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Unravelling Detailed Insights on Phylloplane Bacteria: A Review

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ABSTRACT

A fast-growing field of research focuses on microbial biocontrol within the phyllosphere. Phyllosphere microorganisms possess biocontrol capacity with good range of adaptation to the phyllosphere environment and inhibit the expansion of microbial pathogens, thus sustaining plant health. These biocontrol factors are often categorized in direct, microbe-microbe, and indirect, host-microbe, interactions. This review gives an summary of the modes of action of microbial adaptation and biocontrol within the phyllosphere, the genetic basis of the mechanisms and samples of experiments which will detect these mechanisms in laboratory and field experiments. Detailed insights in such mechanisms are key for the rational design of novel microbial biocontrol strategies and increase crop protection and production. Such novel biocontrol strategies are much needed in today's world to ensure sufficient food production to feed the growing world population.

Key words: Biocontrol, Insights, Phyllosphere, Strategies.

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What is phyllosphere?

The phyllosphere, the above-ground surface of plants, is a complex ecosystem where microorganisms and the host plant interact extensively to create specific, yet dynamic, communities. Microbial communities inhabit both the external surfaces (epiphytes) as the internal spaces (endophytes) and these communities play an important role in protecting the plant against diseases Afzal *et al.* (2019). The term phyllosphere was coined by Dutch microbiologist Ruinen in 1956. It includes leaves (phylloplane), stems (caulosphere), blossoms (anthosphere) and fruits (carposphere). Leaves are the dominant tissues in phyllosphere base on surface area available for colonization. The phyllosphere is inhabited by a complex and dynamic community. The composition of this community depends on which microbes reach the phyllosphere in addition to abiotic factors such as climate, season and surrounding land use, and biotic factors such as leaf characteristics and host plant species Smets *et al.* (2016). Microbes arrive on the phyllosphere rather stochastically via the air, soil, rain or insects. However, only selected taxa successfully colonize the phyllosphere Marie *et al*. (2020).

Microbial communities on leaves

The microbial communities of leaves are diverse and include many different genera of bacteria, filamentous fungi, yeasts, algae and less frequently, protozoa and nematodes. Filamentous fungi are considered transient inhabitants of leaf surfaces, being present predominantly as spores, whereas rapidly sporulating species and yeasts colonize this habitat more actively Thapa *et al*. (2018). In particular, leaf surfaces host a dense population of bacteria (*i.e*., epiphytes) estimated to reach 10^7 bacteria per cm^2 of leaf surface. Despite the high cell density, leaf surfaces are a challenging ecosystem to colonise and grow on. Epiphytes must cope with constant ultraviolet (UV) radiation exposure, low water and nutrient availability and large temperature fluctuations throughout the day, making leaves an extreme environment Schlechter *et al*. (2019).

Factors that influences phyllospheric microorganisms

The leaf surface has been considered as a hotspot for bacterial colonists. The establishment of these microhabitats depends on several factors present on the leaves.

Leaf exudates

Leaf exudates such as amino acids, glucose, fructorse, sucrose *etc*. contains many microbial growth factors that facilitates growth of phyllospheric microorganisms. Moisture released during transpiration provides a favourable environment along with the exudates for growth of microorganisms on leaf.

Position of leaf

Position of leaf also affects phyllospheric microorganisms. The surface of leaf which is directly exposed to sunlight is dry and moisture is less so contains relatively less microorganisms than the other surface of leaf which is shaded. It is due to antimicrobial effect of UV light.

Leaf appendages

Leaf appendages such as trough and veins affect growth of phyllospheric microorganisms. Trough which is the shallow depression on the leaf surface is a favourable spot for growth of phyllospheric microorganisms. Therefore the number of microorganisms is relatively higher on depressed area on the leaf. Moreover veins present in leaf form depression in one surface and hence also influence number of microorganisms.

Stomatal cavities

Water droplet present in stomata provides moisture for growth of phyllospheric microorganisms. Therefore population of microorganisms is relatively high in and around the stomatal cavities.

Age and types of plants

With ageing of the plant, rate of secretion of exudates, number of stomata and hence rate of transpiration is altered that affect growth of phyllospheric microorganisms.

Environmental pollutants

Environmental pollutants like cement and fertilizers deposited on the surface of leaf in the form of dust are antimicrobial and they decrease number of phyllospheric microorganisms.

Insecticides, Antibiotics, herbicides etc

Insecticides, antibiotics, herbicides and other chemical sprayed on leaf on agricultural crops are antimicrobial in nature and decreases the number of phyllospheric microorganisms.

Phytoalexin

Phytoalexins are chemicals produced by plant leaf that inhibits phyllospheric microorganisms as well as other microorganism. Phytoalexin are readily produced by plant in response to certain stimuli called elicitor which are biotic or abiotic in nature. Biotic elictors includes various chemicals produce by pathogenic and other phyllospheric microorganisms whereas Abiotic elicitor includes UV light, cold, tissue damage *etc*.

The phylloplane microflora is not only under the influence of the host but also subject to its own influence. An important aspect is the production of self-inhibitory and self-stimulatory products by microorganisms present in the phylloplane.

Direct interactions

Antibiotic Metabolites

Antibiosis is a biological interaction in which metabolites of one or more microorganisms is detrimental to at least one of them. Many phylloplane microbes have been reported to produce antibiotics in vitro. Bacteria like *Brevibacillus* spp., produce non-ribosomal peptides like marthiapeptideA with antimicrobial activity isolated from *Marinactinospora thermotolerans*. Streptocidin D produced by *Streptomyces* spp., a cyclic decapeptide antibiotic from the tyrocidine family was active against Gram-negative bacteria (Edwards *et al.,* 2001). Phenazines inhibit the expansion of a spread of fungal pathogens, such as *Botrytis cinerea* and *Fusarium oxysporum*. It was demonstrated that phenazines inhibit mycelial growth of *Fusarium graminearum* by interference with fungal histone acetylation, and are involved within the formation of a bacterial biofilm on the hyphae, further decreasing pathogenicity (Chen *et al.,* 2018). Biofilm formation on fungal hyphae may be a widespread trait in soil bacteria (Guennoc *et al.,* 2018). Less frequent inhabitants of the phyllosphere, are *Bacillus* spp. which are often utilized in commercial biocontrol products Ongena *et al*. (2008). *Bacilli* isolated from the phyllosphere often engage in inhibitory interactions with other microbial competitors (Helfrich *et al.,* 2018) and their ability to make resistant endospores facilitates their formulation and shelf life (Ongena *et al.,* 2008). The antipathogenic activity of *Bacilli* has mainly been attributed to the synthesis of non-ribosomal peptides and polyketides (Chen *et al.,* 2009). The three classes of non-ribosomal lipopeptidessurfactin, iturin and fengycin, often act in a synergistic manner. Surfactins produced by *Bacillus subtilis* don't appear to play a task within the antipathogenic activity in vitro, whereas they're necessary for biocontrol in planta (Zeriouh *et al.,* 2011). Surfactins trigger biofilm formation, allowing *B. subtilis* to successfully colonize the phyllosphere in sufficient numbers and to manage the discharge of antimicrobial compounds. Therefore, surfactins are rather adaptation factors than biocontrol factors. Wei *et al*. (2016) confirmed that *B. subtilis* employed in commercial products, readily colonize the leaf surface in sufficient numbers. However, despite successful colonization of the phyllosphere, it is difficult to colonize new leaves *(i.e*., dispersal), which limits the biocontrol potential of this product. Fengycins and iturins are mostly active against filamentous fungi, by interfering with the lipid layers and altering cell wall structures Ongena *et al.* (2007). It also inhibits growth of Gram-negative pathogens *Xanthomonas campestris pv. cucurbitae* and *Pseudomonas carotovorum subsp. carotovorum* Zeriouh *et al.* (2011). *Bacillus amyloliquefaciens* strains are proved as successful biocontrol agents against *B. cinerea* on tomato plants. The genomes of *B. amyloliquefaciens* strains contain several gene clusters encoding for the three *lipopeptides surfactin*, iturin and fengycin, and polyketide compounds, like bacillaene, macrolactin and difficidin (Kim *et al.,* 2015). Chen *et al.* (2009) demonstrated that antimicrobial metabolites of *B. amyloliquefaciens*, the polyketide difficidin and the dipeptide bacilysin, are most vital for control of *E. amylovora* on apple blossoms. Similar results were found by Wu *et al.* (2015) also showed that difficidin and bacilysin from *B. amyloliquefaciens* strain FZB42 controlled pathogens *X. oryzae pv. oryzae* and *X. oryzae pv. oryzicola* by causing changes within the cell membrane of *Xanthomonas* spp.

Hydrolytic enzymes

Production of chitinases, as well as other cell wall degrading enzymes, such as β-1,3-glucanase, is a common defense mechanism of plants. It has been demonstrated that *B. subtilis* J9 strain produces extracellular chitinase and protease that protects the strawberry plants against *B. cinerea* in field Essghaier *et al.* (2012). Certain *Lactobacillus* spp. can inhibit hyphae formation of fungi *in vitro* by producing bifunctional enzymes with chitinase/ peptidoglycan hydrolase activity (Allonsius *et al.,* 2019). Even though *Lactobacilli* are not typical phyllosphere inhabitants, and have low survival rate it decreases disease symptoms of leaf spot on cucumber plants, presumably caused by *Pseudomonas syringae* pv. *lachrymans* (Luo *et al.,* 2019) . Next to the production of hydrolytic enzymes microbes can also induce the production of chitinases in the host plant, a common defense reaction in plants.

Quorum Sensing and Quenching

Quorum sensing (QS) is a communication mechanism between bacteria which helps its survival in phyllosphere with biofilm formation, virulence factor expression, production of secondary metabolites and stress adaptation mechanisms. Pathogenic bacteria use quorum sensing to increase their population size and enters the apoplast or plant cell. Gram-negative bacteria often use N-acyl-homoserine lactones (AHLs) as signaling molecules, which are synthesized by AHL synthase (luxI) and detected by a transcriptional regulator (luxR). Gram-positive rather uses small post-translationally processed peptides as signal molecules or diffusible signal factors. A wide sort of small communication peptides exist which produce other functions like *Lactococcus lactis* and *Bacillus subtilis* produce the antibiotic lantipeptidesnisin and subtilin, respectively, which are involved in quorum sensing that helps their survival in phyllosphere and even have biocontrol characteristics (Wei *et al.,* 2016). On contrary to quorum sensing bacteria can also degrade each other's signals, known as quorum quenching. Strains belonging to the genera *Bacillus, Paenibacillus, Microbacterium, Staphylococcus, and Pseudomonas* are able to rapidly degrade the diffusible signal factor, cis-11-methyl-2-dodecenoic acid. This signal is involved in virulence of *Xanthomonas* spp. and *Xylella fastidiosa* during a quorum-sensing AHL-independent way (Newman *et al.,* 2008). In the quorum-quenching strains, the genes carAB, involved in synthesis of carbamoylphosphateis responsible for rapid degradation of this diffusible signal factor. Bacteria containing the carAB genes could reduce disease incidence and severity of *Xanthomonas campestris pv. campestris* in a detached leaf assay experiment with mustard, cabbage and turnip plants and of *X. fastidiosa* when co-inoculated into the xylem of grape stems. Morohosi *et al.* (2009) screened 109 isolates from the potato phyllosphere for the power to degrade several short-chain and long-chain AHLs, as Gram-negative pathogens use AHLs as a signaling molecule to manage their virulence. They screened the isolates *in vitro* by using AHL biosensors, *i.e.*, bacteria that answer the presence of AHLs by producing a reporter protein. One of the enzymes involved in AHL degradation is AHL lactonase, encoded by the aiiA gene, initially identified in *Bacillus* spp. They concluded that quorum quenching could also be a standard trait among the isolates tested and is most often observed in *Pseudomonas* spp. These *Pseudomonas* isolates with strong quorum quenching activity also showed biocontrol activity against *Pseudomonas carotovorum subsp. carotovorum in vitro* and on potato tubers.

Competition for Nutrients and Space

Deficiency of carbon affects the build up of bacterial communities on leaf surface Mercier *et al.* (2000). Therefore, carbon competition will likely play an important role in build up of a community. Phyllosphere bacteria have developed different strategies to utilize all possible carbon sources available. Methylotrophs, such as Methylobacteria, have specialized in the utilization of single carbon compounds, such as methane and methanol as a result they do not rely much on the available sugars present on the phyllosphere. Methylobacteria even modulate the release of methanol, with expansion of plant cells, by encouraging plant growth *via* the production of plant hormones Kutschera *et al.* (2007). The mxaF gene, which contains the site of a methanol oxidation complex, was found to be highly conserved among methylotrophs and is an appropriate probe to screen for methylotrophy McDonald *et al.* (1997). Methylotrophy is thus a crucial adaptation factor for a few phyllosphere bacteria. Another adaptation strategy is the ability to scavenge carbon sources. The presence of a high variety of TonB receptors in the phyllosphere proteome has been suggested as an indication that the residing species can metabolize a wide variety of carbon compounds Delmontte *et al.* (2009). Indeed, TonB receptors are involved in the transport of carbohydrates, siderophores, and vitamin B12, in Gram negative bacteria Schaeur *et al.* (2011). Blanvillain *et al.* (2007) noted that bacteria expressing a high sort of TonB receptors, but belonging to varied taxonomical lineages, share the power to metabolize a good sort of carbohydrates. The over representation of TonB receptors in *Xanthomonas* spp. appears to facilitate their survival within the phyllosphere by making them competitive nutrient scavengers.

Siderophores

Iron is also a limiting element in build up of phyllosphere microbial communities. Secretion of siderophore by microorganisms helps in binding and transportation of iron into the cell. Siderophore production is not only essential for the epiphytic fitness of *Pseudomonas syringae pv. syringae*, but also helps in control of the pathogen *Pseudomonas syringae pv. glycinea* Michavila *et al.* (2017). In addition to iron scavenging, siderophores also helps in non-iron metal transport, sequestration of toxic metals, signaling, protection from oxidative stress and antibiotic activity. The siderophore enantio-pyochelin, produced by *Pseudomonas protegens* CS1, isolated from the lemon tree phyllosphere, showed antagonistic activity *in vitro* against *Xanthomonas citri subsp. citri* Morohosi *et al.* (2009). The siderophores pyoverdine and enantio-pyochelin, synthetized by *P. protegens*, were responsible for its resistance against the mycotoxin fusaric acid, produced by *Fusarium* genus Nascimento *et al.* (2019). Newman *et al.* (2008) showed that mutations in the iucA and iutA genes, responsible for siderophore and receptor biosynthesis respectively, results in a loss of surface motility of *Pantoea stewartia*, reduction of virulence in sweet corn. This indicates that siderophores also play a role in adaptation by mediating motility.

Indirect interactions

Apart from direct interactions, pathogens can also be controlled through indirect means, by modulating the plant's immune system or hormone levels. Microbe-plant interactions that protect the plant against pathogen infection are discussed as indirect interactions. Plants has a complex immune system to prevent infection by responding with appropriate defense reaction. On the contrary, pathogens keeps on evolving to interfere with plants defense mechanism.

Plant hormones

Recognition of beneficial or pathogenic microbes activates the signaling hormones like salicyclic acid, jasmonic acid, and ethylene, where salicyclic acid and jasmonic acid are considered as antagonistic Ngalimat *et al.* (2021). Jasmonic acid and ethylene are usually involved in the defense mechanism against necrotrophic pathogens while salicyclic acid is involved in the defense mechanism against biotrophic or hemibiotrophic pathogens Ongena *et al.* (2008). A first example on how phyllosphere microbes can modulate plant hormone levels, is through the enzyme 1 aminocyclopropane - 1-carboxylate (ACC) deaminase that degrades the ethylene precursor ACC, has been detected in phyllosphere bacteria, such as several *Methylobacterium* spp. Ongena *et al.* (2007) and *Rhodococcus fascians* Pieterse *et al.* (2007). 1-Aminocyclopropane 1-carboxylate deaminase activity lowers ethylene levels, resulting in reduction of plant's defense responses and thereby facilitating symbiotic microorganisms. It also promotes plant growth, since plants become more resilient against environmental stress such as drought, flooding, salt stress or pathogen pressure Ruiz *et al.* (2015). Levels of phytohormones such as cytokinins and auxins, are also modulated by microbes Kwak *et al.* (2014). Both production and degradation of the indole-3-acetic acid (IAA) have been observed in both plant growth-promoting and pathogenic bacteria Ryffel *et al.* (2016). Degradation of IAA can be advantageous for phyllosphere microbes in two ways. On the one hand, IAA is a good source of carbon and nitrogen. On the other hand, manipulation of IAA levels induces physiological changes in the plant, such as cell wall loosening and the release of nutrients that benefit the survival or colonization of the microbe Saghafi *et al*. (2020). *Pseudomonas putida* 1290 is able to grow on IAA as a sole source of carbon, nitrogen and energy Schaeur *et al.* (2011). This ability of *P. putida* 1290 is encoded by the iac gene cluster. Homologs of the iac gene cluster have been identified in strains from various genera, such as *P. putida* GB-1, *Marinomonas* sp. MWYL1, *Burkholderia* sp. 383, *Sphingomonaswittichii*RW1, *Rhodococcus* sp. RHA, *Acinetobacterbaumannii* ATCC 19606 and *Lelliottia* sp. Schlechter *et al.* (2019) However, IAA degradation is an important adaptation mechanism of bacteria on the phyllosphere.

Induced systemic responses

Detection of a microbe by a plant results in strengthening of plant's defense system at the point of recognition and induce immunity against pathogens in the whole plant body Ngalimat *et al.* (2021). This phenomenon is called induced systemic resistance (ISR). Moreover a systemic response induced by the pathogens, protects other parts of the plants, through systemic acquired resistance (SAR). Differences in gene expression in *Arabidopsis* plants was reported by Smets *et al.* (2016) upon inoculation with *Sphingomonas melonis* Fr1 and *Methylobacterium extorquens* PA1 two model commensal phyllosphere bacteria. Colonization by *M. extorquens* PA1 resulted in little or no transcriptional response from the plant whereas *S. melonis* Fr1 changed the expression of several hundreds of genes. Similar results were shown by Thapa *et al.* (2018) where *S. melonis* Fr1 decreased disease development on *A. thaliana* while *M. extorquens* PA1 did not. The transcriptional response induced by *S. melonis* Fr1 were almost similar to responses induced by a *Pseudomonas syringae* DC3000 Smets *et al.* (2016). A hypothesis was laid down by the authors that plants detect the presence of *S. melonis* Fr1 in a similar way as *P. syringae* and respond with an expression of defense-related genes that are involved in plant protection. More recently, Camalexin production by *S. melonis* Fr1within the host plant was demonstrated by Vanderhoef *et al.* (1981) which may be a typical defense response of Arabidopsis and other plants from the Crucifereae family. Due to its lipophilic nature, camalexin is effective against a good range of bacteria and fungi by interfering with the integrity of membranes (*e.g*., by binding to phospholipids). Production of camalexin by Arabidopsis, triggered by *S. melonis* Fr1 is thus postulated to be the mechanism behind the observed plant protection by this commensal. The host's immune system does not only target bacterial or fungal pathogens, but can also protect against viral diseases Vogel *et al.* (2016). The mechanisms by which microbes are detected by the host and subsequently trigger the host's immune reaction, are similar in both non-pathogenic and pathogenic strains. However, non-pathogenic strains lack additional virulence factors, leading to a milder defense response from the host. Biocontrol agents have the power to trigger the system, resulting in induction of resistance to pathogens.

Classes of epiphytic bacteria

- ✓ Epiphytic bacteria that are plant pathogens.
- ✓ Epiphytic bacteria that are biological disease control agents.
- ✓ Epiphytic bacteria that are ice-nucleation active.
- ✓ Epiphytic bacteria that form biofilms.

Epiphytic bacteria that are plant pathogens - Bacterial pathogens colonize leaf surfaces of healthy leaves of host plants. This phenomenon was first reported in 1959 by Crosse of Italy by the isolation of large numbers of *Pseudomononas syringae pv. morsprunorum* (bacterial canker of stone fruit trees) from healthy leaves of cherry. At times of disease onset and development of severe disease, large populations of epiphytic bacterial pathogens have been observed and have been causally linked Waturangi *et al.* (2008). Disease induction is in turn governed by host genotype and other environmental parameters that prevail. When the bacterial population reaches the threshold size, and if there are changes in the virulence of the pathogen or susceptibility of the host genotype, disease incidence occurs. It has been generally accepted that host genotype more than the environment, determines the outcome of disease or no disease Wei *et al.* (2016).

Epiphytic bacteria as biological control agent against diseases - Epiphytic bacteria present in the leaf surfaces or those introduced as foliar sprays do suppress plant pathogenic bacteria (and fungal pathogens). *Erwinia herbicola*, epiphytic bacterium present in the leaf surfaces of rice was known to lower the pH of the rice leaf and thus made it difficult for the bacterial pathogen (*Xanthomonas oryzae pv. oryzae*) to grow Wensing *et al*. (2010). The diverse kinds of metabolites these epiphytic bacteria produce to suppress rice pathogens or cause enhanced plant growth suggest how well they have evolved in their fitness for performing these vital functions. The mode of action may be varies according to the bacteria it may sometimes positive to Ammonia production, HCN production, Siderophore production, and contributes to the growth promotion activity of the bacteria by secreting Indole Acetic acid in plant system.

Epiphytic bacteria that are ice-nucleation activity - Ice-nucleation active (INA) strains of bacteria are those that have the capacity to catalyze the freezing of supercooled water at temperatures as warm as -1°C Wu *et al.* (2015). This capacity is conferred by a protein present within the outer membrane of the bacterial cell. That they participate during a kind of biological cycle of precipitation whereby they're transported into clouds from plant canopies and incite rain thereby causing favorable conditions for his or her growth on plant surfaces was proposed about 20 years ago Zeriouh *et al.* (2014). Today, sufficient evidence and meteorological tools have emerged to re-ignite interest in bio-precipitation and within the ways during which plants play a task as cloud seeders.

Epiphytic bacteria that form biofilms - Biofilms have been defined as frequently observed assemblages made by plant- associated bacteria that have been referred to as aggregates, microcolonies, and symplasmata. Basically biofilms are assemblages of microorganism adherent to each other and are embedded in a matrix of exopolymers Zeriouh *et al.* (2011). There are three well known models of hydrated biofilm structures, those are as follows: The water-channel model, the mosaic biofilm model and the dental plaque biofilm model. This third model of biofilm has high cell densities and arises in a high nutrient environment where they are bathed continuously in a fluid which has a limited fluid flow. While the first two saturated models have been observed in aquatic plants and plants that are raised in hydroponic systems, the unsaturated biofilms have been observed on roots of terrestrial plants by Zeriouh *et al.* (2011). It is known that the densities of microorganisms occurring in leaf surface biofilms is much lower than those observed in water-unsaturated systems. This is an indication, perhaps for low nutrient availability.

Conclusion and Future Research Perspectives

The phyllosphere harbors a diverse set of microbes. These microbes interact closely with each other and with the host plant. Amongst them are not only pathogens, causing disease in the host plant and reducing yields in agriculture, but also some beneficial microbes present which can be the key to environmentally friendly solutions to protect crops from diseases. The phyllosphere is both scientifically and economically an important habitat to study microbial ecology. Because of the importance of many phyllosphere microbial inhabitants to plant health, there will likely be many practical applications that result from a better understanding of the interactions of microbes with plants and among themselves. Thus, phyllosphere microbiology has much to offer to the field of microbial ecology and promises to contribute to more effective and less environmentally damaging means of plant protection.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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