Phyllosphere microbiology with special reference to diversity and plant genotype

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Summary

The phyllosphere represents the habitat provided by the aboveground parts of plants, and on a global scale supports a large and complex microbial community. Microbial interactions in the phyllosphere can affect the fitness of plants in natural communities, the productivity of agricultural crops, and the safety of horticultural produce for human consumption. The structure of phyllosphere communities reflects immigration, survival and growth of microbial colonists, which is influenced by numerous environmental factors in addition to leaf physico-chemical properties. The recent use of culture independent techniques has demonstrated considerable previously unrecognised diversity in phyllosphere bacterial communities. Furthermore there is significant recent evidence that plant genotype can play a major role in determining the structure of phyllosphere microbial communities. The main aims of this review are (i) to discuss the diversity of phyllosphere microbial populations (ii) to consider the processes by which microbes colonise the phyllosphere (iii) to address the leaf characteristics and environmental factors which determine survival and growth of colonists (iv) to discuss microbial adaptations which allow establishment in the phyllosphere habitat and (v) to evaluate evidence for plant genotypic control of phyllosphere communities. Finally, we suggest approaches and priority areas for future research on phyllosphere microbiology.

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Introduction

The aerial parts of living plants including leaves, stems, buds, flowers and fruits provide a habitat for microorganisms termed the phyllosphere. Bacteria are considered to be the dominant microbial inhabitants of the phyllosphere, although archaea, filamentous fungi, and yeasts may also be important. These microbes can be found both as epiphytes on the plant surface and as endophytes within plant tissues (Arnold, *et al.* 2000; Inacio *et al.* 2002; Lindow and Brandl 2003; Stapleton and Simmons 2006). The global surface area of the phyllosphere has been estimated to total over 4 x 10^8 km^2, supporting bacterial populations in the region of 10^{26} cells (Morris and Kinkel 2002). Furthermore, recent estimates of the diversity of phyllosphere bacteria in the 20 000 vascular plants inhabiting the Brazillian Atlantic forest, suggests the possible occurrence of 2 to 13 million phyllosphere bacterial species in this habitat alone (Lambais *et al.* 2006).

The phyllosphere represents a niche with great agricultural and environmental significance. There is growing evidence for important interactions of phyllosphere microbial inhabitants which may affect the fitness of natural plant populations and the quality and productivity of agricultural crops. Phyllosphere bacteria can promote plant growth and both suppress and stimulate the colonisation and infection of tissues by plant pathogens (Lindow and Brandl 2003; Rasche *et al.*, 2006). Similarly, fungal endophytes of leaves may deter herbivores, protect against pathogens and increase drought tolerance (Arnold *et al.* 2003; Schweitzer *et al.* 2006). Furthermore, interactions in the phyllosphere zone determine the extent to which human pathogens are able to colonise and survive on plant tissues, an area of increasing importance with the rise in cases of human disease associated with consumption of fresh salad, fruit and vegetable produce (Whipps *et al.* 2008).

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There is evidence for functional roles within the phyllosphere microbial community which given the size of the habitat could have global significance. The best studied of these is nitrogen fixation. Measured rates of bacterial nitrogen fixation in the phyllosphere vary widely, but in the phyllosphere of trees in some tropical habitats has been reported at rates of over 60 kg N ha^{-1}, although amounts fixed in the phyllosphere of temperate trees is generally considerably lower (Freiberg 1998). Furthermore, N_2 fixation or the presence of N_2 fixing bacteria has been reported in the phyllosphere of many crop plants (e.g. Murty 1983; Miyamoto *et al.* 2004). Other environmentally important microbial processes for which there is evidence in the phyllosphere include methanol degradation (Corpe and Rheem, 1989; Van Aken *et al.* 2004) and nitrification (Papen *et al.* 2002), although the rates of these process and their ubiquity within the phyllosphere remains to be elucidated.

Most knowledge of the structure and activities of phyllosphere microbial communities has been established using culture-dependant methods. However, these are recognised to significantly underestimate diversity, with only 0.1-3 % of environmental bacteria considered culturable (Wagner *et al.* 1993). Data gathered using these methods therefore relate only to culturable members of the community and provide no information on the vast majority of microbes present in samples. As in other areas of environmental microbiology, the recent application of culture-independent methods based on the characterisation of small subunit rRNA gene sequences for microbial community analysis is providing new insights into the complexity of phyllosphere microbial communities and their interactions with plants and the wider environment.

In the current paper we , review the extent to which the use of culture independent approaches has changed our understanding of the structure and diversity of phyllosphere communities. A variety of plant, microbial and environmental factors control establishment of microbial communities in the phyllosphere, but recently there has been recognition of the role

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that plant genotype plays in selecting phyllosphere communities. The evidence for the different factors which regulate the structure of phyllosphere communities is discussed with special reference to the role of plant genotype. Finally, we suggest approaches and priority areas for future research on phyllosphere microbiology. Although much of the phyllosphere literature is concerned with interactions between plant and plant pathogens (bacteria and fungi that cause diseases in plants), in the current review emphasis is placed on studies of microorganisms that live in the phyllosphere without causing obvious damage to the plant, as absence of disease is the normal situation in nature.

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Microbial diversity in the phyllosphere

The microbial communities of the phyllosphere are diverse, supporting numerous genera of bacteria, filamentous fungi, yeasts, algae and in some situations protozoans and nematodes (Morris *et al.* 2002; Lindow and Brandl 2003). Bacteria are the most numerous and diverse colonists of leaves, with culturable counts ranging between 10^2 to 10^{12} cells g leaf (Thompson *et al*. 1993; Inacio *et al.* 2002). Culture-based studies of sugar beet over the whole of the growing season have found more than 78 bacterial species representing 37 known bacterial genera (Thompson *et al.* 1993). Similar studies in wheat have revealed 88 bacterial species representing 37 known bacterial genera (Legard *et al.* 1994).

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Recent studies have demonstrated that profiling of phyllosphere communities based on culture dependent methods is likely to be inaccurate and to underestimate diversity (Rasche *et al.* 2006b). In the case of the phyllosphere, use of culture independent approaches has shown that although assumptions regarding the dominant inhabitants are largely correct, the diversity of phyllosphere communities is far greater than previously recognised. Analysis of 16S rDNA cloned directly from leaf samples has demonstrated that proteobacteria are the dominant group found on leaves (Table 1), confirming data obtained using culture-dependant methods

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(e.g. Thompson *et al.* 1993). α and γ- proteobacteria are generally the dominant bacterial inhabitants of the phyllosphere, with bacteroidetes also usually important. β-proteobacteria and firmicutes can also form a large part of the bacterial community in some situations, with acidobacteria, actinobacteria and cyanobacteria occurring infrequently (Kadivar and Stapleton 2003; Idris *et al.* 2004; Lambais *et al.* 2006; Rasche *et al.* 2006b,c).

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In a study of phyllosphere bacterial communities in a tropical Brazillian forest, 97 % of bacterial sequences were from previously undescribed species with phyllospheres of different plant species supporting from 95 to 671 bacterial species (Lambais *et al.* 2006). The extent to which such diversity occurs in other plant species is unclear. Those sequences showing 95 % or less homology to known bacterial species database entries comprised 15.2 % of sequences obtained from *Thlapsi geosingense* (Idris *et al.* 2004), and 7.9, 2.3, 3.5 and 1.2 % of those sequences obtained from Crocus, potato, pepper and maize respectively (Kadivar and Stapleton 2003; Rasche *et al.* 2006 a,b, Reiter and Sessitsch, 2006). However, in a study of a range of temperate agricultural crop species, 5 of 17 bands cut from 16S rRNA denaturant gradient gel electrophoresis gels had less than 90 % similarity to database entries, suggesting that in some situations phyllospheres of crop plants may support large numbers of novel bacteria (Yang *et al.* 2001). The number of sequences investigated in the culture independent studies conducted to date has been limited, so that only dominant members of the community are likely to have been detected, and the true extent of bacterial diversity in the phyllosphere therefore remains to be determined.

Yeasts are the major epiphytic fungal group in the phyllosphere with filamentous fungi largely occurring as dormant spores rather than active mycelia except on older leaves (Andrews and Harris 2000; de Jager *et al.* 2001). Culturable yeast populations can range between 10 and 10^{10} colony forming units g leaf (Thompson *et al*. 1993, Inacio *et al*. 2002). The diversity of culturable yeasts appears to be mostly limited to the genera *Cryptococcus,*

Sporobolomyces and Rhodotorula, although total species number can reach over 40, with multiple species of each coexisting in the phyllosphere, together with a number of other genera which occur less frequently (Thompson *et al.* 1993; Inacio *et al.* 2002; Glushakova and Chernov 2004).

Filamentous fungus population sizes can range between 10^2 and 10^8 colony forming units g leaf. *Cladosporium* and *Alternaria* are usually considered the most abundant fungi found on leaves, although several other genera, including *Penicillium*, *Acremonium*, *Mucor* and *Aspergillus* are also found (Thompson *et al*. 1993; Inacio *et al.* 2002). Filamentous fungi appear to occur ubiquitously as endophytes, the diversity of which may be substantial, particularly in long lived tropical leaves. Using culture dependant approaches, over 340 genetically distinct taxa were recovered from individuals of 2 tropical forest understory plant species at 2 sites. Furthermore, there was evidence for host preference within the endophyte community (Arnold *et al.* 2000). Culture independent approaches have not yet been used to characterise fungal diversity in the phyllosphere.

There are various developing technologies which show promise to significantly increase throughput of analysis to provide a finer resolution of understanding about the diversity and structure of phyllosphere communities and to link diversity with functioning. Culture-independent analysis using phylogenetic specific primers represents a powerful method to investigate the dynamics and distribution of specific bacterial groups of interest (Sessitsch *et al.* 2002; Miyamoto *et al.* 2004). Additionally multiplex TRFLP, in which several phylogentic groups or functional genes can be analysed at the same time provides an opportunity to improve throughput of samples in a cost effective manner (Singh *et al.* 2006). However, these techniques remain time consuming, and future developments will depend on high throughput methods. Phylogenetic microarrays clearly provide a way forward, allowing the presence and amount of thousands of microorganisms to be determined simultaneously, and could also be

used to detect novel members of phylogenetic groups. Similarly, functional gene arrays provide a means of characterising activity of the phyllosphere community, and when used with phylogenetic microarrays, for linking community structure to function (Sessitsch et al., 2006).

In order to understand and predict the diversity and structure of phyllopshere communities, it is necessary to understand the biological and environmental factors which control the establishment and dynamics of microbial communities on the leaf surface. This is the focus of the remainder of the review.

Sources of microbes colonising the phyllosphere

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The sources of microorganisms on the phyllosphere can be manifold. Epiphytic filamentous fungi, yeasts and bacteria may arrive on the leaf surface through insect-, atmosphere-, seed- or even animal-borne sources. Tree buds, seeds of annual plants and the debris from previous crops are likely to be the most important sources for the colonization of new plants and leaves as they are a major source of bacteria already adapted to the phyllosphere (Manceau and Kasempour 2002).

Those microorganisms that show no or limited multiplication in the phyllosphere are considered transient epiphytes whereas those with the capacity for multiplication in the absence of wounds are known as residual epiphytes (Suslow 2002). Microbial populations can vary in size among and within plant species over short periods of time (Hirano and Upper 1989) as well as over the growing season (e.g. Thompson *et al.* 1993; Legard *et al.* 1994; Inacio *et al.* 2002), with few epiphytic bacteria present on leaves shortly after emergence from buds or seeds, but increasing in quantity subsequently (Hirano and Upper 1993). There is a

general succession of microbial populations on leaves over the growing season with bacteria dominating initially, followed by yeasts and finally filamentous fungi (Kinkel 1997).

The atmospheric microflora can vary in composition and concentration diurnally and seasonally as well as in response to environmental events such as rainfall and high wind (Kinkel 1997; Zak 2002), directly influencing the immigration of microorganisms to the phyllosphere. Local vegetation, and in areas of crop production, agricultural practices such as harvesting and cultivation, also influence atmospheric microbiology and colonisation of nearby plants (Lindemann *et al.* 1982; Lacey 1996; Lighthart 1997). Immigration of microorganisms to leaves from the atmosphere can take place through impaction onto the leaf surface, sedimentation or rain splash as well as from contamination with soil (Venette and Kennedy 1975; Lacey 1996).

There is increasing evidence that microorganisms on seeds or roots can become endophytic in the roots, enter the vascular system and be transferred internally to the aerial parts of plants where they establish as phyllosphere endophytes (Lamb *et al.* 1996; Wulff *et al.* 2003). Endophytes can also arise from ingression into the internal leaf spaces following colonisation by epiphytes, suggesting that epiphytes and endophytes are really part of a continuum in the phyllosphere (Wilson *et al.* 1999; Beattie and Lindow 1999).

Once microorganisms have arrived in the phyllosphere they have to become established and colonise the leaf to become a residual epiphyte. The pattern of distribution of microorganisms on leaves is not even. The most common sites of bacterial colonisation are in the epidermal cell wall junctions (Blakeman 1985; Davis and Brlansky 1991) especially in protected sites in grooves along the veins (Mansvelt and Hattingh 1987; Leben 1988; Mariano and McCarter 1993), at stomata (Mew and Vera Cruz 1986; Mariano and McCarter 1993), and at the base of trichomes (Mew and Vera Cruz 1986; Mansvelt and Hattingh 1987; Mariano and McCarter 1993). They are also found under the cuticle (Corpe and Rheem,

1989), in depressions in the cuticle (Mansvelt and Hattingh 1987), near hydrathodes (Mew *et al.* 1984) and in specific sites that only occur on particular plants such as stomatal pits in oleander and pectate hairs in olive (Surico 1993). In general, greater numbers of bacteria are found on lower than upper leaf surfaces (Leben 1988; Surico 1993) possibly due to the lower leaf surface having a greater density of stomata or trichomes, or a thinner cuticular layer (Beattie and Lindow 1999).

Bacterial populations in the phyllosphere can differ in distribution over very small scales, as little as 0.1 mm^2 (Kinkel *et al.* 1995) and are often well-described by a log-normal distribution (Hirano *et al.* 1982; Ishimaru *et al.* 1991) whereas yeasts and filamentous fungi may be better described by a normal distribution (Kinkel *et al.* 1989). Microorganisms may occur individually on the leaf surface but frequently, they occur as aggregates or biofilm-like structures containing bacteria, (Kinkel *et al.* 1995; Morris *et al.* 1997, 1998; Jacques *et al.* 2005), yeasts (Last 1955) and filamentous fungi (Bernstein and Carroll 1977).

Clearly, not all microorganisms that arrive in the phyllosphere are able to colonise and grow. To some extent this reflects processes of emigration through dispersal mechanisms such as rain splash, wash-off, bounce-off, water movement or removal by insects (Kinkel 1997). Ability to survive and grow are dependent on the environmental, physicochemical and genetic features of the plant and specific properties exhibited by the phyllosphere microorganisms, which together determine the structure and diversity of the microbial community. Evidence for such selection is supported by the findings of Miyamoto *et al.* (2004), in which 16S rRNA-Terminal Restriction Fragment Length Polymorphism (TRFLP) with Clostridia specific primers was used to show the presence of diverse Clostridia populations within *Miscanthus sinensis*, which were shown to be distinct to those Clostridia populations inhabiting soil around plants. Furthermore, since a substantial proportion of those bacteria inhabiting the phyllosphere appear to be novel to this habitat there have been

suggestions that some may be unique or specialists to this habitat (Yang *et al.* 2001; Lambais *et al.* 2006).

There are a number of areas relating to the colonisation of phyllosphere which require more complete understanding. The transmission of microorganisms from roots to aerial parts of plants appears to have been a neglected area of research and the importance of this environmentally protected phyllosphere colonisation route needs to be elucidated. This could be particularly important for soils contaminated with human pathogens.

Leaf characteristics and environmental factors controlling phyllosphere microbiology

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Following arrival of microbial cells or propagules on the leaf surface, a variety of factors determine whether cells are able to colonise the leaf, and where cells become localised. Establishment is determined by interaction between leaf and environmental characteristics which interact to control conditions prevailing in the phyllosphere habitat. The first point of contact of microbial cells immigrating to the phyllosphere is the cuticle (Beattie 2002). This waxy surface, often microcrystalline in nature, serves several functions: a diffusion barrier, reducing water and solute loss and aqueous pollution ingress; as a reflectant to minimise temperature fluctuations; conferring water repellancy; and providing protection from pathogens (Beattie 2002). The water repellancy is particularly important in preventing immigration of microorganisms to the leaf surface. This is so especially on young leaves where the cuticle is intact relative to older leaves because as the cuticle erodes, wettability increases (Beattie 2002). In addition, cuticle-mediated limitation of nutrient loss from the leaf is particularly important in supporting epiphytic microbial populations. Use of whole cell bacterial biosensors for sucrose, fructose and glucose have revealed that these sugars are present only in discrete localised sites on the leaf (Leveau and Lindow 2001; Miller *et al.*

2001). This, and recent microscopical evidence (Monier and Lindow 2005), suggests that most microbial immigrants to the phyllosphere are exposed to nutrient poor environments and that only few cells randomly land in zones of relatively abundant nutrients that support growth. Other discrete sites of nutrient loss such as wounds or glandular trichomes (Monier and Lindow 2005), or sites of nutrient enrichment including pollen (Diem 1974) or honeydew (Dik et al. 1992) also provide sites for microbial growth. Other nutrients such as N-sources or iron are not considered as growth limiting to microorganisms on the phyllosphere as C-sources (Lindow and Brandl 2003). Interestingly, when the resurrection fern, *Polypodium polypodioides*, is exposed to rainfall after a period of desiccation, the complex phyllosphere community undergoes changes in overall structure and activity, reflecting use of labile organic substrates in the form of an enrichment culture (Jackson *et al.* 2006). Whether this occurs with other plants is unknown.

Plant leaves also release a wide range of volatile organic compounds into the boundary layer around leaves. These can include small molecules such as CO_2 and acetone, medium sized molecules including terpenoids and a number of aldehydes and alcohols, as well as large molecules such as long-chain hydrocarbons and sesquiterpenoids; sulphides and nitrogen-containing compounds also occur. It is unclear whether these could be nutrient sources directly, but there is evidence that some of these compounds can be inhibitory or toxic to some fungi (Mechaber 2002). Similarly, some proteins secreted by glandular trichomes can inhibit some pathogens (Shepherd *et al.* 2005). There are also data to suggest that plants can release a number of compounds in response to damage that not only promote microbial development but can selectively inhibit microbial growth as well (Dingman 2000).

Characteristics of the plant species themselves may also influence the microbial carrying capacity of the leaf. The total number of culturable bacteria from broad leaf succulent herbaceous plants such as cucumber, lettuce and bean can be significantly higher than that

from grasses or waxy broad-leaved plants such as cabbage and citrus (O'Brien and Lindow 1989; Lindow and Andersen 1996; Kinkel *et al.* 2000). Culture independent approaches have demonstrated that community structure on leaves from individuals of the same species is similar, but varies significantly between species (Yang *et al.* 2001). Lambais *et al.* (2006) showed that just 0.5 % of bacterial species recorded in tropical tree canopies were common to all tree species. Furthermore, both bacterial and fungal population size on leaves has been correlated with leaf position, plant architecture and height in the canopy (Wildman and Parkinson, 1979; Oliveira *et al.* 1991; Jacques *et al.* 1995). We would suggest that microbial diversity and community structure are also influenced by these factors, although this remains to be shown.

Information is needed to characterise the arrangement and dynamics of communities to time and space, especially at the landscape scale. In particular the relative importance of environmental factors, location and plant species in determining the composition and dynamics of phyllosphere communities needs to be addressed. Biogeographical approaches to the analysis of microbial communities show potential to elucidate these fundamental relationships (Ramette and Tiedje, 2007).

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Microbial adaptations to the phyllosphere habitat

In addition to plant and environmental factors, properties of the microbial colonists themselves determine the extent to which they are able to establish on the leaf surface. For some microorganisms this reflects their inherent ability to survive in the existing habitat whereas others are capable of modifying the environment to ameliorate the levels of stress they are exposed to.

Culture independent analyses have indicated that tolerance to UV radiation is likely to be an important selection pressure for survival and growth this habitat (Kadivar and Stapleton,

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2002; Stapleton and Simmons, 2006), and most isolated phyllosphere microorganisms are capable of withstanding high UV radiation levels on the leaf surface (Sundin 2002). In fungi, dark melanin-type pigments are thought to play a key role as protective pigments along with UV-B induced hyphal-wall thickening, the latter protecting lower levels of the fungal colony (Fourtouni *et al.* 1998; Sundin 2002). Interestingly, the most UV-B tolerant strains of bacteria from the peanut phyllosphere were those that produced pink or orange pigments (Sundin and Jacobs 1999) and so multiple UV-B protectant mechanisms may be exhibited by phyllosphere microorganisms.

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A low level of water availability and nutrients are key limiting factors for microbial growth in the phyllosphere so epiphytes display a variety of mechanisms to overcome these limitations. For example, some epiphytic *Pseudomonas* spp. can release surfactants that increase the wettability of leaf surfaces making it easier for microorganisms to use water and increasing solubilisation and diffusion of nutrients, thereby increasing substrate availability to epiphytic bacteria (Bunster *et al.* 1989). A number of phyllosphere bacteria have recently been shown to increase permeability of the cuticle enhancing water and nutrient availability in the phyllosphere (Schreiber *et al.* 2005). Another, potentially related, mechanism to increase nutrient availability may relate to the ability to produce toxins that affect ion transport across plant cell plasma membranes (Quigley and Gross 1994; Hutchison *et al.* 1995). Plant pathogenic *Pseudomonas syringae* pv. *syringae* secrete the toxin syringomycin which eventually leads to cell lysis. Nevertheless, low levels are produced by non-pathogenic epiphytic strains of *P. syringae* pv. *syringae* such that necrosis and disease do not occur although release of plant nutrients is still stimulated (Hutchison *et al.* 1995). Interestingly, syringomycin also acts as a surfactant providing two possible mechanisms to enhance nutrient availability in the phyllosphere.

Another, perhaps more widespread mechanism, is the production and release of plant growth regulators. Production of indole-3-acetic acid (IAA) is common among bacterial epiphytes (Glickman *et al*. 1998; Brandl *et al.* 2001) and is associated with enhanced nutrient leakage and microbial fitness (Brandl and Lindow 1998; Manulis *et al.* 1998). Lindow and Brandl (2003) have also made the suggestion that presence of a functional type III secretion pathway in *Pseudomonas fluorescens* and *Pseudomonas putida* (Preston *et al.* 2001), which provides the capacity to modify the local habitat, may be needed for growth and survival in the phyllosphere. Production of pili and flagellae may also be important in allowing bacterial attachment and colonisation of the phyllosphere (Romantschuk *et al.* 2002). A whole range of genes and gene products that are important for phyllosphere colonisation are now being identified using molecular techniques (Gal *et al.* 2003; Gourion *et al.* 2006) and may provide further insights into mechanisms involved in epiphytic growth.

As mentioned earlier, bacterial distribution on the leaf surface is not uniform and frequently, aggregates of cells occur (Morris and Monier 2003). The presence of these aggregates may provide the epiphytes with an ability to colonise and survive in the phyllosphere and modify the local environment. The production of extracellular polysaccharides (EPS) by bacteria may protect the bacteria from water stress and help anchor the cells to the leaf surface (Morris *et al.* 1997; Gal *et al.* 2003). By analogy with biofilms (Morris *et al.* 2002; Morris and Monier 2003), these aggregates may also protect from UVR, predation and bacteriocides, moderate pH and gas exchange, enhance genetic exchange particularly through plasmid transfer, and allow cell density – dependent behaviour. The latter, often mediated by accumulation of diffusible molecules such as *N*- acyl homoserine lactones through quorum sensing may have numerous effects on microbial behaviour including EPS and antibiotic production as well as pathogenicity traits (Swift *et al.* 1994; Greenberg 1997). Interestingly, if signalling controls the formation and functioning of aggregates, it may be possible in future to manipulate the

microbial populations on the phyllosphere if the molecular signals and receptors essential for aggregate behaviour can be identified (Morris *et al.* 2002).

Plant genotype and phyllosphere microbiology

It is clear that microbial populations in the phyllosphere can vary markedly in size and composition both spatially and temporally on the same plant, differ between different plants and parts of plants in the same place, and even differ on the same plant species in different places (Lindemann *et al.* 1984; Morris and Lucotte 1993; Lindow and Andersen 1996; Kinkel 1997). Much of the variability must reflect the environmental conditions prevailing at the time and place of sampling, thereby influencing the processes of microbial immigration, emigration, growth and death. However, the microbial population that does develop must relate to a large extent to the phenotypic characteristics exhibited by the plant, controlled ultimately by its genetic make-up. Certainly, there are "hot-spots" of microbial growth on the leaf associated with specific sites and it would be expected that these would similarly be under the influence of plant genetic characteristics. We suggest that within plant species, genotype has a key role in determining colonisation and establishment of microbial communities in the phyllosphere. However, few studies have addressed the relationship between plant genetic control of phenotypic characteristics and their concomitant effects on microbial populations in the phyllosphere, despite its potential importance.

Several studies have used culture-dependent approaches to investigate the impact of plant genotype on phyllosphere microbiology. Adams and Kloepper (2002) showed that endophytic bacteria population sizes and structure differed between 9 cotton cultivars, and in pea 5 of 11 cultivars were found to contain endophytic bacteria with one showing a higher colonisation level than the others (Elvira-Recuendo and van Vuurde 2000). In a gnotobiotic system with

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The occurrence of large populations of microorganisms on the phyllosphere allows the possibility of extensive microbe-microbe interactions providing cells are in relatively close proximity. Besides the studies of aggregates already mentioned, there have been numerous investigations of other microbial interactions, largely concerned with biological control. For example, some bacteria on the phyllosphere exhibit ice nucleation activity (Ice^+ bacteria), and when in high numbers, these bacteria reduce the ability of the plant cells to supercool and avoid ice formation (Lindow 1995; Hirano and Upper 2000). Consequently, pre-emptive applications of Ice^- bacteria when Ice^+ bacteria were at low levels resulted in effective control of the problem, apparently acting largely through competition for carbon compounds as antibiotic production appears rare in bacterial interactions in the phyllosphere in general (Lindow 1988). Another successful case of bacterial biocontrol involves the use of non-pathogenic strains of *P. fluorescens* and *Pantoea agglomerans* to control the fireblight pathogen, *Erwinia amylovora* on flowers of apple and pear (Lindow and Leveau 2002). Again, pre-emptive colonisation, in this case of the stigma, by the non-pathogenic strains lead to competitive exclusion of the pathogen and disease control although antibiotic production may have been involved to some extent in this system (Stockwell *et al.* 2002). ¶
Fungal-fungal interactions on the phyllosphere can also provide disease control. For instance, on onion leaves, *Gliocladium catenulatum* and *Aureobasidium pullulans* appeared to control *Botrytis aclada* by antibiotic production whereas *Ulocladium atrum* acted through competitive exclusion (Köhl *et al.* 1997). The other most widely reported mode of action involves parasitism of fungal pathogens by other fungi but often this mode of action is accompanied by antibiotic production as well (Bélanger and Avis 2002) indicating that multiple modes of action for biocontrol are likely to occur in the phyllosphere overall.¶

tomato, one cultivar out of four supported fewer *Pseudomonas* sp. on the shoot exterior following application of the bacterium to the seed (Pillay and Nowak 1997). Differences in ability to support populations of *Pseudomonas syringae* pv. *syringae* were also found between different cultivars of snap bean (Hirano *et al.* 1996; Upper *et al.* 2003). However, no differences in occurrence of native, epiphytic mycoparasites were observed between the 3 main coffee cultivars or between clones of the same group (ten Hoopen *et al.* 2003). Similarly, no differences were found between epiphytes on three cultivars of apple (Becker and Manning 1983) or in endophytes in three cultivars of wheat (Larran *et al.* 2002).

Culture-independent community profiling approaches have been particularly valuable for elucidating interactions between plant genotype and phyllosphere microbial community structure. Several studies have indicated that different cultivars of the same plant species exhibit different phyllosphere microbial populations. Phyllosphere populations of bacteria were found to differ between cultivars of sweet pepper and tomato (Correa *et al.* 2007; Rasche *et al.* 2006b) and both endophytes and epiphytes differed between varieties of potato (Sessitch *et al.* 2002; Rasche *et al.* 2006a, c). Plant genotype differences may affect some microbial communities more than others. For example, phyllosphere bacterial community structure was shown to vary between wheat cultivars, although there was found to be no difference in archaeal communities (Stapleton and Simmons 2006). Recently, lettuce cultivar was shown to affect colonisation of leaves by *Salmonella enterica* serovars (Klerks et al., 2007), with significant serovar-cultivar interactions demonstrated. Furthermore, diversity of endophyte bacterial populations varied between the three lettuce cultivars used, and data suggested that the degree to which *Salmonella enterica* serovars were able to colonise plants endophytically was in part determined by competitive interactions with the natural endophyte bacterial community.

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Culture dependent analysis showed that genetic modification of potato with an antibacterial peptide, magainin, failed to influence the number or structure of phyllosphere bacterial or fungal populations even though maganin-expressing potato tubers did exhibit lower total numbers of bacteria than unmodified plants (O'Callaghan *et al.* 2004). In contrast, genetic modification of potato with a gene producing anti-bacterial T4-lysozyme or attacin/cecropin, was shown to induce greater difference in phyllosphere microbial community structure to the parent line, relative to variations between three cultivars (Rasche *et al.* 2006a, c). However, field site and plant growth stage had greater effects on bacterial community structure than either cultivar or genetic modification.

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Furthermore, microbial communities selected by different genotypes can show differing responses to environmental variables. Rasche *et al.* (2006b) showed that chilling sweet pepper plants altered endophyte bacterial community structure, with the extent of the effect differing between cultivars, and dependant on cultivar chilling tolerance. Similarly, in a study of wheat cultivars, it was shown that the response of phyllosphere bacterial communities to UV-B radiation depended on host genotype. However it was not clear whether these differences reflected direct effects on the bacterial community or indirect effects associated with differences in the plant responses to UV-B (Stapleton and Simmons 2006). Furthermore, plant genotype can influence colonisation and survival of microbial inoculants in the phyllosphere. Correa *et al.* (2007) showed that the survival of a plant growth promoting *Azospirillum* inoculant differed in the phyllosphere of contrasting tomato genotypes, and that the response of the phyllosphere bacterial community to inoculation varied between genotypes.

In the case of fungi, there is limited data on plant genotype-diversity relationships, although several studies have demonstrated differences in the nature of endophytes associated with contrasting host genotypes. For example, distinct communities of endophytes were shown to

be associated with different *Populus* hybrids, with the percentage condensed tannins in bark implicated in directing these differences (Schweitzer *et al.* 2006).

Although plant genotype appears to be an important factor determining the structure of phyllosphere microbial communities, the mechanisms controlling these interactions remain to be elucidated. Various plant science resources are available which show potential for examining plant genotype-phyllosphere microbiology interactions. In particular recombinant inbred mapping populations (Asins, 2002) have the potential to identify plant genes controlling leaf microbiology.

Conclusions and future directions

Although culture-independent molecular analysis of microbial populations in the phyllosphere is still in its infancy, it is clear from recent studies that phyllosphere microbiology is greatly more complex than previously understood. Although progress has been made in elucidating the structure and distribution of microbial communities in the phyllosphere, much less is known of the functional consequences of the community or its composition for the fitness of individual plants, the quality and microbiological safety of fresh produce, and wider environmental processes. Microbes reach the phyllosphere by atmospheric deposition from plant and soil sources, but may also colonise plants through the roots, and become transported to aerial parts. The relative importance of these mechanisms remains to be determined. Although microbial establishment and colonisation has long been recognised to be the result of interplay between plant and environmental factors, and the physiological characteristics of microbial colonists, it has now been clearly demonstrated that within plant species, contrasting genotypes can support different microbial communities. This understanding provides opportunities to understand the molecular mechanisms by which plants control microbial populations in the phyllosphere. Such studies could provide methods

to manipulate phyllosphere communities via plant genotype, providing exciting opportunities to manage applied aspects of phyllosphere microbiology, such as the survival of human pathogens or the activity of beneficial microbes.

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Most understanding of phyllosphere microbiology is derived from culture-based studies and although the culture-independent molecular analysis of microbial populations in the phyllosphere is still in its infancy, it is clear from recent studies that phyllosphere microbiology is greatly more complex than previously understood. Although progress has been made in elucidating the structure and distribution of microbial communities in the phyllosphere, much less is known of the functional consequences of the community or its composition for the fitness of individual plants, the quality and microbiological safety of fresh produce, and wider environmental processes. ¶
There are various developing technologies which show promise to significantly increase throughput of analysis to provide a finer resolution of understanding about the diversity and structure of phyllosphere communities and to link diversity with functioning. Culture-independent analysis using phylogenetic specific primers represents a powerful method to investigate the dynamics and distribution of specific bacterial groups of interest (Sessitsch *et al.* 2002; Miyamoto *et al.* 2004). Additionally multiplex TRFLP, in which several phylogentic groups or functional genes can be analysed at the same time provides an opportunity to improve throughput of san ... [1]

Deleted: There are a number of areas relating to the colonisation of phyllosphere which require more complete understanding. The transmission of microorganisms from roots to aerial parts of plants appears to have been a neglected area of research and the importance of this environmentally protected phyllosphere colonisation route needs to be elucidated. This could be particularly important for soils contaminated with human pathogens. Information is needed to characterise the arrangement and dynamics of communities to time and space, especially at the landscape scale. In particular. the relative importance of environmental factors, location and plant species in determining the composition and dynamics of phyllosphere communities needs to be addressed. Biogeographical approaches to the analysis of microbial communities show potential to elucidate these fundamental relationships (Ramette and Tiedje, 2007).

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Table 1 Percentage frequency of bacterial groups in 16S rRNA gene clone libraries prepared from DNA extracted directly from the phyllospheres of different species.

Figures in brackets give total number of sequences obtained

	Thlapsi geosingense[1] (76)	*Trichilia catigua*[2] (109)	*Trichilia clausenii*[2] (153)	*Campomanesia xanthocarpa*[2] (166)	*Zea mays*[1,3] (30)	*Capsicum annum*[1,4] (39)	*Solanum tuberosum*[1,5] (137)	*Crocus albiflorus*[1]
α-proteobacteria	20.0	10.9	7.8	32.0	30.0	30.8	5.8	15.8
β-proteobacteria	29.0	0.9	1.4	2.4	6.7	17.9	25.5	10.5
γ-proteobacteria	12.0	75.2	63.7	11.4	23.3	25.6	38.6	60.5
Bacteroidetes	17.0	12.9	23.7	20.6	16.7	0.0	2.2	0.0
Cyanobacteria	0.0	0.0	0.0	14.5	0.0	0.0	0.0	2.6
Actinobacteria	4.0	0.0	0.0	1.2	0.0	5.3	8.0	5.3
Firmicutes	12.0	0.0	0.0	13.9	23.3	20.5	19.8	5.3
Acidobacteria	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Reference	Idris *et al.* 2004	Lambais *et al.* 2006	Lambais *et al.* 2006	Lambais *et al.* 2006	Kadivar and Stapleton 2003	Rasche *et al.* 2006b	Rasche *et al.* 2006a	Reiter and Sessitsch 2006

[1] DNA extracted from surface sterilised shoot

[2]DNA extracted from bacterial cells washed from leaf

[3]Field grown, UV and no UV treatments combined

[4]Non chilled and chilled milder Spiral and Ziegenhorn Bello varieties combined

[5] Flowering and senescent Desire and Merkur cultivars combined

Most understanding of phyllosphere microbiology is derived from culture-based studies and although the culture-independent molecular analysis of microbial populations in the phyllosphere is still in its infancy, it is clear from recent studies that phyllosphere microbiology is greatly more complex than previously understood. Although progress has been made in elucidating the structure and distribution of microbial communities in the phyllosphere, much less is known of the functional consequences of the community or its composition for the fitness of individual plants, the quality and microbiological safety of fresh produce, and wider environmental processes.

There are various developing technologies which show promise to significantly increase throughput of analysis to provide a finer resolution of understanding about the diversity and structure of phyllosphere communities and to link diversity with functioning. Culture-independent analysis using phylogenetic specific primers represents a powerful method to investigate the dynamics and distribution of specific bacterial groups of interest (Sessitsch *et al.* 2002; Miyamoto *et al.* 2004). Additionally multiplex TRFLP, in which several phylogentic groups or functional genes can be analysed at the same time provides an opportunity to improve throughput of samples in a cost effective manner (Singh *et al.* 2006). However, these techniques remain time consuming, and future developments will depend on high throughput methods. Phylogenetic microarrays clearly provide a way forward, allowing the presence and amount of thousands of microorganisms to be determined simultaneously, and could also be used to detect novel members of phylogenetic groups. Similarly, functional gene arrays provide a means of

characterising activity of the phyllosphere community, and when used with phylogenetic microarrays, for linking community structure to function (Sessitsch et al., 2006).

Although many studies have demonstrated that plant genotype is an important factor determining the structure of phyllosphere microbial communities, the mechanisms controlling these interactions remain to be elucidated. Various plant science resources are available which show potential for examining plant genotype-phyllosphere microbiology interactions. In particular recombinant inbred mapping populations (Asins, 2002) have the potential to identify plant genes controlling leaf microbiology. Such studies could provide methods to manipulate phyllosphere communities via plant genotype, providing opportunities to manage applied aspects of phyllosphere microbiology, such as the survival of human pathogens or the activity of beneficial microbes.