deinococcus-Thermus							
Deinococci							
Deinococcus				1		1	
Firmicutes							
Bacilli							
Bacillus	1			1			
B. aminovorans		1					
B. cereus			1				
B. megaterium	1	1	1				
B. mycoides			1				
B. pumilus		1			1		
B. subtilis	1						
Clostridium						1	
C. bifermentans					1		
Enterococcus							
E. faecium	1						
Exiguobacterium						1	
Kurthia							
K. zopfii		1					
Lactobacillus							
L. plantarum	1						
Leuconostoc							
L. dextranicum	1						
Paenibacillus				1		1	
P. pabuli		1	1				
P. polymyxa		1					
P. validus		1					
Sporosarcina							
S. globispora		1					
Staphylococcus				1			
S. haemolyticus		1					
S. hominis		1					
Weissella							
W. hellenica						1	

Phylum / *Class* / *Genus* / *Species*	References						
	1	2	3	4	5	6	7
Proteobacteria						1	
Alphaproteobacteria						1	
Acetobacter							
A. aceti	1						
Agrobacterium							
A. rubi		1					
A. tumefaciens		1	1				
Blastomonas				1		1	
Brevundimonas				1			
B. vesicularis		1					
Caulobacter				1		1	
Gluconobacter							
G. oxydans	1						
Methylobacterium				1			
Ochrobactrum							
O. anthropi		1					
Orientia							
O. tsutsugamushi							1
Paracoccus				1			
Rhizobium				1			
R. radiobacter		1					
Sphingomonas			1	1		1	
S. adhaesiva					1		
Zymomonas							
Z. mobilis	1						
Betaproteobacteria						1	
Acidovorax				1			
A. delafieldii	1						
Alcaligenes							
A. faecalis	1						
Chromobacterium							
C. violaceum	1						
Comamonas							
C. testosteroni		1					
Deftia							
D. acidovorans		1					
Hydrogenophaga							
H. pseudoflava		1					
Janthinobacterium							
J. lividum		1					

Phylum / *Class* / *Genus* / *Species*	References						
	1	2	3	4	5	6	7
Proteobacteria							
Betaproteobacteria						1	
Massilia				1			
Oxalobacter				1			
Roseateles							
R. depolymerans						1	
Zoogloea				1			
Deltaproteobacteria							
Desulfurominas							
D. choroethenica					1		
Gammaproteobacteria						1	
Acinetobacter		1		1	1	1	
A. calcoaceticus		1					
A. haemolyticus					1		
A. lwoffii		1					
Aeromonas							
A. punctata		1					
A. salmonicida		1					
A. sobria		1					
Alishewanella				1			
Buchnera						1	1
B. aphidicola							1
Enterobacter							
E. asburiae					1		
E. amnigenus		1					
Erwinia	1			1			1
E. amylovora					1		
E. chrysanthemi		1					
E. persicinus			1				
E. rhapontici		1			1		
Escherichia							
E. coli	1						
Hafnia							
H. alvei		1					
Hamiltonella							
Candidatus H. defensa							1
Kluyvera							
K. intermedia		1					
Marinobacter							
M. hydrocarbonoclasticus					1		
Morganella							
M. morganii		1					

Phylum / Class / Genus / Species	References						
	1	2	3	4	5	6	7
Proteobacteria							
Gammaproteobacteria						1	
Pantoea				1		1	1
P. agglomerans	1	1			1		1
P. ananatis			1				
Pectobacterium							
P. carotovorum	1						
Pseudomonas				1			1
P. aeruginosa	1						
P. argentinensis							1
P. chlororaphis		1					
subsp. aureofaciens		1					
P. cichorii		1					
P. corrugata		1					
P. flavescens			1				
P. fluorescens	1	1	1				
P. marginalis		1					
P. mendocina		1					
P. oleovorans					1		
P. oryzihabitans		1					
P. poae			1				
P. pseudoalcaligenes		1					
P. putida		1			1		1
P. stutzeri		1					
P. syringae	1	1	1				
P. viridiflava		1	1				
Raoultella							
R. planticola	1	1					
R. terrigena	1	1					
Regiella							
Candidatus R. insecticola							1
Rheinheimera				1			
Serratia							
S. liquefaciens		1					
S. marcescens	1						
S. plymuthica		1					
Stenotrophomonas				1		1	
S. maltophilia		1					
Xanthomonas							
X. campestris	1		1				
Yersinia							
Y. enterocolitica		1					

A.1.2 Occurrence of Bacterial Classes in the Phyllosphere

Table A.2: Richness (bottom) and proportions (top) of leaf-associated bacterial classes identified in a selection of articles numbered from 1 to 7 (see Table A.3).

	Phylum	References						
	Class	1	2	3	4	5	6	7
Taxonomic proportion of bacteria	*Actinobacteria*	21	30	46	48			
	Bacteroidetes							
	Flavobacteria		4	4	4		11	
	Sphingobacteria		1					
	Deinococcus				2		6	
	Firmicutes (Bacilli)	21	13	14	7	15	22	
	Proteobacteria	(58)	(52)	(36)	(39)	(85)	(61)	(100)
	Alphaproteobacteria	10	6	7	15	8	22	9
	Betaproteobacteria	10	5		9		11	
	Deltaproteobacteria					8		
	Gammaproteobacteria	38	41	29	15	69	28	91
Number of different bacteria		29	79	28	46	13	18	11

A.1.3 Literature Used for the Phyllosphere Characterisation

Table A.3: List of articles used for the estimation of the phyllosphere composition. The extraction and identification methods as well as the plant used are listed.

Ref - Authors	Extraction Method	Identification Method	Host
1 - Ercolani (1991)	Culture	Phenotypic characterization	Olive tree
2 - Thompson *et al.* (1993)	Culture	Microbial identification systems Fatty acid methyl esters	Sugar beet
3 - Kniskern *et al.* (2007)	Culture	16S rDNA	*Arabidopsis thaliana*
4 - Enya *et al.* (2007)	Culture	16S rDNA	Tomato
5 - Yang *et al.* (2001)	DGGE	16S rDNA	Citrus
6 - Kadivar and Stapleton (2003)	Clone library	16S rDNA	Maize
7 - Gu *et al.* (2010)	Clone library and DGGE	16S rDNA	Wheat

A.2: PHYLOGENETIC ANALYSES OF CULTURABLE BACTERIA

A.2.1 Phylogenetic Analysis of Isolated *Pseudomonas* Bacterial Species

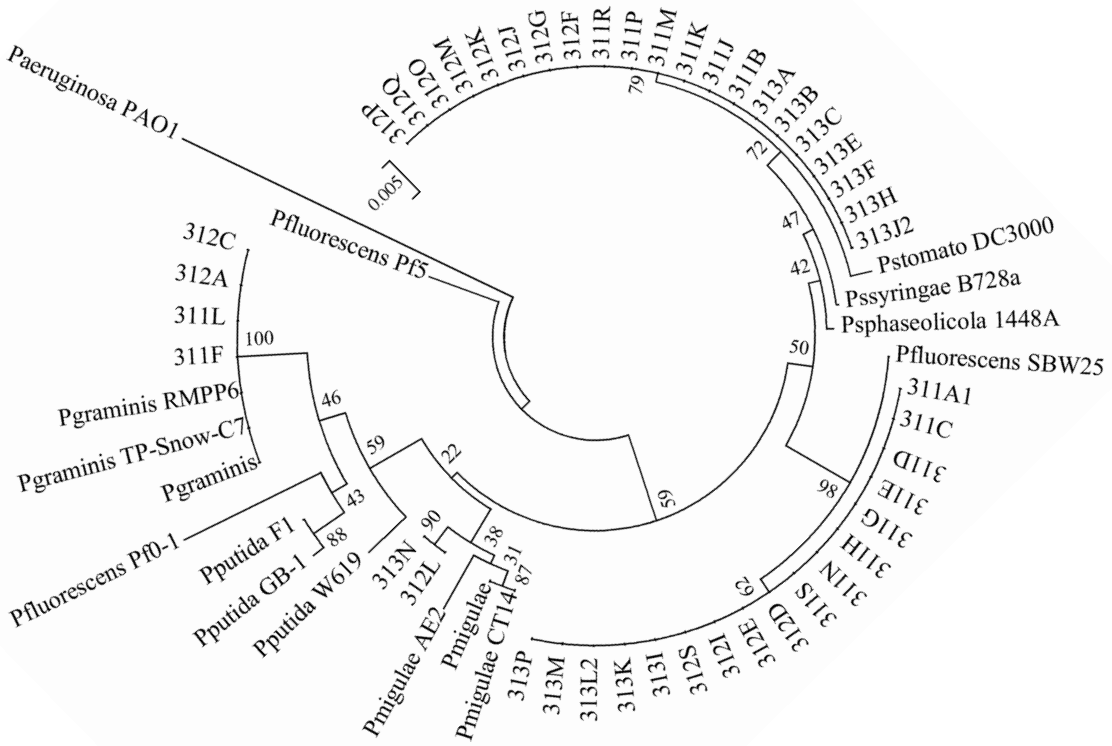

Figure A.1: Phylogenetic comparison of the 44 epiphytic bacterial isolates identified as belonging to the *Pseudomonas* genus with various known *Pseudomonas* species. The phylogenetic tree was carried out using the maximum-likelihood algorithm (bootstrap = 100) and rooted with *P. aeruginosa*.

Table A.4: List of the *Pseudomonas* (*P.*) species used for the phylogenetic analysis and their 16S rDNA accession number.

Bacterial species	16S rDNA accession number	Bacterial species	16S rDNA accession number
P. aeruginosa PAO1	NC_002516	*P. migulae* AE2	EF528261
P. fluorescens Pf0-1	NC_007492	*P. migulae* CT14	EU111725
P. fluorescens Pf-5	NC_004129	*P. putida* F1	NC_009512
P. fluorescens SBW25	NC_012660	*P. putida* GB-1	NC_010322
P. graminis	DQ059301	*P. putida* W619	NC_010501
P. graminis RMPP6	GU396281	*P. s. phaseolicola* 1448A	NC_005773
P. graminis TP-Snow-C7	HQ327116	*P. s.syringae* B728a	NC_007005
P. migulae	AF074383	*P. s . tomato* DC3000	NC_004578

A.2.2 Phylogenetic Analysis of Isolated *Erwinia* Bacterial Species

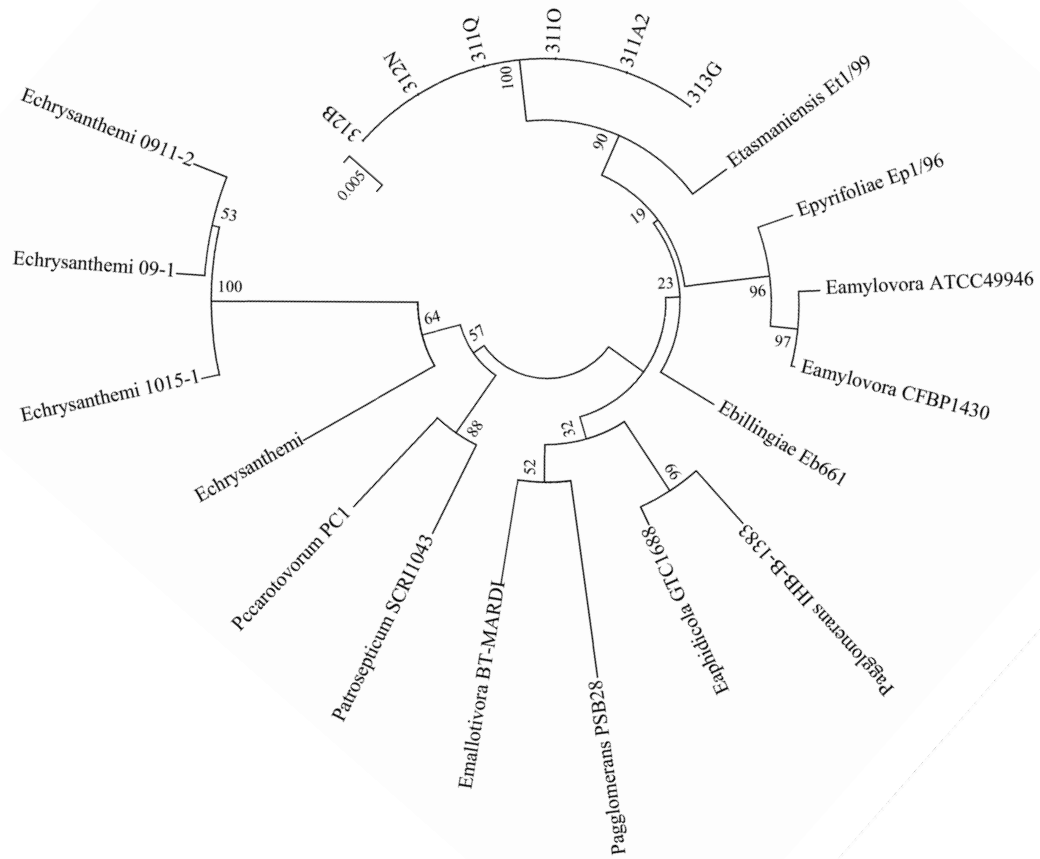

Figure A.2: Phylogenetic comparison of the six epiphytic bacterial isolates identified as belonging to the *Erwinia* genus with various known *Erwinia* species. The phylogenetic tree was carried out using the maximum-likelihood algorithm (bootstrap = 100) and rooted with *E. chrysanthemi*.

Table A.5: List of the *Erwinia* (*E.*), *Pantoae* (*Pa.*) and *Pectobacterium* species (*Pe.*) used for the phylogenetic analysis and their 16S rDNA accession number.

Bacterial species	16S rDNA accession number	Bacterial species	16S rDNA accession number
E. amylovora ATCC49946	NC_013971	*E. mallotivora* BT-MARDI	HQ456230
E. amylovora CFBP1430	NC_013961	*E. pyrifoliae* Ep1/96	NC_012214
E. aphidicola GTC 1688	AB273744	*E. tasmaniensis* Et1/99	NC_010694
E. billingiae Eb661	NC_014306	*Pa. agglomerans* IHBB1383	GU186121
E. chrysanthemi	EU526397	*Pa. agglomerans* PSB28	HQ242741
E. chrysanthemi 09-1	HM222417	*Pe. atrosepticum* SCRI1043	NC_004547
E. chrysanthemi 0911-2	GQ293898	*Pe. carotovorum carotovorum* PC1	NC_012917
E. chrysanthemi 1015-1	GQ293897		

A.3 ORIGINAL RISA PROFILES

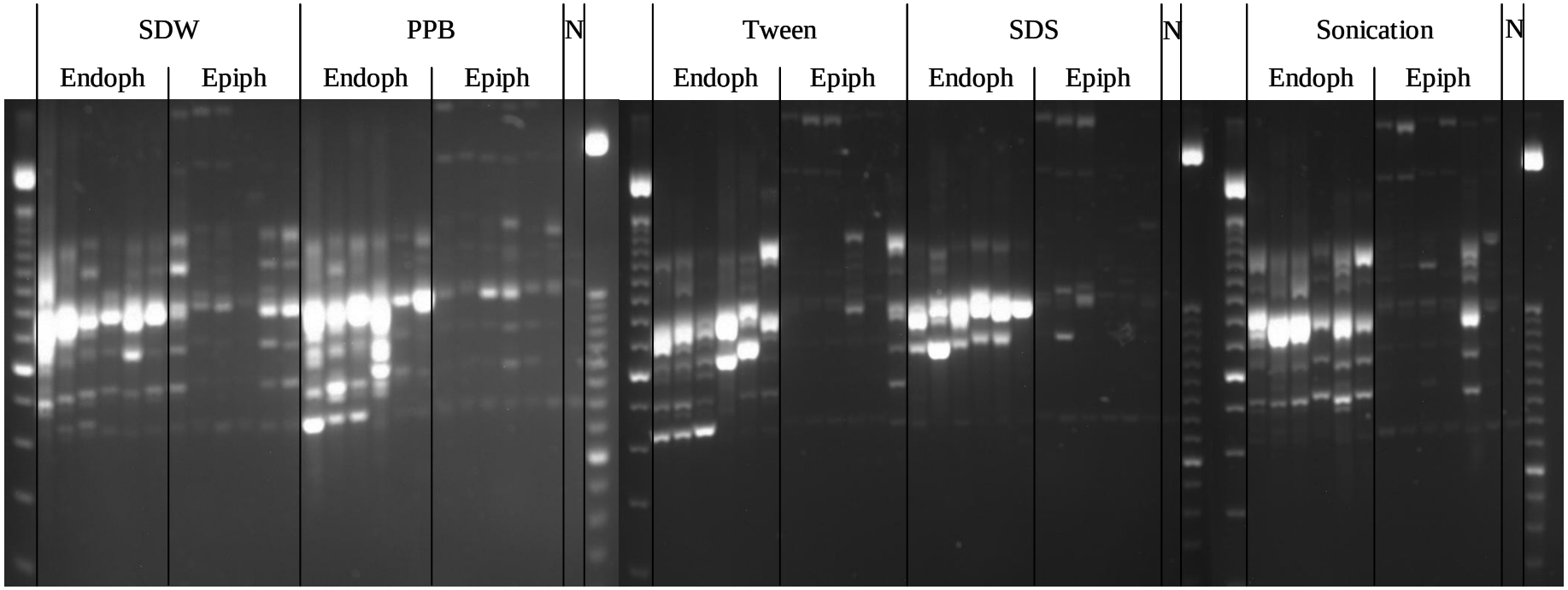

Figure A.3: RISA profiles of the leaf-associated endophytic and epiphytic communities of field-grown Sumo. The communities were extracted using the five same methods used for the T-RFLP analysis: washing with water alone, washing with PPB, washing with PPB and Tween (0.001% v/v), washing with PPB and SDS (0.1% w/v) and sonicating with PPB. A negative control (N) was run alongside each gel.

A.4 COMPLEMENTARY T-RFPL DATA

A.4.1 Complementary Data on the Effects of the Removal Technique and Sample Size on the Epiphytic T-RFLP Analysis

Table A.6: OTUs' relative abundance and loading vector values associated with the first principal component of PCA analysis of the effect of the removal technique on epiphytic communities of field-grown barley leaves (see Figure 3.5). Marked OTUs (§) were significantly affected by the removal technique (see Figure 3.6).

OTU	Loading Vector	Relative Abundance	OTU	Loading Vector	Relative Abundance
365§	-0.289	0.131	68	0.015	0.002
62§	-0.262	0.166	467	0.016	0.002
86	-0.258	0.027	55	0.020	0.054
192	-0.255	0.006	498	0.033	0.001
293	-0.254	0.003	201	0.064	0.001
82	-0.198	0.026	60	0.067	0.007
93	-0.163	0.062	175	0.073	0.002
363	-0.161	0.002	180	0.080	0.008
80	-0.142	0.027	70	0.087	0.000
155	-0.132	0.000	206	0.098	0.019
73	-0.122	0.001	337	0.102	0.001
217	-0.105	0.005	94	0.136	0.019
163	-0.050	0.003	139	0.154	0.060
57§	-0.041	0.048	204§	0.170	0.116
157	-0.035	0.000	177	0.191	0.011
492	-0.035	0.001	299	0.201	0.003
244	-0.031	0.001	89	0.212	0.022
77	-0.017	0.046	368	0.212	0.090
495	-0.008	0.002	472	0.237	0.004
369	-0.006	0.015	340	0.248	0.001
166	-0.003	0.002	120	0.288	0.004

A.4.2 Complementary Data on the Effects of the Removal Technique and Sample Size on the Endophytic T-RFLP Analysis

Table A.7: Ecological indices (richness, Simpson and Shannon diversity indices and evenness) of endophytic communities isolated from sonicated washing solutions using from 1, 5 or 20 leaves.

Number of leaves	Richness (S)	Diversity (1-D)	Diversity (H)	Evenness (E)
1	16.7	0.894	2.494	0.734
5	16.0	0.873	2.366	0.674
20	18.3	0.901	2.574	0.719

Table A.8: Ecological indices (richness, Simpson and Shannon diversity indices and evenness) of endophytic communities from leaves washed using five methods: washing with SDW (S), washing with PPB (B), washing with PPB and Tween (T), washing with PPB and SDS (S) and sonicating with PPB (N).

Treatment	Richness (S)	Diversity (1-D)	Diversity (H)	Evenness (E)
SDW	15.7	0.844	2.255	0.715
PPB	14.5	0.809	2.135	0.685
Tween	15.5	0.841	2.235	0.727
SDS	19.3	0.860	2.448	0.709
Sonication	11.0	0.834	2.063	0.778

A.4.3 Complementary Data on the Effects of the Cultivar Genotype and the Agro-Chemical Treatments on Endophytic Communities.

Table A.9: Endophytic bacterial Shannon diversity of eight field-grown barley cultivars treated with either conventional fungicides or a mixture of elicitors or nothing. The diversity was significantly affected by the cultivar (P = 0.007, lsd = 0.171). The effect of the treatments on the bacterial diversity was also affected by the cultivars (P < 0.001, lsd = 0.344).

		Treatment			
		Control	Elicitor	Fungicide	Average
Cultivar	Igri	1.572	2.425	2.607	2.201[a]
	Flagon	2.258	2.227	2.487	2.324[ab]
	Sumo	2.127	2.313	2.577	2.339[ab]
	Maris Otter	2.576	2.357	2.364	2.432[bc]
	Saffron	2.581	2.363	2.493	2.479[bc]
	Manitou	2.613	2.294	2.562	2.490[bc]
	Pearl	2.518	2.531	2.429	2.493[bc]
	Leonie	2.531	2.521	2.492	2.515[c]
	Average	2.347	2.379	2.502	

Table A.10 (following page): ANOVA results of OTUs significantly affected by either treatments (T), cultivars (C) or the combination of the two (T x C). The ANOVA results are defined by the degree of freedom (df), the sum of squares (SS) and probability (P). Significant effects are notified with one to three asterisks, representing various degrees of probabilities. The OTUs, which are annotated with their relative abundance (r. a.), are sorted based on their loading vector of the PC1 (see Figure 5.2).

OTU (r. a.)	197 (0.039)			78 (0.015)			204 (0.074)			178 (0.006)		
Loading vector	-0.225			-0.222			-0.068			0.021		
Source of variation	df	SS	P	df	SS	P	df	SS	P	df	SS	P
T	2	0.003	*	2	0.001		2	2E-04		2	3E-04	
Residual	4	0.001		4	0.001		4	0.004		4	4E-04	
C	7	0.007		7	0.002	**	7	0.014	*	7	0.001	*
T x C	14	0.007		14	0.002	*	14	0.042	***	14	0.001	
Residual	35	0.022		33	0.002		33	0.021		35	0.002	
Total	64	0.189		62	0.029		62	0.145		64	0.004	

OTU (r. a.)	73 (0.009)			294 (0.012)			271 (0.010)			135 (0.063)		
Loading vector	0.090			0.140			0.144			0.171		
Source of variation	df	SS	P	df	SS	P	df	SS	P	df	SS	P
T	2	4E-05		2	0.003		2	0.001		2	0.026	
Residual	4	1E-04		4	0.005		4	0.007		4	0.042	
C	7	0.001		7	0.002	*	7	0.001	*	7	0.019	*
T x C	14	0.002	**	14	0.002		14	0.001		14	0.030	*
Residual	34	0.002		35	0.004		33	0.002		33	0.030	
Total	63	0.008		64	0.015		62	0.012		62	0.150	

OTU (r. a.)	113 (0.014)			93 (0.121)			164 (0.034)			55 (0.070)		
Loading vector	0.179			0.227			0.276			0.282		
Source of variation	df	SS	P	df	SS	P	df	SS	P	df	SS	P
T	2	3E-04	*	2	0.006		2	0.001		2	0.003	
Residual	4	7E-05		4	0.010		4	0.001		4	0.005	
C	7	0.001		7	0.005		7	2E-04		7	0.001	
T x C	14	0.002	*	14	0.018	*	14	0.002	*	14	0.008	*
Residual	35	0.002		33	0.018		34	0.002		34	0.008	
Total	64	0.006		62	0.057		63	0.006		63	0.022	

OTU (r. a.)	90 (0.113)			241 (0.043)			85 (0.021)		
Loading vector	0.297			0.306			0.314		
Source of variation	df	SS	P	df	SS	P	df	SS	P
T	2	0.007		2	0.003		2	2E-04	
Residual	4	0.006		4	0.002		4	4E-04	
C	7	0.004		7	0.002	*	7	1E-04	
T x C	14	0.020	*	14	0.007	***	14	0.001	*
Residual	35	0.024		33	0.004		33	0.001	
Total	64	0.061		62	0.015		62	0.003	

A.4.4 Complementary Data on the Effects of the Association with a Root Endophyte on Leaf Epiphytic Communities.

Table A.11: Ecological indices (richness, diversity and evenness) of leaf-associated epiphytic and endophytic communities of barley seedlings infected with *P. indica* or mock-treated.

		Populations	
	Treatment	Epiphytes	Endophytes
Taxa (S)	Mock	15.8	30.0
	+ *P. indica*	16.5	32.3
Simpson (1-D)	Mock	0.878	0.928
	+ *P. indica*	0.884	0.934
Shannon (H)	Mock	2.373	3.063
	+ *P. indica*	2.440	3.169
Evenness (E)	Mock	0.690	0.715
	+ *P. indica*	0.705	0.737

A.5 COMPLEMENTARY STATISTICAL DATA

A.5.1 ANOVA Raw Data of the Yield of Various Treated WB

Table A.12: ANOVA of repeated measurement (over two years) of the yield from eight WB cultivars treated with various agro-chemical compounds (fungicides, an elicitor mixture or nothing).

Source of variation	Degree of freedom	Sum of squares	Probability
Cultivar	7	2.66	0.005
Treatment	2	6.00	< 0.001
Cultivar x Treatment	14	1.22	0.696
Residual	48	5.43	
Time	1	44.61	< 0.001
Time x Cultivar	7	2.86	0.036
Time x Treatment	2	0.16	0.633
Time x Cultivar x Treatment	14	0.69	0.993
Residual	48	8.23	
Total	143	71.85	

A.5.2 ANOVA Raw Data of *R. commune* Infection Percentage on Various Treated WB

Table A.13: ANOVA of repeated measurement (over two years) of the infection percentage of *R. commune* on eight WB cultivars treated with various agro-chemical compounds (fungicide, an elicitor mixture or nothing).

Source of variation	Degree of freedom	Sum of squares	Probability
Cultivar	7	196.47	< 0.001
Treatment	3	236.97	< 0.001
Cultivar x Treatment	21	228.62	< 0.001
Residual	56	157.29	
Time	1	1.97	0.39
Time x Cultivar	7	68.37	< 0.01
Time x Treatment	3	34.87	< 0.01
Time x Cultivar x Treatment	21	95.96	0.06
Residual	56	148.85	
Total	175	1015.42	