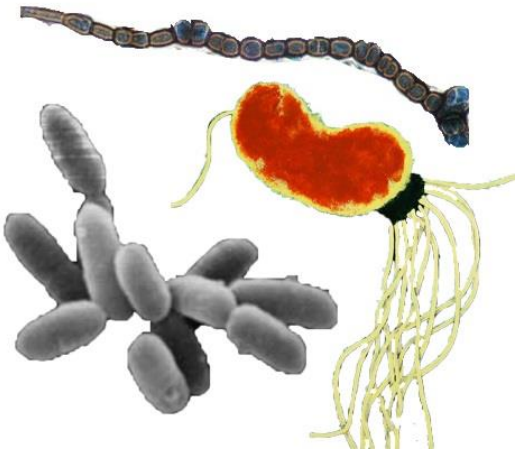
Working with the plant: perspectives for chassis selection in accordance with the phyllosphere

IGEM IONIS APPLIED DESIGN REPORT



iGEM IONIS 2017
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This report aims at giving an insight of the wonderful ecosystem developing on plant leaves and raise awareness for its consideration in case of any microbiological application planned in the future. Written for Softer Shock, it is also done for other iGEM teams willing to apply any compound on leaves (living or not). This report also discusses the choice of the microorganism species for the project Softer Shock and gives additional details on metagenomics, a very interesting aspect of genome sequencing that could be of crucial use for Softer Shock and other projects.
This is the second of a series of reports for the applied design of Softer Shock :

1) Foliar applications: nourishing and protecting from the leaves
2) Working with the plant: perspectives for chassis selection in accordance with the phyllosphere
3) Protective compound choices: mechanism of action at low and high temperatures
4) Biosafety: Killswitch and contamination-limiting diffusion
5) Risk assessment: Toxicity & ecotoxicity studies
6) Final engineering and application : a Softer Shock story

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We really want to express, through this report, how interesting is the **phyllosphere** and how we want to work in **harmony** with it rather than ignoring its fantastic characteristics.
By working **with** the plant rather than **for** the plant, we hope to show that synthetic biology can also be done with an important amount of care for the environment.

The question is: why do we want so much to focus on the plant leaf microbiota for our project, and choose a species of this microbiota as a chassis rather than a classical strain of biocontrol like *Bacillus Thuringiensis*?

Our strategy will consist in a **first application** of our organism on the crops, and then its **sustain with its synthetic amino acids** over time (see the biosafety report).
What we want for our product, is an **organism that develops well on leaves** and can potentially be **sustained** through successive applications of nutrients. We then asked ourselves: **why not just look at the leaf microbiota?**

We should find some **interesting organisms** that are **adapted to leaves** and can indeed be sustained and resist to this environment over long periods of time !

Another aspect of this specific leaf microbiota chassis is **biosafety**. Our logic is the following: if we manage to find an organism **adapted only to leaves** and not to environment such as the soil and the water, we might **reduce the contamination** of our organism in these environment after the application, since it will not be adapted to these ecosystems and die.

Furthermore, as we will see, microorganisms of the phyllosphere have some interesting **phyto-stimulating** and **protecting** capacities that help the plant grow and fight pathogens.

Why not **choose** one of these organisms and **combine** its protective and stimulating capacities with the one acquired with our modification in Softer Shock? This is our reasoning, and this is why we want to present you the **phyllosphere**.

Introduction : What is the phyllosphere?

"**Microbial jungle**" of which the name derives from the more famous **rhizosphere** ("the region of soil surrounding plant roots in which the chemistry and microbiology are influenced by the roots' growth, respiration, and nutrient exchange") (Bulgarelli et al., 2013), the **phyllosphere** can be given the exact same definition at the subtle difference that it applies to the **leaves** rather than roots.

In scientific literature, it seems that experts tend to give **different definitions** of the phyllosphere. Some will say "The phyllosphere is the microenvironment extending from the leaf surface outward to the outer edge of the boundary layer surrounding the leaf and inward into the leaf tissues" (Morris 2002), others will say it implies "**the aerial parts of plants**" as a whole (Verholt 2012), which is nevertheless dominated by leaves.

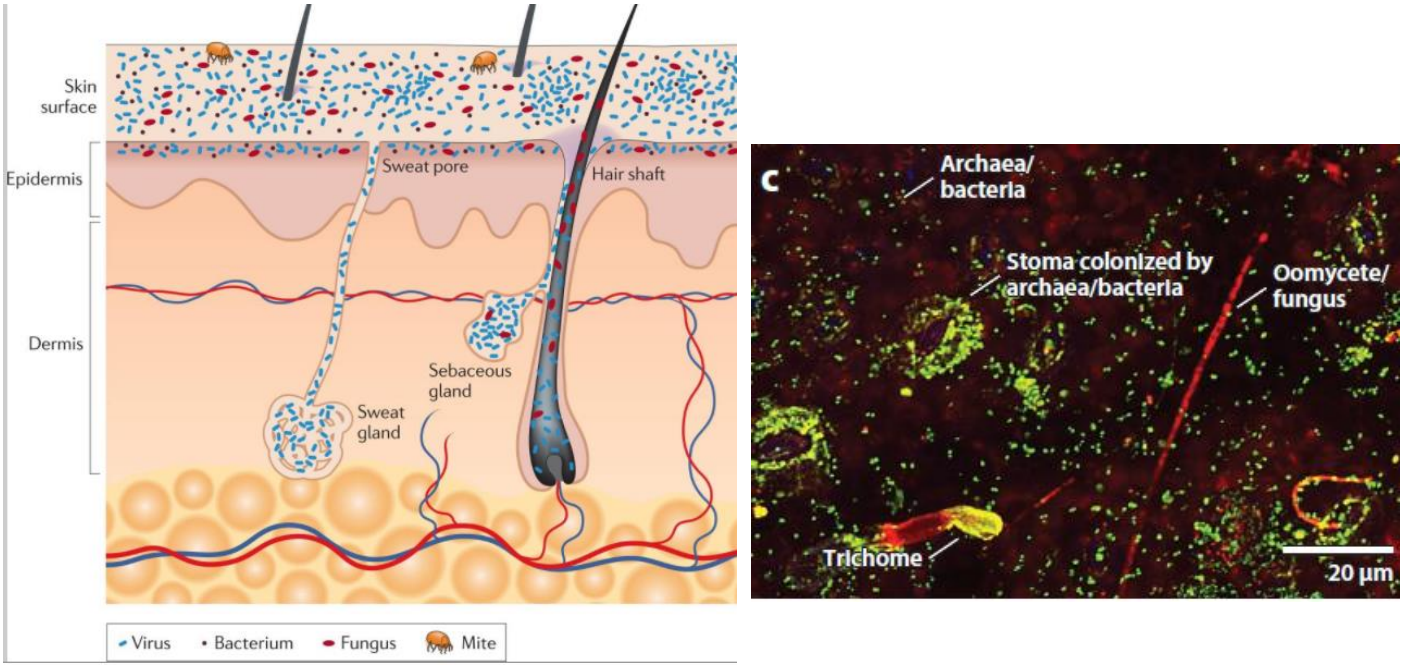
Such **confusion** can be misleading, therefore for this report we will focus on the **phyllosphere of the leaves** rather than the one on the **fruit or barks**, which can be **different**. This choice was made because of the **major surface** that leaves cover and the **better characterization** leaves microbiota are subjected to usually. Note however that **ignoring the microbiota of fruits and bark would be an** error from us, we will notably treat the fruit microbiota when treating the grapevine later.

Hence when the word "phyllosphere" is used here, we will use the definition given by C.Vacher, C Morris and J.Ruinen : "**The external surface of the leaf, as an environment for microorganisms**" (Vacher et al., 2016)

Just like the rhizosphere, the phyllosphere is **very complex** and is home to many **microorganisms of all the living kingdoms** (Eukaryotes, Bacteria and Archaea) .

Its potential **size** (plants inhabit around 90% of terrestrial surface and the total area occupied by plant leaves is estimated to be 1,017,260,200 km²) makes it **one of largest microbial habitat on the Earth surface**, highlighting its importance and showing how crucial it is to understand it (Vacher et al., 2016, Vohrolt 2012).

Think of the **leaves** just as our **skin**: they have **their own microbiota** (Davis 1996)(set of organisms inhabiting a given habitat) (Bulgarelli et al., 2013), even though naturally we don't really pay attention to it. Just as skin **diseases** can emerge from our microbiota, plant diseases like **rusts and mildews** (Morris 2002) caused by **deregulation of the phyllosphere** are very common. Similarly, it has been proven that both human skin and leaves microbiota play an **essential role** in the **protection** of their respective hosts and through different interactions (Davis 1996, Vacher et al. 2016).



Comparison between the human skin microbiota (From Grice et al., 2011) and the leaf microbiota (Vacher et al., 2016). Stoma and trichomes are visible (see "Foliar applications" report for more information on these structures).

The phyllosphere concept **seems to be very recent**, but this topic has been studied since the 1950's, even though recent progress in what is called **metagenomics** (see part 3) had highlighted its role and diversity (Vacher et al., 2016).

It is important to differ organisms that live **on** the plant organs and others that can be found **in** the plant organs.
Microorganisms living **ON** the plant organs, are called epiphytes. and belong to the **phyllosphere and the Phyllosphere Microbial Community (PMC)**. Microorganisms that live **IN** the leaves are called **endophytes** and belong to the **endosphere**, which is the microbiota of the inside of the plant, from the leaves to the roots (Vacher et al., 2016).

As much as the definition of the phyllosphere is **complex**, it is important to precise that it is **far from being totally characterised**.

The phyllosphere is divided into **two domains** :

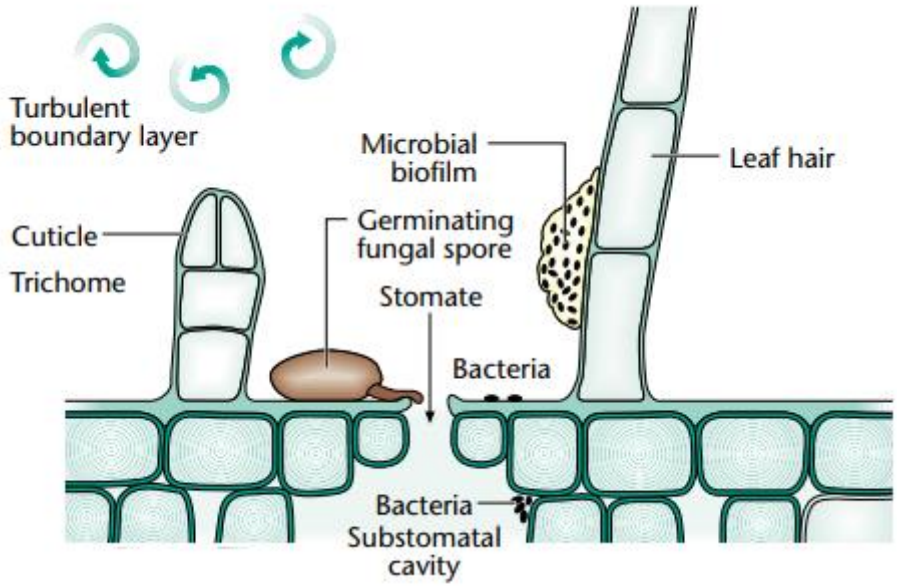
-**The phylloplane**, corresponding to the landscape of the leaf, which means its **cuticle** and its topography governed by structures such as **trichomes or stomata**. (See the Foliar Application report for more information on these structures).

-**The phyllotelma**, corresponding to its waterscape, a thin water film at its surface or "collective body of water that is attached to and influenced by a leaf surface in which phyllosphere microorganisms would reside planktonically" (Doan 2015).
The phylloplane is as thin as 1µm or less most of the time (Vacher et al., 2016) and is **heavily influenced** by phenomenon such as **rainfalls, condensation and evaporation** (Morris 2002).

Remember that a plankton is an organism incapable of swimming against any form of current. These "**planktons" of the phyllotelma** are completed of course by the **microorganisms of the phylloplane**, solidly attached to the leaf cuticle, and **both form the leaf microbiota** (Vacher et al., 2016).

During an interview with **Corinne Vacher**, author of a very useful review for this report, she mentioned the **phyllotelma** as being definitively **the best way** for microorganisms **to "navigate"** across the phyllosphere.

However, it is possible that some, such as **filamentous fungi**, do not require such locomotion, as they simply develop into **mycelium** that progresses through the medium by cell division (Summerbell 2005).



Schematic representation of the major components of the phyllosphere. Note the presence of the cuticle (phylloplane) but the absence of the phyllotelma. Stomate = Stoma . From Morris 2002

For those organisms, of which specificities as well as species will be treated in the part 2, the phyllosphere is indeed a microbial jungle, **influenced by many parameters** and needs to be considered as a **highly dynamic system**.

It is crucial to take the phyllosphere into account for the great development of Softer Shock and to anticipate how the organism we will choose will progress through this vegetal maze and how it will interact with the plant, other microorganisms, and the leaf microclimate.

1) The leaf environment: Paradise and hell on the vegetal mountains

The **surface of the leaf**, as discussed above and in other reports, can be described as an environment on itself, **haven** and **terrible jungle** at the same time, harboring **multiple structures** and **organisms**, and of course, its **own climate and physico-chemical** properties.

As in every spatial exploration in science-fiction movies, when humans want to land on a planet, it is **better to understand it** the best way possible **to be prepared**. We can consider Softer Shock like this, so we better understand first what is the environment our microorganism will interact.

In the report “Folliar application”, we discussed already the anatomy of the leaf, its topography, and the properties of the cuticle and other organs such as stomata and trichomes, and assessed that parameters such as temperature, pH and humidity, as well as sunlight could influence the efficiency of a foliar application of nutrients and microorganisms.

Now we will focus on the **characteristics of a leaf** such as it is **naturally**, and what are the **different stresses** and **conditions** our **microorganism will face** once applied efficiently. Most of the points here have already been mentioned in the “Foliar application” report, but here we will give more insight into them and really try to **describe life at the plant leaf surface**.

If we continue in the space exploration metaphor, consider each **leaf** as having its **own atmosphere** or “**boundary layer**” of 1mm to 1cm that **isolates** it from the external environment (Morris 2002).

As much as planets are different from one another, and that different conditions can be found at the surface on the same planet, the **leaf microclimate** (called **phylloclimate**, even though the term engulfs other aerial plant organs as well (Chelle 2005)) has rather same properties. **Phylloclimates** will therefore **vary according to plant species**, the **position on the leaf**, and be even **different from one leaf to another** of the same plant (Morris 2002, Vacher et al., 2016), so **be careful not to generalise the information that will be given here.**

A) Physico-chemical characteristics of the phylloclimate

Here are summarised the **physico-chemical** parameters of the **phylloclimate**, followed by an interesting curve from Chelle's 2005 review, displaying both variation of temperature and PAR (photosynthetically active radiation):

	Variable	Plant functions affected
Radiation	Spectral irradiance	Photosynthesis
	Ultraviolet	Photomorphogenesis
	PAR (blue, red)	Stomatal opening
	Near infrared	Energy budget
	Thermal infrared	
Surrounding air	Wind speed	Photosynthesis
	Temperature (T_a)	Thigmomorphogenesis
	Humidity	Stomatal opening
	CO_2 content	Energy budget
	Pollutants content	
Organ temperature	Surface temperature (T_s)	Growth and development
	Internal temperature (T_i)	Photosynthesis
		Pathogens development
On-leaf water	Quantity	Pathogens development
	Wetness duration	Pollutant deposition
		Energy budget

The phylloclimate, integration of variables influencing the plant physiology and the phyllosphere, from Chelle 2005. Thigmomorphogenesis = Growth in response to mechanical stimuli. Photomorphogenesis = Growth in response to light.

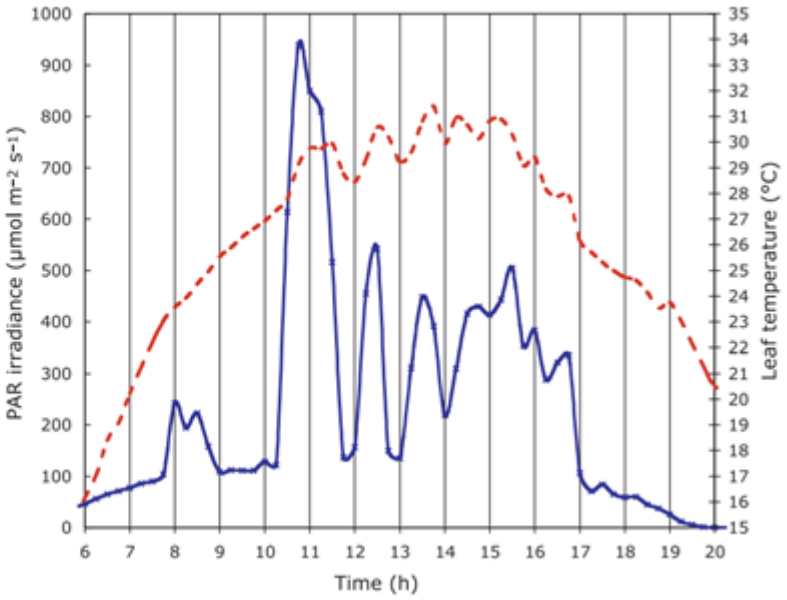


Fig. 2 Temporal variation of 15-min-average incident PAR (solid line) and leaf temperature (dashed line) of a maize leaf. Measurements were made using a Si cell (Solems, Palaiseau, France) and a type-T thermocouple (TC SA, Dardilly, France) in the middle part of leaf number 12 of an adult maize plant (cv Déa) (08/01/2000, Grignon, France) (M. Chelle, M. Chartier & A. Fortineau, UMR EGC, INRA, unpublished data).

Temperature and light radiation are two parameters presenting high degree of variation in the phylloclimate showing the ephemeral state of these variables, from Chelle 2005,

Always remember that **phylloclimate** can be considered as a **very capricious entity**:
“Phylloclimate measurement consists of monitoring irradiance, temperature, water quantity and air features (wind speed, humidity, CO2 content, etc.) for each organ in a canopy using a relatively short time-step (shorter than 20 min).” From (Chelle 2005).

This **ephemeral state** makes direct measurement on the field **very complicated** and modelling has been proposed as a solution (Chelle 2005). We will not give more details here and **we encourage** the reading of **Chelles review** if you are interested in phylloclimate modelling. It might be the **only decent solution for Softer Shock** because we aim at vineyard protection, which are huge crop plantations.

We will treat the specificities of grapevine later in this report for Softer Shock. To that extent, we advise every team to do the same for the plant species they want to work with in their project

a) Temperature in the phyllosphere

Microorganisms of the phyllosphere are considered to have their “**body temperature**” similar to **the one they perceive**, which means the **temperature of the leaf surface** (Bernard 2013).

Temperature of the phyllosphere is influenced by the **radiation uptaken** from the environment and the temperature/pressure **gradients** between **air** and the **leaf surface** (Bernard 2013). A leaf will always, as any other organ, employ different mechanisms to reach temperature equilibrium (heat loss, scattered light, transpiration).

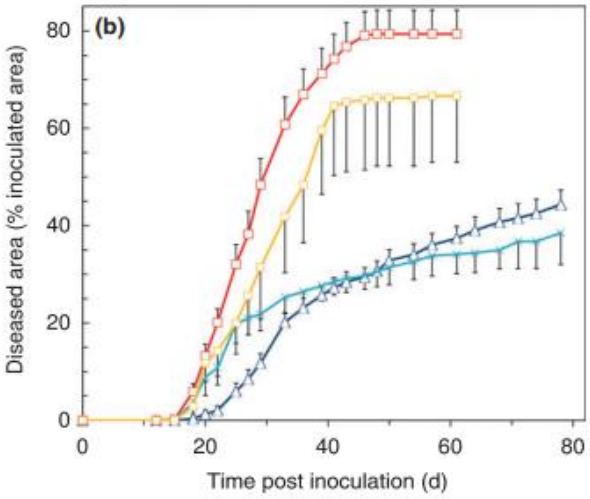
The **phylloclimate temperature** is **different** from the **microclimate temperature** at the surface of the **canopy** (surface of the plant), termed the **mesoclimate**/ This is due to complex interactions between the canopy and energy fluxes in leaves (Bernard 2013).

As said previously, the **differences of temperatures** between the **ambient air** and the **phylloclimate** temperature can therefore reach up to 7°C (Morris 2002), this is very important for our choice of organism and our project.

What is called a **Thermal Performance Curve** can be used to predict the behavior of an organism at a given temperature, and establish its **optimal conditions of development** (Bernard 2013).

As for our planetary metaphor, to insist on that very point, in one single *Tilia platyphyllos* (Linden tree), it has been reported that the phylloclimate temperature of different leaves could vary from 25°C to 37°C, whether the leaves were exposed to the sun or not, hence according to their disposition in the canopy (Leuzinger & Korner 2007).

In an experiment, Frédéric Bernard and his team showed the variation in the development of a disease caused by a fungi called *M.graminicola* on leaves of *Triticum aestivum* (Wheat) according to various temperatures, here is a sample of the results they got :



(b) Optimal and suboptimal leaf temperature classes C1 (triangles), C2 (crosses), C3 (yellow squares), and C4 (red squares). Error bars are confidence interval (95%), for the sake of clarity, only one-half of the error bars are shown.

Thermal Performance curve of M.graminicola on Triticum aestivum leaves.from Bernard 2013 ***C1*** *= 10-12°C* ***C2****=12-14°C* ***C3****= 14-16°C* ***C4*** *= 16-18°C*

Here through this example we can clearly see that **a variation of 6 °C can influence the development** (corresponding to the diseased areas) **of an organism on the phyllosphere**, highlighting the **crucial role of the phylloclimate temperature**.

As the variations of temperature will increase in the future due to climate deregulation, potential impact on the PMC is rather grim and some catastrophic disorders could happen for plants and their associated microorganisms.

For Softer Shock, as grapevines are rather small, and because the human mind tends to organise things very symmetrically, it is possible that **across a vineyard, the phylloclimate temperature will not vary as much between plants and leaves**. The leaves that will receive most of our spray will be the one that are the most exposed to the sun, logically, but we will still need to do some **empirical tests** at different ambient temperatures in the future.

Typical vineyard *showing symmetry and wide exposition to sunlight.*

b) Light impacting the phyllosphere

The phyllosphere is quite logically considered **one of the environment the most exposed to light**. Of course, **shaded leaves**, as much as the **abaxial surface** are not exposed to sunlight, but most of the **PMC** will be affected to **UV** radiations and the **damages** they can cause to the DNA.

Remember that the leaf surface can be considered as a **forest of structures** like trichomes, that might **shelter the microorganisms** from the sun at a microscopic scale (Morris 2002). As seen above, the PMC is also exposed to other radiations than **UV, like PAR or Near-infrared radiations**, but the **UV radiations** are the ones that are the most cited in scientific literature as being a **source of concerns** for microorganisms in the phyllosphere (Vacher et al., 2016).

Moreover, **UV radiations** can also be **deadly to spores**, and can **affect the activity of certain proteins** (Satinder et al., 2006). Microorganisms of the phyllosphere have developed a range of **protection mechanisms** against UV radiations, and **ways to exploit other light radiations** (Vorholt 2012).

These properties will be treated in the part 2, and highlight even more the reason why we should choose an organism already adapted to the phyllosphere for Softer Shock, since it will be less likely to be damaged by the UV radiations and take advantage of others.

c) Other parameters

Humidity is crucial however, because of the direct impact it can have on the **state of the phyllotelma**, its width and solute concentration. **High humidity** will certainly **favor microorganism growth and dispersal** on the leaves, and **increase accessibility of nutrients** (see below) (Morris 2002). The **relative humidity** perceived by the **phyllosphere** is also **generally greater than the one of ambient air** (Morris 2002).

PH is a **crucial parameter** for organism growth. In the phyllosphere, the **pH of the phyllotelma** is **dependent on the origin of the water film** (guttation dew, precipitation, irrigation). **Acidic rains** for example, can hence induce the **lowering of the phylloplane pH**, and influence organism growth and development (Andrews 1991).

As much as it can be important for plant physiology and influences canopy architecture, **windspeed influence** had been described as being **close to null** in the phyllosphere, except in the case of extreme events such as gusts. The PMC is believed to be protected by the so-called **boundary layer** and the **leaf topography**, hence wind speed does not have direct impact on the phyllosphere microorganisms (Morris 2002). Wind can induce **leaf fluttering**, which **decreases temperature and sunlight uptake** (Morris 2002).

Here has been shown the complexity of the phylloclimate and the conditions in which the PMC lives with.

We can't insist on this point more, but these parameters are really difficult to anticipate and are very capricious. The leaves could actually be seen as multitude of ever-changing planets, vast forests shaken by multitudes of tempest and harsh conditions if we finish with our planet exploration metaphor.

B) Ressources available in the phyllosphere

To grow and prosper in the phyllosphere, the **PMC** (Phyllosphere Microbial Community) **needs nutrients** and **essentials elements**. Although it is considered **usually poor in resources** (**oligotrophic**) as compared to other systems such as the rhizosphere, nutrients under different form are available in the phyllosphere, but **heterogeneously** (Vacher et al., 2016, Vorholt 2012).

a) Carbohydrates,

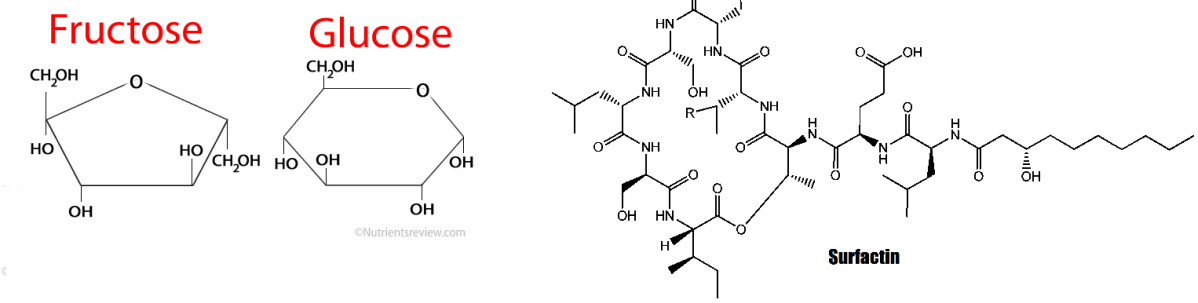
Essential for numbers of metabolic pathways, **carbohydrates** in the phyllosphere **originates from the photosynthesis** undergone majoritarily in the leaves cells of the adaxial surface forming the palisade mesophyll. The pathway permitting the presence of carbohydrate in the phyllosphere is termed the **apoplastic pathway** (Vacher et al., 2016).

The **apoplast** is the extracellular space located into the leaf. In the apoplast, carbohydrates can be found after their secretion by the cells undergoing photosynthesis. These **carbohydrates are usually re-located** to the **plant tissues that need it the most** through phloems, but it has been shown that **some of it can diffuse** (**leaching**) across the cuticle, and be accessible to the PMC.

The carbohydrates found in the phyllosphere will more likely be **simple sugars** such as **glucose and fructose**, whereas sucrose will predominate inside the leaf. **Leaching** has been shown to **correlate positively with the leaf age and with the leaf external wettability** (Vacher et al., 2016).

Interestingly enough, **microorganisms** of the phyllosphere (especially bacteria) will **"play"** with such correlation and secrete **biosurfactants** (much like the adjuvants mentioned in the report "Foliar application"), like the **viscosin** of *Pseudomonas Fluorescens* (Satpute et al., 2010).

These **molecules** (lipopeptides, glycopeptides, secondary metabolites…) have been suspected to play **important roles** for increasing the wettability of the leaf therefore **increasing leaching of carbohydrates**, and even **improving motility** on the leaf (Burch 2011). Some even, have **antibiotic properties** (Hamley 2015).



Fructose, glucose (carbohydrates) and a biosurfactant (surfactin, Hamley 2015), three molecules playing role in the survival of microorganisms in the phyllosphere

Biosurfactants are really interesting molecules and are very diverse, we encourage readers to further explore this topic as it will not be treated with more details here.

It would be interesting to select a **chassis secreting biosurfactants** for Softer Shock as it could make the **addition of adjuvant** in our spray **less important**, lowering the cost and the potential environmental impacts. **Care must be taken** though because some of these molecules affect the cuticular waxes and could damage the plant (Burch 2011). It is with the help of the syringafactin, a biosurfactant produced by the pathogen *Pseudomonas Syringae*, that this bacteria manages to penetrates into leaves (Vacher et al., 2016).

b) Volatile organic compounds (VOCs)

Emitted mostly by the stomata, these families of compounds are **by-products of plant metabolism** (Verholt 2012) and includes **aldehydes, terpenes, alcohols and esters**. The most abundant are **isoprene and monoterpenes**, believed to protect plant against temperatures (Vacher et al., 2016).

One VOC, **methanol**, has been documented as being a **primary carbon source** for bacteria of the family ***Methylobacterium*** and yeasts (*Candida Boidinii*) (Verholt 2012), which can metabolize it, and are ubiquitous in the phyllosphere (Morris 2002), showing yet another **adaptive feature of the PMC**.

Some of these compounds might though have antimicrobial properties, and can diffuse through the cuticle in addition to their emission from the stomata (Morris 2002).

c) Carbon dioxide and oxygen

These two compounds are of course found naturally in the atmosphere surrounding the phyllosphere, but **major fluxes** are present around the **stomata**, which are master regulators of plant gaseous exchanges (Vacher et al., 2016).

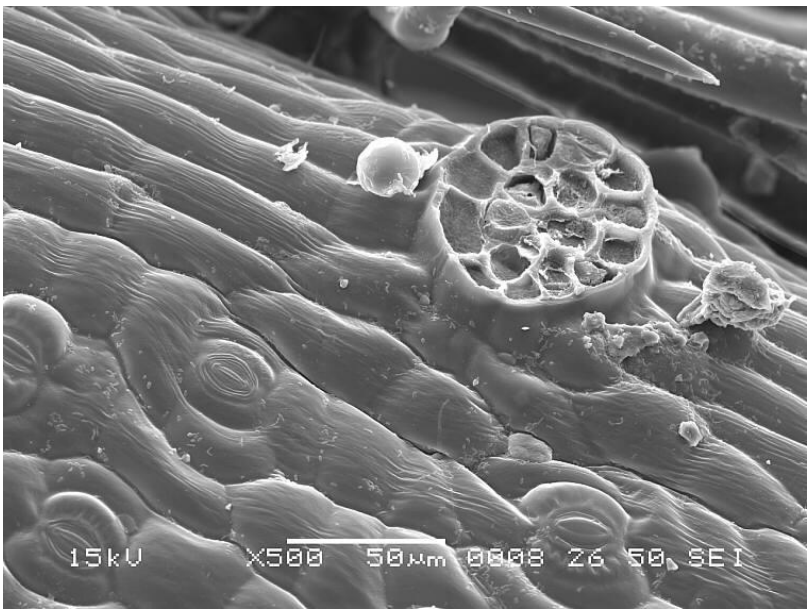
Even though **CO2** has not been documented as an influencing factor for the PMC, **O2** can be metabolized into **Reactive Oxygen Species** (ROS), signaling molecules that c**an cause damages to cells** at high concentration (Vacher et al., 2016). O2 can also be used of course for **aerobic respiration** by the PMC.

d) Other nutrients

Numerous metabolites other than VOCs can be found in the phyllosphere. **Amino acids, organic acids** and **essential oils** are also present and accessible for the PMC (Morris 2002). Amino acids and ammonia are suspected to be used a nitrogen sources, and some bacteria have the ability to fix the atmospheric N2, just as the legume nodule bacteria in the rhizosphere (Verholt 2012).

Such **nutrients** are present around **trichomes** as well, as they contain naturally those substances (glandular trichomes) and they can **leak out** and be accessible to the PMC.
Macro and micro-nutrients (see "Foliar application report) such as **phosphate and sulphate** are also very important for the PMC (Verholt 2012), they are naturally present on the leaf surface at various concentration, and foliar applications of nutrient sprays can directly interact with the phyllosphere quite logically.

In the iGEM Ionis, we love metaphor, as you might have noticed. In the abyssal part of the ocean, nutrients are very scarce, and when a giant mammal such as a whale dies, its body falls to the bottom of the ocean and becomes a major source of nutrients for bacteria and organisms of this ecosystem (**whale falls**) (Mladenov 2013). Well the whales of the phyllosphere could be the trichomes. When a **trichomes breaks**, it liberates a wide variety of nutrients that are available for the PMC (Morris 2002). The comparison needless to say, stops here.



A broken trichome, "whale fall" of the phyllosphere

Here was then described the global environment of the phyllosphere, nutrients and physico-chemical variables. We are certain that researches in this domain will progress in the near future and that more and more resources will be added to the already available ones today. It is now important to describe the inhabitants of such hostile and interesting environment.

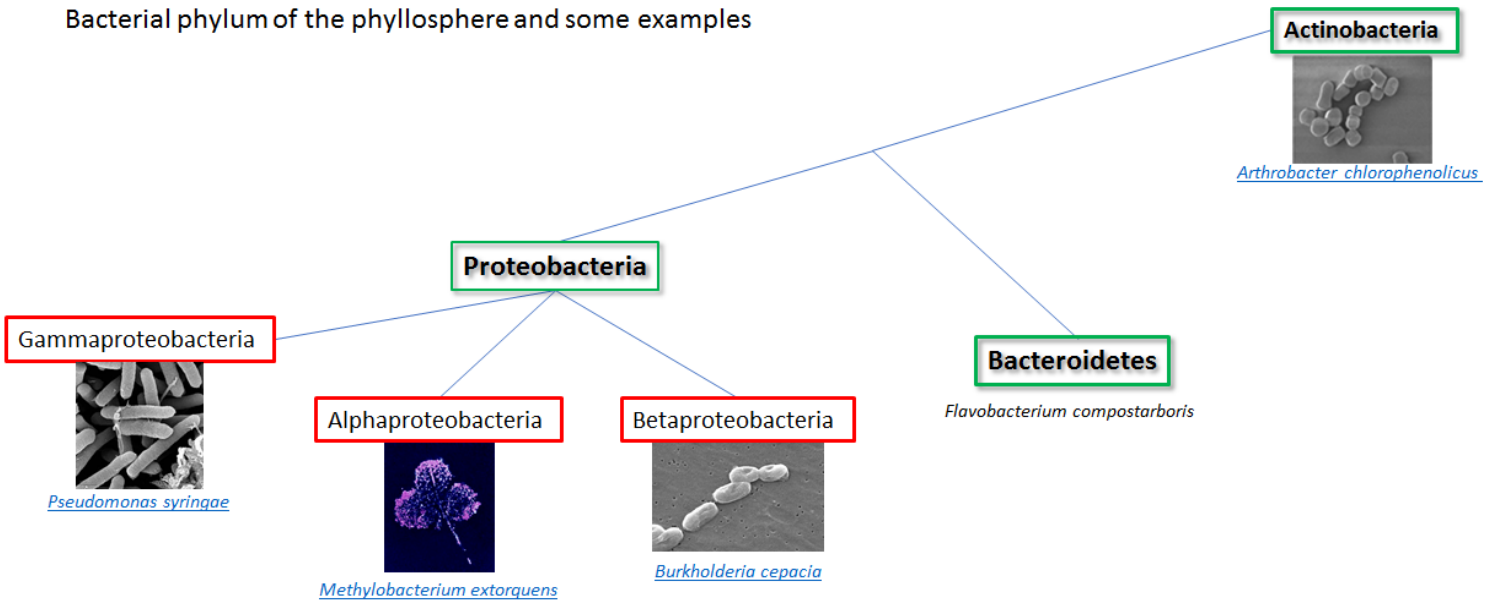
2) A dynamic population of different organisms interacting closely with its host

Considered **less numerous** than their rhizosphere counterpart (Bulgarelli 2013, Morrris 2002), the **microbial species of the phyllosphere** are nevertheless **various** and engulf **all the living kingdoms**.

Here will be described the microbial population of the phyllosphere, these population **vary in identity over time**, **space**, and **species**, but generalisation can be made, depending on the phylogenetic level (especially for genus and order (Verholt 2012)). Later on this report we will, for Softer Shock, focus on grapevine phyllosphere microbial community.

A) Global analysis of the microbial population

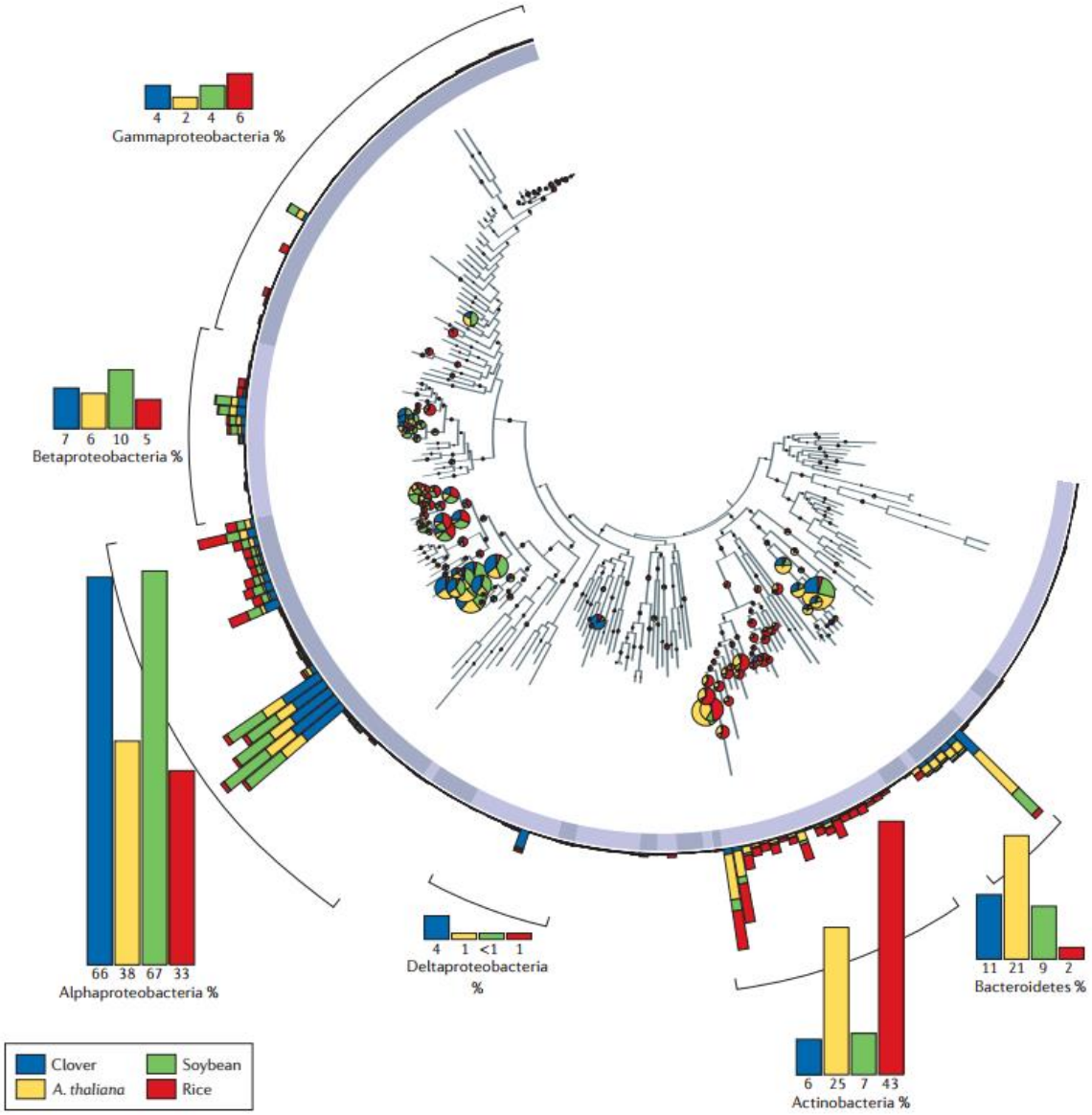
The **PMC (Phyllosphere Microbial Community)** is **diverse** and for this report, the important aspect we want to show is this very property. What will follow is hence not a precise study of each species in the phyllosphere, but rather a global view of the **phylums** and the interesting adaptive properties of some examples.
Following, a brief recapitulative tree of **phyllosphere bacteria**:



Bacteria of the phylloshere in brief, adapted from Vehrolt 2012

It is needless to say that bacterial population is much more diverse that those five species, but as we roamed across scientific literature, it seemed correct to provide a visual simplified table for everyone.

In the Verholt's review of 2012, a great representation of bacterial phyllosphere community complexity was showed :



Bacterial diversity of the Clover/A.Thaliana/Soybean/Rice phyllospheres, from Verholt 2012

As seen, this population **varies** more in **term of proportion** than in term of phylums, this is **highly linked** to the **metabolism of the plant** and the **environment** it lives in.

Keep in mind also that a **phylum can contains hundreds of different species**, and that it is common to find for example a Pseudomonas (Gammaproteobacteria) species on a plant phyllosphere, and a totally different Pseudomonas species on another plant's phyllosphere. This particularity concerns of course the two other kingdoms (Eukaryotes and Archaea) and highlights the **close interaction between the phyllosphere and its host plant** (Verholt 2012).

We can just draw another comparison with the skin microbiota, because the latter varies according to the individuals and their lifestyles.

Knowing the phyllosphere of the target plant before any application is hence very important due to these variations, and this is where **metagenomics** is crucial (see later in this report).
Bacteria of the phyllosphere **have not been studied very precisely** until now, so our knowledge about them is **very limited**, except for some species belonging to *Pseudomonas* or *Methylobacterium* for example (Vacher et al., 2016).

Additionally to bacteria, the phyllosphere is also richly populated by **filamentous fungi and yeasts** (*Ascomycota, Basidiomycetous*) (Vacher et al., 2016). Just like bacteria, these population may **vary** according to the plant **species, age, season, and surrounding environment**.

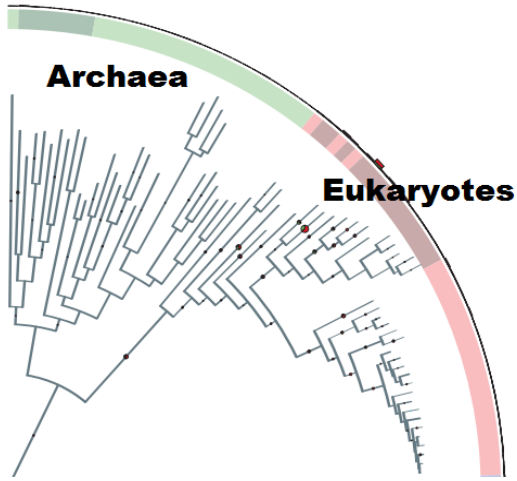
Some are nevertheless very common, just like ***Aureobasidium Pullulans***, a yeast-like fungus that can be found on both leaves and fruits of grapevines (Vacher et al., 2016), and that can account for about **90%** of the fungal population of the phyllosphere. This species has been used by the INRA as a **biocontrol agent** against the pathogen *Botrytis cinerea* (Pestourie 2014).

Some of the **fungal population** of the phyllosphere is however potentially **composed** of **opportunistic pathogen**, even though protective properties have been highlighted, like antibiotic secretion (Vacher et al., 2016).

In the literature we roamed across, it seemed that much more interest was given to bacteria as compared to fungi and archaea (the latter occupy only 1% of the total PMC), this is mostly explained by the fact that **bacteria account for most of the microbial population of the phyllosphere** (Verholt 2012, Bulgarelli 2013).

Nevertheless, fungi are not to be ignored, it has been shown that yeasts can be as numerous as 500 cells/cm2 (Vacher et al., 2016), and that an important part of the fungal population of the phyllosphere is in **“dormant state”**, waiting for the leaf to **decay** and start nourishing on its remain (such organisms are called **saprotrophs**) (Vacher et al., 2016).

Here is an interesting tree showing the diversity of both archaea (Green arc) and eukaryotes (Red arc) in the phyllosphere, of which fungi are part:



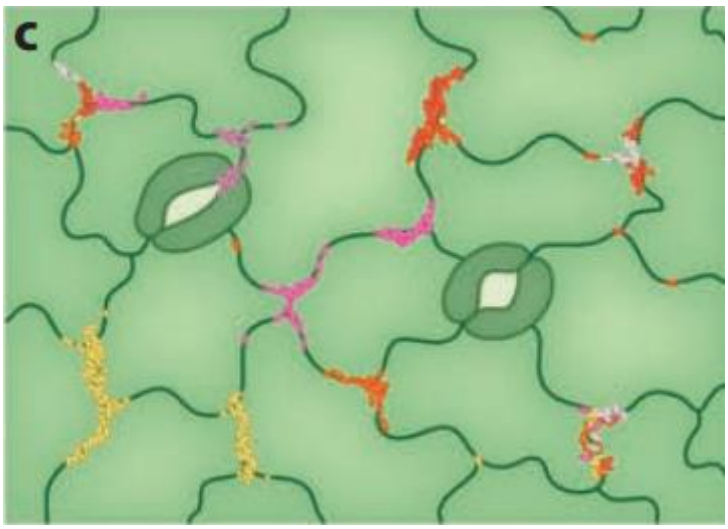
Diversity of the eukaryotes and archaea in the soybean/rice/clover/A.Thaliana phyllospheres, from Verholt 2012

We did not treat **archaea** with much details here, as they are usually **poorly studied**. Archaea are organisms that are sometimes very **well adapted** to **rough environment**, so this is not surprising to find them in the phyllosphere. Maybe some **incredible properties** could be found in archaea, but only the future will tell if this is true, maybe they could be the **"unsung heroes"** of the phyllosphere, who knows?

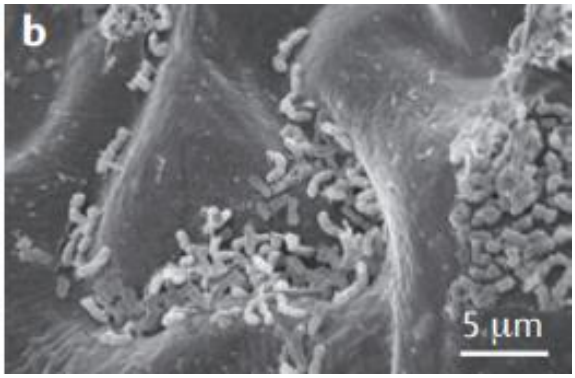
We described the environment, its inhabitants, but apart from some details, did not cover where these organisms are located exactly. This will be treated in the next part.

B) Where to live in the phyllosphere?

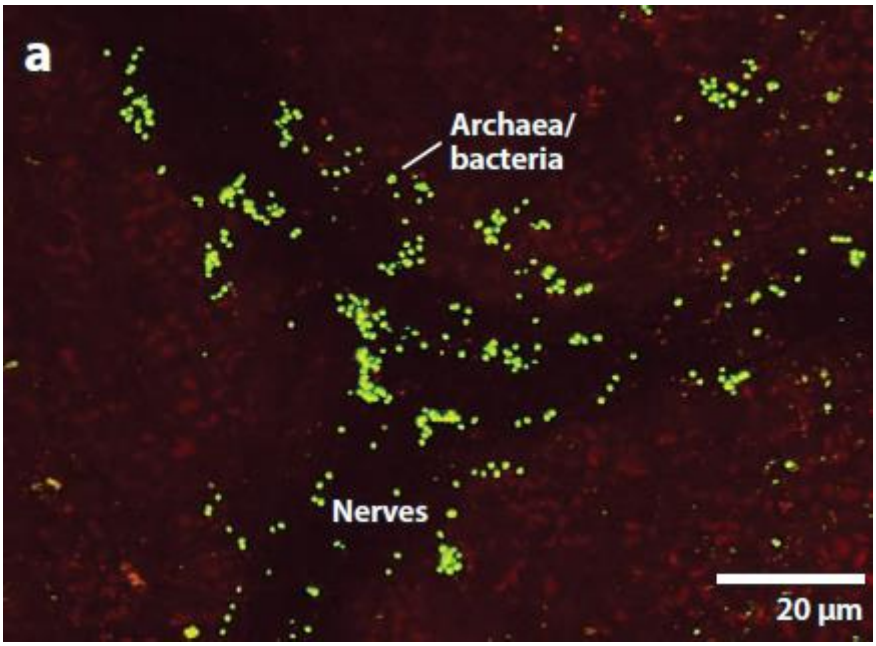
To give a visual insight, here are some pictures we found in the literature :



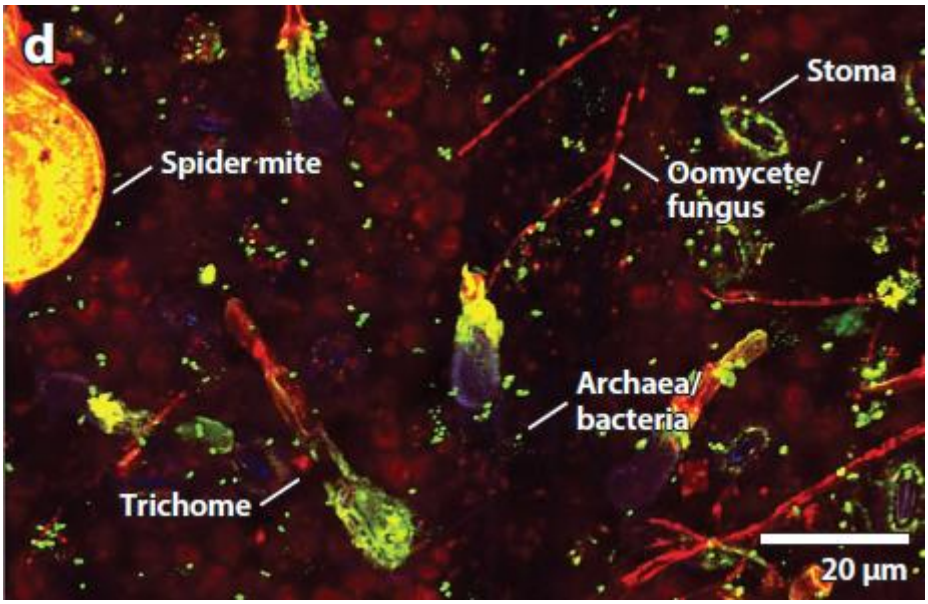
The abaxial leaf surface, populated by microbial aggregates, from Muller 2016



Bacteria favoring the junctions of leaf epidermal cells, where nutrients and leaching carbohydrates can be found, from Verholt 2012



Archaea and bacteria also aggregates on leaf nerves, "nutrients pipes" and phyllosphere mountains, from Vacher et al., 2016



Leaf structures like trichomes and stoma are favored by the PMC, pioneer filamentous fungi extending their mycelium in quest for more nutrients. A spider mite is also detected, from Vacher et al., 2016

One clear trend comes out of these pictures: **where there is nutrient, there is life**. This is rather logical, and this applies to every ecosystem.

As said before, the phyllosphere is an **oligotrophic system,** meaning the repartition of nutrients is **heterogeneous** (Morris 2002). The **PMC repartition follows the same pattern**: it locates around the **strategic points** such as the **trichomes** (fallen or glandular); the **interspace between the leaf epithelium cells**, chimney of carbohydrates and plant metabolites; the **stomata** and **hydathodes**, exchange points between the inner leaf and the surrounding environment; and the **leaf nerves**, where nutrients are certainly leaching even more (Verholt 2012).

Of course, **stresses** like **temperature and UV** will also force the organisms to organise near **shaded points** like leaf hair and trichomes, and favor the secretion of **Extracellular Polymeric Substances** (EPS), that keep the population hydrated and concentrate detoxification enzymes (Verholt 2012).

The PMC is not organised in a "single cell" model, but rather, as shown in the pictures, as **aggregates of 1000 or more cells** of different species (regulated by **quorum-sensing**). Their size correlates with water and nutrient availability (Verholt 2012). This shows that **life in the phyllosphere is everything but randomly organised**, and follows a **clear survival pattern**.

These points are **absolutely crucial** for Softer Shock: **how will we manage to create a uniform layer of proteins against sunlight if we expect our organism to develop only on certain points? For the cold application**, this will be less problematic, as the main sources of ice crystals are microorganisms, and our chassis will most likely locate at the same points as them (see the report "Choice of proteins" for more information).

This however give an insight about our potential final strategy and chassis choice : we will **need an organism** that can be found in **as many leaf spots as possible**, and **encapsulation methods** could permits its development **outside of those nutrients oasis** (just like a spaceship).

C) The super-powers of the PMC

To **face the harsh phylloclimate**, **competition**, and **exploit its environment** the best way possible, the organisms of the PMC express **different proteins and compounds**:

-**To counter the effect of Reactive Oxygen Species** emerging from high concentration of O2 around stomata for example, enzymes such as **photolyases**, **Dps** (Dna protection during starvation proteins), **catalases and superoxide dismutases**, that protect DNA from damages, can be found in **high concentration in the PMC** (Verholt 2012). These enzymes also protect the DNA from UV damages.

-**To face desiccation** (drought), as said above, the PMC species will tend to **aggregate** and surround themselves with **EPS**, **keeping them hydrated**. They will also secrete **biosurfactants** that increase **leaves wettability** (Verholt 2012). **Osmotic stress** due to variation in water availability is being dealt with by the expression of compounds such as **trehalose and choline** (Verholt 2012), the former can also protect the **cells against freezing damages** (Termont 2006).

-To **protect themselves from pathogens**, enhance their competitivity, bacteria of the PMC have been found to **secrete antibiotics** such as the **2,4-DAPG** of *Pseudomonas Fluorescens* (Raaijmakers 2012). These compounds can also be secreted by fungi and **shape the overall microbial community as well as protecting the plant** (Muller 2016).

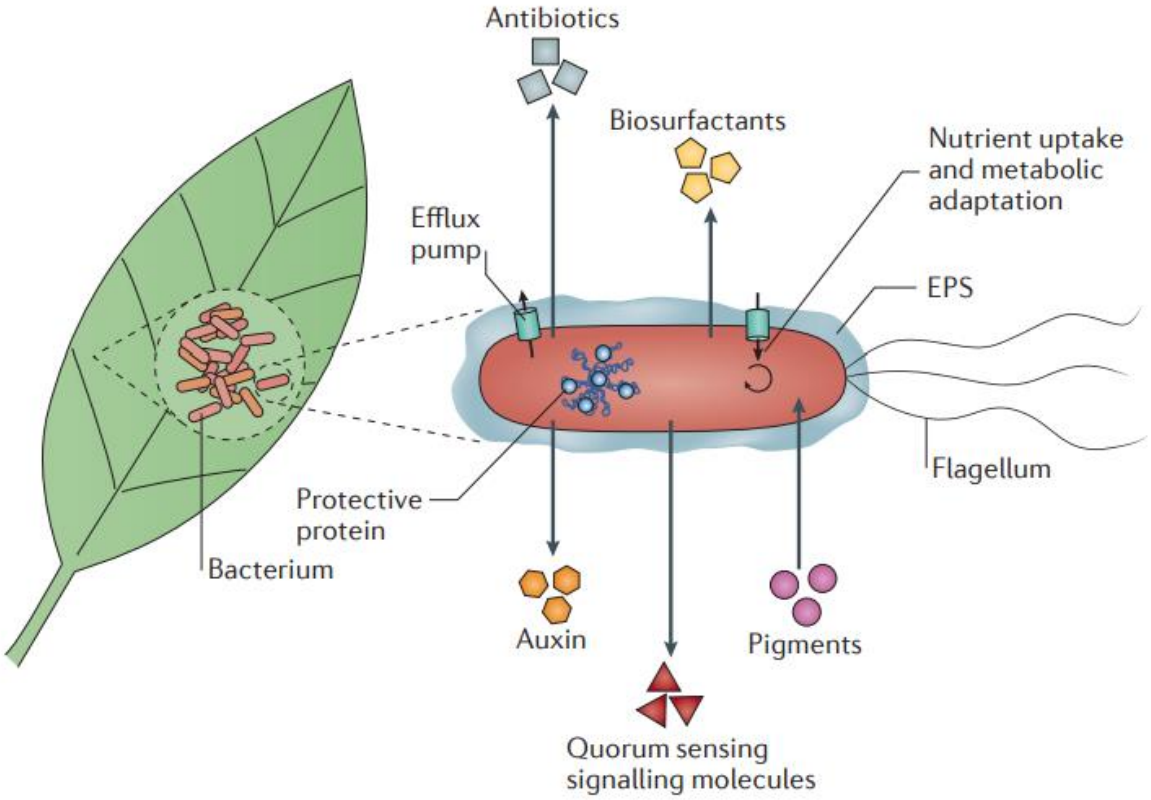
The mechanism of antibiotic secretion can hence be seen as a **"beneficial and structural war"** between the organisms of the PMC and the plant itself.

Some **pathogens** present in the phyllosphere, like the famous ***Pseudomonas Syringae***, counter the plant **immune system** recognition that is triggered by **PAMPS** (Pathogen associated molecular patterns) and usually induces stomatal closure. They indeed secrete **coronatine**, re-opening the plant's stomata and **forcing their entrance** in the vulnerable apoplast (Vacher et al., 2016)

-Some organisms of the PMC are suspected to **counter wash-off** by **rainfall** by secreting **adhesion molecules** (like proteins of the **hemagglutinin** family), helping them to settle and **favoring aggregation and stress resistance** in an **EPS matrix** (Verholt 2012).

-To cope with **nutrient scarcity**, organisms of the PMC have shown **adapted metabolisms**, such as **methanol or methyl-chloride** use as a **carbon source**. Additional to **biosurfactants,** that increase nutrient **leaching**, they can also produce **plant hormones** such as auxin (Verholt 2012) and enzymes like **cutinases** (Morris 2002) that loosen the plant cell wall and waxes and **increase availability of nutrients** even more. **Nitrogen fixation**, **light-using bacteriorhodopsin**, as well as **over-expression of mono/di saccharides** and **plant metabolites transporters** have also been recorded, showing **adaptation** to the nutrients and resources in the phyllosphere (Verholt 2012, Bulgarelli 2013)

Here is a recapitulation of these traits in a phyllosphere bacterial model:



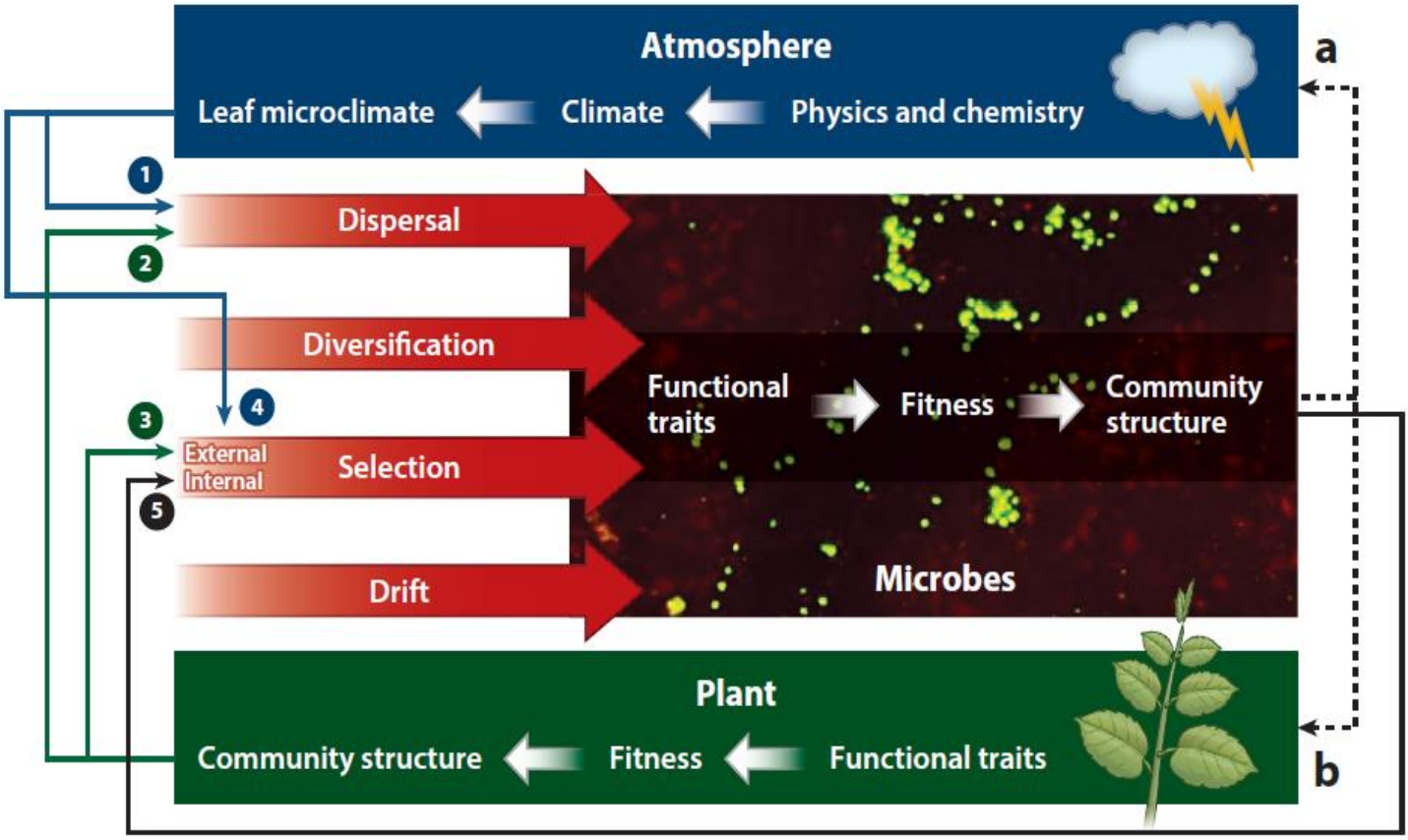
Proposed bacterial traits involved in adaptation to the phyllosphere, from Verholt 2012

Keep in mind that t**hose traits are most likely found in Fungi and Archaea**, but as bacteria are the most studied organisms in the PMC, most of the information provided by the literature comes from their study. Maybe more “super-powers” will be found in the future by studying these two other kingdoms.

Inhabited with **gifted microorganisms**, the phyllosphere is nevertheless **very dynamic** and the **PMC will vary according to various mechanisms**, this will be treated in the next part.

D) The dynamics shaping the Phyllosphere Microbial Community

Four mechanisms have been described influencing the **dynamic of the PMC** and explaining its **variability across the year**. These mechanisms are **highly linked to the phylloclimate** and the **host plant** characteristics:

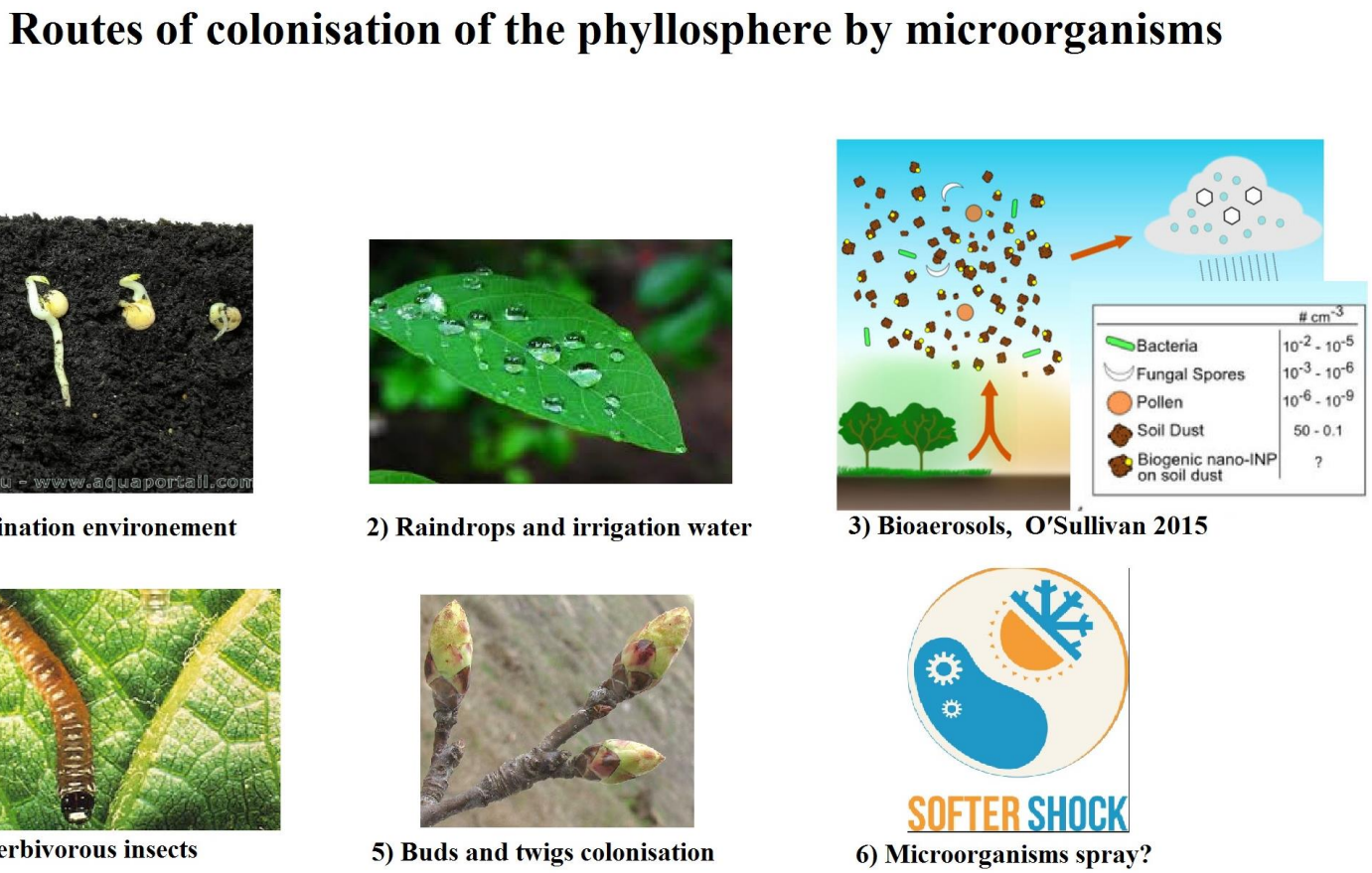


The PMC is at the interface of diverse biotic and abiotic interactions, from Vacher 2016

As seen here, **the plant and phylloclimate influence directly the shaping of the PMC**, which in turn influences them both. It has been theorised that the plant and its PMC form what is called a **holobiont**, an ecological unit of different species interacting together. This **holobiont PMC-Plant** is believed **to evolve together to favor survival of both** (Vacher 2016).

As seen in the picture above, the four mechanisms of evolution of the PMC are:

-**Dispersal**: The way the organisms arrive originally on the plant. They can arrive by five (six?) different ways :



1) Germination environement

2) Raindrops and irrigation water

3) Bioaerosols, O'Sullivan 2015

4) Herbivorous insects

5) Buds and twigs colonisation

6) Microorganisms spray?

The five routes of colonisation of the PMC, supplemented by a sixth? Adapted from Vacher 2016

We are not going to treat those six points with more detail (except the sixth, which is quite logical), but we advise other teams to go more in depth into these routes, especially the **bioaerosols**, which are organisms (dormant or not) in suspension in the air, that can impact many environments (*Pseudomonas Syringae* is for example responsible for raindrops) (Vacher et al., 2016).

-**Evolutionary diversification** is also a mechanism of evolution of the phyllosphere. The PMC is believed, with its pool of Archaea species and fantastic bacterial and fungal diversity, to be **a reservoir of very interesting genes** that are under UV stress, ROS, and potential damages, **causing mutations and creating diversity**.

The spearhead of this diversification is of course **horizontal gene transfers** between species, possibly common in the **cell aggregates** discussed above (Vacher et al., 2016). **The gene transfers and UV mutations** are pression absolutely **crucial for Softer Shock**, and we need to assess them if we want a durable and protected system.

-**Natural selection** is of course shaping the PMC. On one hand, the **external pressure applied by the phylloclimate and the plant** itself favorites organisms that can fix efficiently to the cuticle, survive UV radiations and temperature shifts, and grow efficiently.

On the other hand **internal selection** is regulated by the **sacred rules of biological interaction**s like **parasitism , commensalism, mutualism, predation and amensalism** (Vacher et al, 2016). Traits like **antibiotic expression** can in this case be very valuable for example. This sum of external and internal pressures are described in the next picture :

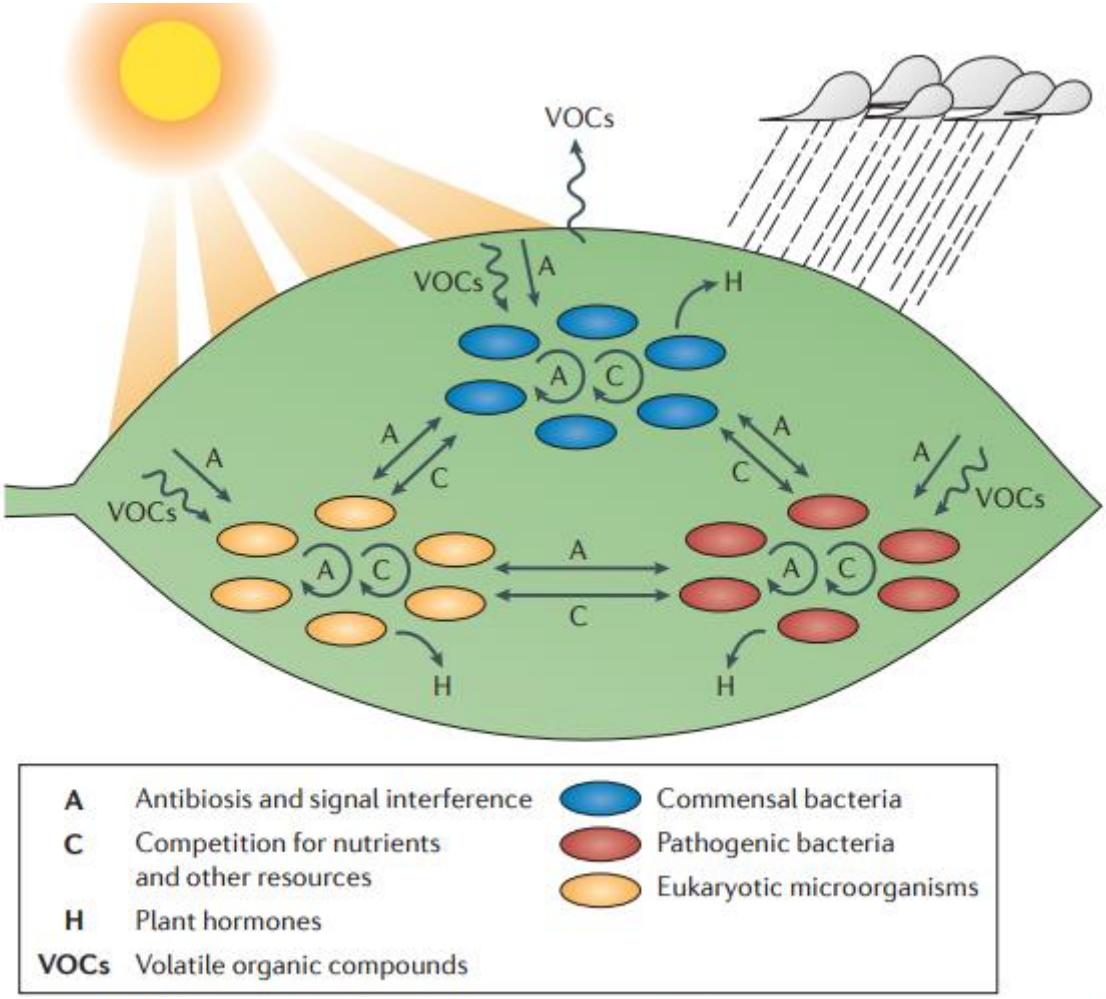


Figure 5 | **Multipartite interactions occur in the phyllosphere among commensal and pathogenic microorganisms and between microorganisms and the plant.**

From Verholt et al., 2012. Antibiosis refers to the interaction of two organisms in which one inhibits the growth or kill the other. We can find the VOCs and plant hormones discussed above. Eukaryotes are mostly fungi but can also be organisms like acarine (see the spider mite in the previous pictures).

Crucial is the notion of priority effect : the first organisms that colonise the phyllosphere can determine the number of other taxa that will be later in the PMC (Vacher et al., 2016). This is incredibly important for Softer Shock, what if we could identify early on the organisms in the PMC of a plant to better choose a suitable chassis? What if we could incubate early seeds with our organism to favorise its development in a sustainable way?

This will probably not happen for grapevines, as most of the vineyards are already settled and few new germinations occur, but such information could be very relevant for other projects or other plants if we want to broaden our application.

-Finally, **drifting** influences the **shaping of the PMC**. “Ecological drift causes species abundances to fluctuate randomly, lowering diversity within communities and increasing differences among otherwise equivalent communities” (Gilbert 2017).

In the phyllosphere as well as in many ecosystems, **few species occupy most of the microbial population, and a lot of species occupy very few proportion of the total population**. As said previously, *Aureobasidium Pullulans* can at a certain time of the year, represent 90% of the total fungal species on grapevine (From Corinne Vacher’s Interview). Hence, if these **rare taxa** are submitted to **drifting**, they could simply **disappear** from a PMC of a certain plant.

All these mechanisms (we can call them the **3DS -Drift, Diversification, Dispersal and Selection**), although described with few details here, shape the PMC and **could heavily influence Softer Shock in term of strategy.**

We need to be **aware** of the **evolution of the phyllosphere according to environment, plant, and time of year**, to know fully where we are going. As mentioned in the picture above, the PMC of course interacts with the plant itself. The nature of these interactions and their relevance will be discussed in the next part.

E) Interaction between the Phyllosphere Microbial Community and the plant

It has been established that **organisms of the PMC (Phyllosphere Microbial Community)** can be **commensal** (like E.Coli in the human microbiota of the gut), **parasites/pathogen** (like Pseudomonas Syringae) whether **opportunist or not**, or **saprotroph** (waiting for the leaf to decay).

As contrarily to the rhizosphere, the positive impact of the PMC is suspected highly but more studies need to be done in the future to bring proofs to statements like “plants wear their guts on the outside” by Mr Janzen (Verholt 2012). For now, the **beneficial impact of the PMC has been demonstrated** by experiments on plants like **Arabidopsis Thaliana**, showing:

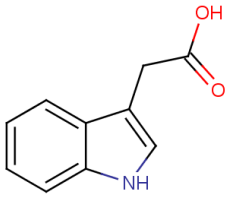
-That the **PMC can increase the availability of nutrients for the plants**. This consistent with our “Folliar application” report in which we showed it was possible for plants to uptake nutrients for the leaves. Indeed, with **biosurfactant production** for example, the PMC can indeed increase leaching out of the leaf, but also the **uptake in the leaf of important nutrients** (Muller 2016).

-That the **PMC helps the plant against pathogens**. As we saw **the dynamic of the PMC** population is regulated by **number of phenomenon**, such as **antibiotic synthesis**, and leads to the “beneficial war”.

This conflict makes the colonisation of the phyllosphere by new species, as well as the deregulation of the population of the present species much less likely (Muller 2016).

The PMC could also act as a **"physical barrier"** against pathogens, exactly like the gut microbiota, **complicating access of nutrients** and **points of entry** like stomata and hydathodes for pathogens (Verholt 2012). It has also been shown that **sterile leaves are more susceptible to diseases and infections** (Vacher et al., 2016, Verholt 2012).

-That the **PMC stimulates plant growth**, by metabolites secretion such as **plant hormones** like auxin and cytokines. These **growth promotions** have been shown to have **influences even on leaf numbers and leaf area** in Maize (Vacher et al., 2016). This aspect has been poorly studied for now, but as the rhizosphere is influencing plant growth in many ways (Bulgarelli 2013), it is logical to suspect the same activity for organisms of the phyllosphere.



Indole-3-acetic acid, or auxin, is believed to be produced by the PMC and stimulates plant growth.

As great as those properties are, they remain poorly studied and need further proofs. The interaction is of course contrasted by the opportunism of some pathogens (especially fungi) and the dormant state of the saprophytes that live in the phyllosphere (Vacher et al., 2016).

We truly believe that the PMC hides many interesting properties like the abyss of our oceans. This part shows the interest of using an organism instead of simple proteins produced in bioreactors. **The organism we will use in Softer Shock, if chosen wisely, will be able to bring additional qualities that supplement the temperature protection aspect**, making our product **more interesting economically and ecologically** (because it could replace other existing products).

We are nearly at the end of our journey into the phyllosphere jungle. Cleaved vines after cleaved vines, the light appears slowly before our eyes. But lastly, let's talk briefly about the impact of other products used in plant care as well as pollutants on the PMC.

F) Impact of external compounds on the equilibrium of the phyllosphere

The **potential compounds** that the PMC will encounter are :

-**Air pollutants** : compounds like **sulfur dioxide, nitrogen dioxide, ozone, heavy metals, black carbon and polycyclic aromatic hydrocar**bons are emitted by humans on a daily basis (Vacher et al., 2016).

Some of these compounds like phenol can actually **be used** by some organisms of the PMC as a **carbon source** (Sandhu et al., 2007); but **most of them are likely to impact leaf physiology and cuticle composition**. As we saw in this report, those two parameters can impact greatly the PMC, hence **air pollutants are likely to be drivers of PMC modifications**.

-Pesticides of course are probably the **main concern for Softer Shock**, as we will deal with crops that are likely to be already treated. Pesticides can be **fungicides or bactericides**, like azoxystrobin, epoxiconazole or streptomycin.
It could seem rather logical that such product impact hugely the PMC, but few studies proved this hypothesis, and it seems that **the microorganisms of the phyllosphere can indeed be damaged by these products, but seem to recover** (Vacher et al., 2016).

Of course, **before using Softer Shock**, we will need to **evaluate the resistance of our organism against classically used pesticides**, as well as anticipate "**cocktail effects**" caused by the simultaneous use of these substances. Let's also not forget about **biopesticides** like Bt that can bring **other species** to the PMC **in addition** to Softer Shock.

Compatibility lists can be made for different products, it could be crucial to look after the ones that suit the organisms we and you will choose:

Trade name (formulation)	Active agent	concentration of active agent	Appl. conc. (%)	compatible with Boni Protect Boni Protect forte Botector BlosP Comp. B	compatible with Blossom Protect + Buffer Protect
Fungicides					
Aktuan (WP)	cymoxanil + dithianon	100 g/kg + 250 g/kg	0.2	no	no
Aktuan Gold	dimethomorph + Dithianon	150+350	0.16	no	no
Alex pk	potassium phosphite	300g/kg	0.3	yes	yes
Amylo X (WG)	*Bacillus amyloliquefaciens* strain D747	5x1010 Sporen/g (250 g/kg)	0.15	yes	no
Aprovia	Benzovindiflupyr (Solatenol) (SDHI)	102,7 g/L	0,077	no	no
AQ10 WG	*Ampelomyces quisqualis*	5E+09 Sporen/g	0.035	yes	yes
Armicarb	potassium-hydrogen-carbonate	850g/kg	0.5	no	no
Aviso (WP)	cymoxanil + metiram	80 g/kg + 670 g/kg	0.15	no	no
Bayfidan WG 5	triadimenol	50 g/kg	0.05	yes	yes
Bellis (WG)	boscalid + pyraclostrobin (F500)	252 g/kg + 128 g/kg	0.055	no	no
Benocap WG 20	flusilazol	200 g/kg	0.012	no	no
Bordoflow (SC)	bordeaux mixture	124 g/l	0.5	concentration !	no
Bordoflow (new)	copper (copper sulfate with lime)	124 g/L	0,4	conzentration !	no
Cabrio Top (WG)	pyraclostrobin + metiram	50 g/kg + 550 g/kg	0.15	no	no

An example Compatibility of Boni Protect, Boni Protect forte, Blossom Protect or Botector with plant protection agents, plant strengtheners, fertilizers or additives. The list is huge and contains references to products such as fertilizers, insecticides and growth regulator (as well as biopesticides). Made by the company Bioferm, 2017

Here finally ends this part of endless, and we hope not boring, talk about the phyllosphere and its fascinating microbial community**. As we mentioned earlier, the PMC varies according to plant species and plants themselves**, so let's now **focus on grapevines** and investigate into what we know about its PMC to try to find a suitable chassis.

3) Metagenomics relevance and the grapevine phyllosphere

From now on, we focused on the phyllosphere of plants in general, but **if we want the specificity required for the safety of our project, there is a need to know the grapevine phyllosphere.** What is problematic although, is that **the PMC of the grapevine, even if it has been studied (as we are going to show), remains poorly characterised**.
A good chassis choice for Softer Shock would **need much more precise studies** with the help of what is called **metagenomics**.

A) Metagenomics to the rescue

As we understand more and more the **relevance of microbial ecosystems**, including our dear phyllosphere, there is a **recent need for techniques and disciplines to study them**. This approach and way of thinking gave rise to **metagenomics**, a set of **genetic** and **computational methods** capable of **sampling microbial ecosystems from diverse origins**.

“Metagenomics, still a very new science, has already produced a wealth of knowledge about the uncultured microbial world because of its radically new ways of doing microbiology. All metagenomics studies take the same first step: DNA is extracted directly from all the microbes living in a specific environment. The mixed sample of DNA can then be analyzed directly, or cloned into a form maintainable in laboratory bacteria, creating a library that contains the genomes of all the microbes found in that environment.” (CMCFA 2007)

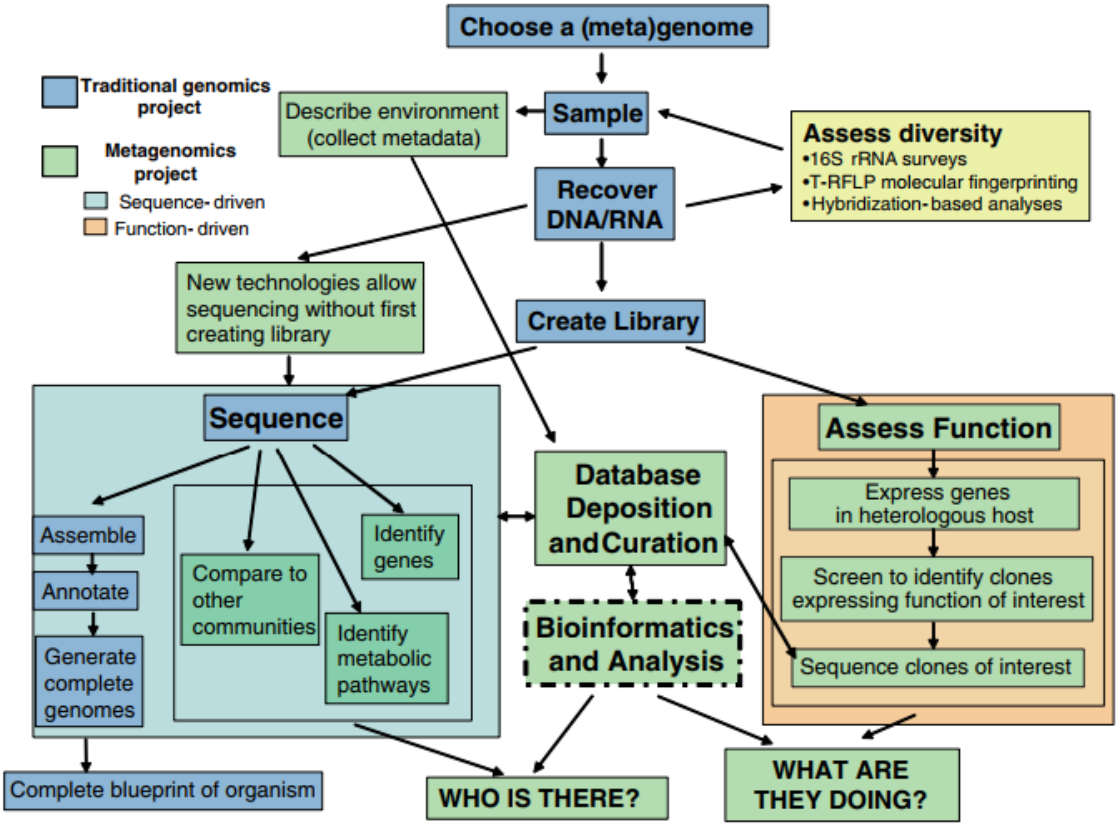
Such discipline is of course **very welcome for Softer Shock**, as it provides exactly what we need.

Recent contact with the **French Agriculture Reasearch Institute (INRA)** and **Nicolas Aveline**, in charge of the project **Resaq Vitibio** that aims at protecting grapevines from the pathogen *Botrytis cinerea* using the fungus *Aureobasidium Pullulans* proved us that there was a **real will to advance in this field and characterise microbial ecosystems**.

With its **recent program called “MEM Metaprogramme”**, the INRA wishes “to improve the understanding and services provided by microbial ecosystems, in the different fields of INRA's activities.”, by relying on **metagenomics** (genome), **metaproteomics** (proteins), **metabolomics** (metabolism) and **metatranscriptomics** (transcriptome) (INRA 2017).

Very important to know is the fact that **metagenomics is not the first science to sample and try to characterise microbial presence in a sample**. Indeed, technics like **pyrosequencing** are much older and were created for the same purpose (Farkruddin et al, 2012).

These techniques were **not as complete as metagenomics** to study the role of the organisms and their diversity, and are now eclipsed by metagenomics:



The relevance of metagenomics as compared to older traditional techniques of genome sequencing , from CMCFA 2007

We are not going to cover metagenomics with huge precision, as it **is overall very complex** (see the CMCFA report if you want the full details), but this is briefly how a classical study works:

-**Microbial habitat selection**: Before any metagenomics study, it is crucial to know the environment you are going to work with.
Here, by reading this report, you became an expert in the phyllosphere undoubtedly, so interpreting the results you can have from a metagenomics study coming from a sample of leaves will be much easier and provide much more information. In its report, the CMCFA mentioned a metagenomics study of acid mines:

" Knowledge of the nitrogen budget of the site (the mine, editor's note) impelled the researchers to seek nitrogen-fixation genes in the metagenome. When they did not find candidate genes in the dominant members, they examined the minor components of the community and discovered that one of the least abundant members of the community, *Leptospirillum ferrodiazotrophum*, carried the nif operon" (CMCFA 2007).

This nif operon was responsible for the **nitrogen fixation recorded in the microbial ecosystem of the mine.** And a non-abundant species was the carrier ! This is in accord with what we said previously about the "**unsung heroes**" of the phyllosphere !

This shows how important is is to understand the environment you are doing a metagenomics study on before any analysis.

-Sampling: before any analysis, it is quite logical to **make samples of what you want to work on**. Sampling is **very hard** because of the need of **reproductive results**. We saw previously that the **phyllosphere and the phylloclimate were very heterogeneous** and **capricious entities** displaying **high variations**. It is the same for many environments, so how to deal with such difficulties?

“The answers to most of these questions depend on the complexity of the community, the heterogeneity of the habitat over time and space, and the fineness of the distinctions that need to be made. As biological and computational methods become more efficient, it will be possible to draw more robust conclusions from more complex communities in more variable habitats. No matter the power of the methods now or in the future, it is essential to consider sampling issues and limitations at the beginning and throughout any metagenomics study of a complex community, and the sampling scheme must inform the interpretation of results” (CMCFA 2007).

The excellent CMCFA report provided a table for the sampling methods:
from CMCFA 2007

TABLE 4-1 Sampling Considerations in Metagenomic Analyses

Sampling Considerations	Questions
Scale	What is the size of the habitat? What is the size of the sample of the habitat? How representative of the habitat is the sample?
Biological variation	How is biological variation in the site accommodated in the sampling scheme? On what scale is the variation (subsample to subsample, sample to sample, site to site)? How much replication is needed to represent the full variety of properties of the site? How flexible is the community? If very flexible, then what does it mean to take a snapshot in time?
Experimental variability	Where is the experimental variability in process sampling? In extracting DNA? In cloning? In storage of samples? How does the experimental design maximize replication to account for experimental variability?
Reproducibility	If patterns are detected, are they reproducible?
Coordinates of place and time	Is detailed information about the site and time of sampling recorded?
Repository	Can the samples be stored for future analysis? Can they be placed in a central repository?
Singletons	What is the significance of a singleton (a unique sequence or other data point)? If it is never found again, how should its relevance be assessed?

-**Recovery of molecules of interest**: For a genomic analysis, the macromolecule of interest is of course DNA. DNA extraction is not as easy as it seems. The DNA is harvested through cell lysis and some members of a microbial community in a sample will be more susceptible than others to certain lysis methods.

Let's take an example : in a sample we have **one fungi** (Aureobasidium Pullulans) and **one bacteria** (Pseudomonas Syringae). We choose to use a lysis method that destroys the bacterial gram- envelope, and this method does not affect the fungus membrane. The DNA of the bacteria is released, and another lysis method is used to extract the DNA from the fungus. Well this method can simply damage the DNA of the bacteria already in the sample !

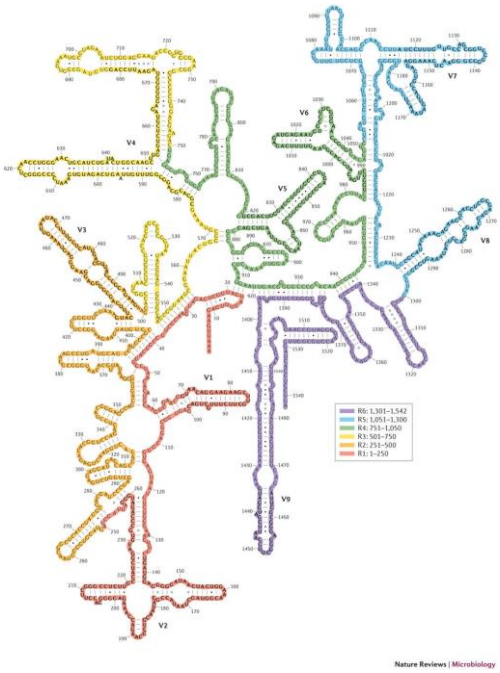
Such problems are multiplied when we refer to complex microbial ecosystems which contain, as we saw, hundreds of different species susceptible to different methods, so the extraction of DNA is actually a difficult task. **To counter such problems, separation methods are classically used, but standardisation of methods for all the ecosystems is difficult** (CMCFA 2007).

-The analysis of data can be done in several ways. The **useful 16S rRNA genes**, common to all bacteria, are crucial. **These sequences have been conserved throughout the whole Evolution process and present variations between species** (CMCFA 2007).

In a sample of 15 different bacteria species, for example, fifteen 16S rRNA sequences will be found and **amplified with a PCR using specific primers**. The fragments obtained from such amplification, or **amplicons**, are then **digested with restriction enzymes with the method called "terminal-restriction fragment length polymorphism "or T-RFLP** (CMCFA 2007).

Each sequence from **each species** will give **different amplicons** fragments and comparison will be made with the **help of databases** such as:

-RDP : http://rdp.cme.msu.edu
-Silva : https://www.arb-silva.de



The 16S rRNA, spearhead of genetic analysis and metagenomics of bacteria

These techniques are also useful for fungi and eukaryotes (using the **18S rRNA**), and archaea (using the **16S rRNA** as well). The only **problem** with such approaches is that **they do not give any information of the function of the species in the microbial ecosystem sampled** (CMCFA 2007).

Different methods using other markers and relying on powerful informatic tools are always under development, this field of study is hence **very promising** and will more likely **overcome all the difficulties** it faces right now.
Therefore, **there is a need to assemble whole genomes from the sample collected, instead of just relying on universal sequences that do not provide enough information**. **Shotgun sequencing** strategies are hence used on the identified species, to analyse their whole genome.

Genes obtained from such methods are **afterward compared to the ones of databases such as the NCBI**. Unknown genes of course cause problems and will always need further characterisation (CMCFA 2007).

Nowadays **number of societies** such as Illumina and Second Genomes provides **efficient and interesting sequencing technologies**. The later one has made microbiota of the human gut its identity : “Our development pipeline is fueled by novel technologies for identifying, screening, and scientifically validating product candidates through a comprehensive understanding of host-microbe interaction and the resulting implications in driving disease.” (from their website).

Once **the genome of the organisms in the sample is completed** (keep in mind that this takes a lot of time and is very complex), results such as the identification of the nitrogen fixing bacteria of the acid mine can be made possible, and **metagenomics can prove its relevance.**

This part was too short to describe the true potential of metagenomics, **we encourage the readers to explore more**, as the field is **growing very quickly** and a **bright future** awaits it. Let’s now explore the grapevine phyllosphere and discuss about the chassis and what we want to do for Softer Shock.

B) The grapevine phyllosphere: what do we know about it?

A shadow of ignorance shrouds our knowledge of the grapevine phyllosphere. A shame for sure, given that **it has been proven that the grapevine PMC (Phyllosphere Microbial Community) plays a considerable role in the quality of the yield the plant can provide** (Martins et al., 2013).

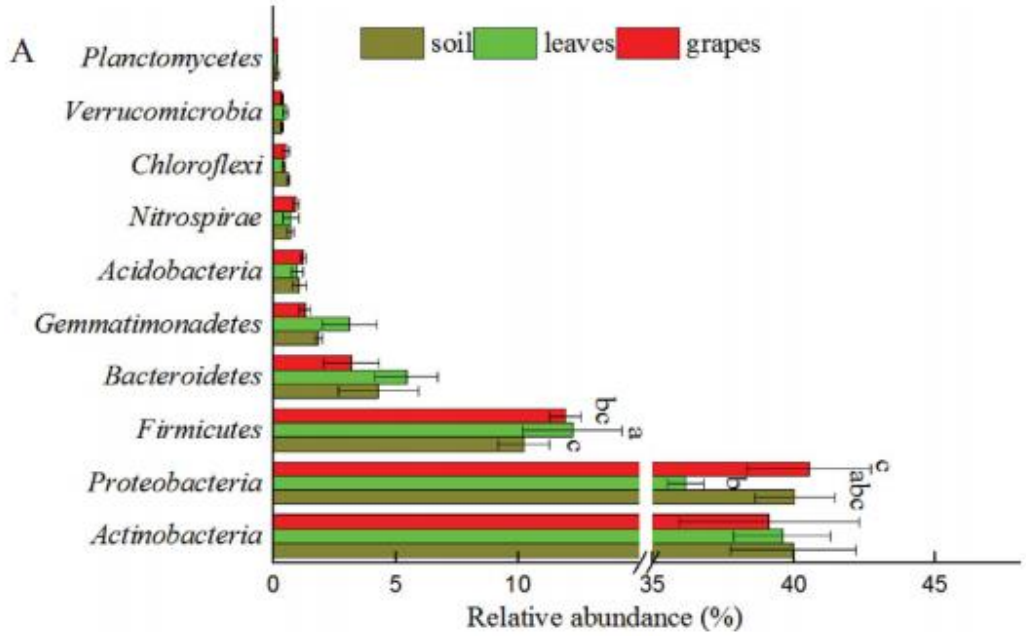
The **two environments in question** are **the leaves** and of course **the grapefruit**. **Until now in this report we proposed that the phyllosphere we talked about all along was the one of the leaves, but fruits possess a microbiota at their surface too, as well as a cuticle and even stomata** (Zhang et al., 2017).

The **phyllosphere of the fruits can be different from the one of the leaves**, but will **most likely behave the same way** (which means aggregation of numerous species at key nutrient sources and display of stress resistance traits).
In Softer Shock, **we want to target the leaves in priority**, but **excluding the fruits and their microbiota could be a problem** and break the very logic of our approach, so we must be careful.

From now on we will precise if we talk about the leaf phyllosphere or the fruit phyllosphere.

The articles of **Zhang and Martins** furnish good example of genomic studies of the phyllosphere of the grapevine leaves and its comparison with the one of barks (indeed barks do have phyllosphere too) and grape fruits, as well as the well-known rhizosphere.

Here are some of their results:



The main bacterial phyllum in the grapevine microbiota, leaves phyllosphere, from Zhang et al., 2017

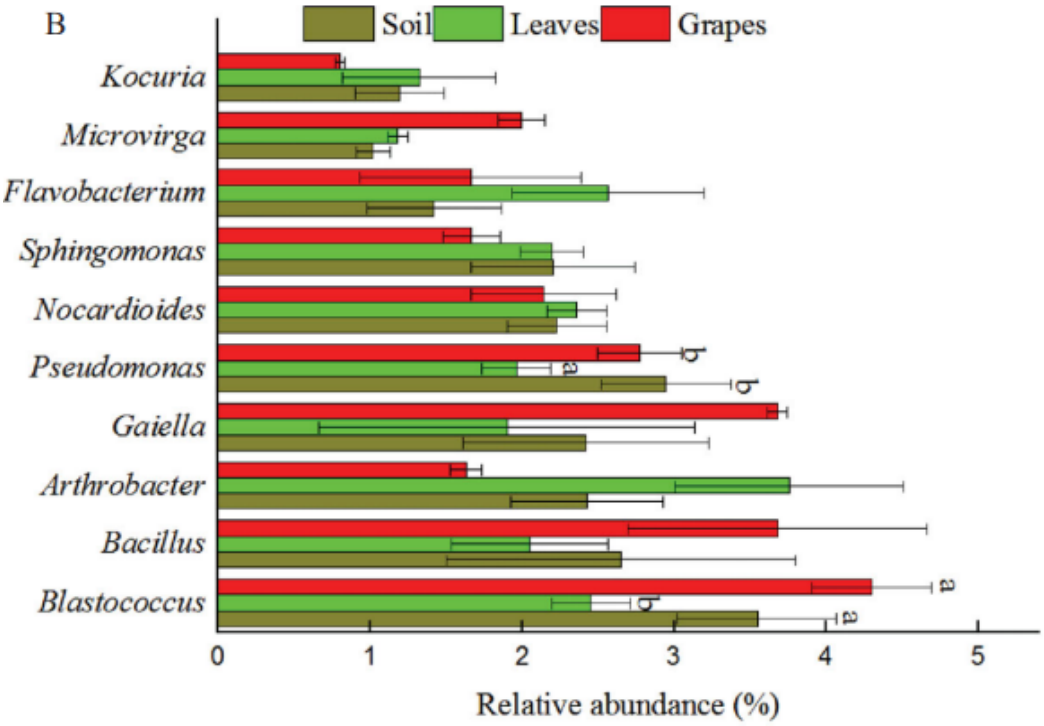
Notice the general domination of ***Proteobacteria*** and ***Actinobacteria***, which is **consistent with the general results obtained from soybean/rize/A.thalianna/clover.**

Interestingly enough, in the four other plants presented **in the Verholt article, the proteobacteria occupied a much greater part of the total bacterial population of the PMC** (Verholt 2012).

Notice as well the **proximity** but **slight differences** between the **rhizosphere** and the **phyllosphere of the leaves and grapes**. This has been attributed to one of the potential **route of colonisation** we mentioned earlier, which is the **inoculation directly by the soil** when the plant germinates and growths (Martins 2013).

Grapes, leaves and soil, three microbiota with a close relationship

Moving on to the genus level :



The most abundant bacterial genus in the plant microbiota, including the leaf phyllosphere, from Zhang 2017

Here we have a **much more precise approach**. The scale says it all, the **most represented** bacterial genus of the grapevine phyllosphere (leaves) would then be ***Arthrobacter*** (an ***Actinobacteria*** genus) (Zhang 2017).

The variation is here easier to notice, but the scale can fool our perception. If we compare this graph with the one above it is clear that the variation is approximately the same across the different environments.

Well, we said enough about how **capricious** the phyllosphere can be and how **heterogeneous** it is according to multiple factors.

What did Martins found in his 2013 article? **According to his study, the most abundant genus of the phyllosphere of grapevine leaves were *Pseudomonas* and *Sphingomonas*, two genera belonging to the Proteobacteria phylum (Martins 2013).**
No Actinobacteria was recorded, which is the big point that shows how incredibly hard it is to characterise such microbial environment (Martins 2013).

Why is one study reporting the immense abundance of Actinobacteria on the grapevine leaf phyllosphere and another one did not record a single one species of this phylum?

The way the sampling was done, the way the DNA was obtained, the phylloclimate and climate (one study happened in Benjiing, the other one in France), the age (not mentioned in both articles) as well as the treatment undergone by the vineyards, the air pollution, the health of the plant; **so many parameters could explain such differences**!

We do not wish to compare entirely the articles, even though a lot is to be said, but this shows **how hard it is to provide great and precise information** about the PMC of the grapevine for now on.

Zhang also did the analysis of the fungal community of the grapevine microbiota, but we are not going to expand further into it as it could be irrelevant. **What was said however in both articles and that can be generalised is**:

“The main bacterial genera, *Blastococcus*, *Bacillus*, and *Arthrobacter*, and the main fungal genera, *Alternaria*, *Guehomyces*, and *Cladosporium*, were present in all samples, but in different amounts, whereas some other genera were sample-specific. Cluster analysis defined generally two major groups: (1) the microbial communities on grapes and leaves, and (2) those originating from the soil. The results suggested that there was a correlation between the microbial communities on the below- and aboveground grape tissues.” (Zhang et al., 2017)

“The identification of isolates revealed that some genera – *Pseudomonas*, *Curtobacterium*, and *Bacillus* – were present in all ecosystems, but in different amounts, while others were ecosystem-specific. About 50% of the genera were common to soil and bark, but absent from leaves and grapes. The opposite was also observed: grape and leaf samples presented 50% of genera in common that were absent from trunk and soil. The bacterial community structure analyzed by T-RFLP indicated similarities between the profiles of leaves and grapes, on the one hand, and bark and soil, on the other, reflecting the number of shared T-RFs. The results suggest an interaction between telluric bacterial communities and the epiphytic bacteria present on the different grapevine parts”. (Martin 2013)

Both studies showed then that the microbiota of the phyllosphere of leaves and grapes as well as the one of the soil were closely related, and that **two groups** could be formed between the **shared species found in all the microbiota** and the one that are **specific to each**.

This is **great for us** in the way that even though **both studies struggled to find similar results**, they showed that there was the **specificity** we were searching and demonstrated that **our strategy of finding a specific chassis was right**. Nevertheless, the **PMC of the grapevine** leaves and fruits remains **very far from being characterised** precisely and efficiently.
This is **rather problematic** for Softer Shock and our will to achieve specificity and increase biosafety of our product. We know many things: the benefits of the PMC, how it globally behaves and evolves, what are its main phylums of bacteria, archaea (which were forgotten by both articles), and eukaryotes.

The grapevine phyllosphere remains a poorly characterised ecosystem

However **so much more needs to be assessed**, and **we might for now on choose a less specific and more characterised species already used in biocontrol**. What we plan for our chassis choice will be definitively assessed in the next part.

4) Strategy of chassis selection for Softer Shock

The report finally ends to this part that will be a conclusion and opener to future perspectives for our strategy.
Let's remind ourselves **why we are focused on this strategy of having a specific chassis** adapted to the grapevine phyllosphere:

-Because it will be **more easily sustainable on leaves**
-Because it **might guarantee biosafety** due to its specificity and only adaptation to the phyllosphere
-Because it can **provide additional phyto-stimulating and protecting capacities**

We have a lot of criteria to take in account, so let's sum them up in the following part.

A) Properties wished for the Softer Shock Chassis

We know for sure what are the **properties we wish for our chassis** :

-**Low nutrients need**, to make its sustain as cheap as possible on the leaves and reduce the cost of the spray.
-An **ability to survive in the phyllosphere** and its “beneficial war”, the phylloclimate and of its stresses (UV, temperature).
-To be **as neutral as possible for the phyllosphere**, so putting a high population of the species in this microbiota will not result in an imbalance of the latter.
-To **secrete plant hormones** and other **beneficial agents** for the plant.
-To **express compounds like biosurfactants** that will lower the number of adjuvants in the spray and limit its cost.
-To be an **effective biocontrol agents** against pathogens of the grapevine.
-The fact that it is **naturally avoiding stomata and hydathodes** on the leaves, to lower the risk that it could penetrate the plant.
-To **survive at low and high temperatures**, thermal performance curves will be needed here.
-If possible, the fact that it is **found only on grapes and leaves** and not in soil, to maximise biosafety
-To **NOT induce ice-nucleation if we choose to express Anti-freeze proteins** (this seems obvious) and to **INDUCE ice-nucleation if we choose to express Ice-nucleation proteins.** (see “Compound choice” report).
-A **great ability for protein expression**
-To **stay on the leaves** and **not propagate** in the external environment through aerosols and rainfalls
-To be **easy to store in a dormant state before application** and to be **easily applied** (see Foliar Application report).
-**No or few interactions with already used products** by the farmers.
-**No harm for human health and environment** in general (see Biosafety and Toxicology reports).
-**An ability to be motile on the leaves** (phyllotelma/phylloplane) to enhance its search for nutrients and its survival.

Here we can clearly see that finding an organisms, fungus, archaea or bacteria, that has all these properties will be impossible.

Sacrifices or “trade-off” will be needed. In our interview with **Corinne Vacher**, she clearly said that if **we wanted to impact the phyllosphere the least possible, the best would be to choose an organism that is already abundant on the leaves,** so bringing an additional population of this species will not change the natural balance of the phyllosphere.

The problem is that species that are abundant on the leaves, she said, **are certainly present in the soil as well**. **Will we favorise the efficacy of the treatment and lower its impact on the phyllosphere in exchange for the biosafety that could bring leaf-specific chassis?**

This is the type of question we ask ourselves a lot and further answers will be brought in the future, **either with metagenomics, either with lab studies**.
Maybe a **specific archaea** species which is **not abundant on the phyllosphere** of the leaves **could be perfect**; but lab tests as well as proof of concept for its low impact on the PMC if sprayed in huge quantity will need to be done.
And this, sadly, we can't afford to do now, either by **lack of data and time**. Furthermore, **finding a constant chassis as we said for the whole project in actually in total contradiction with the whole report.**

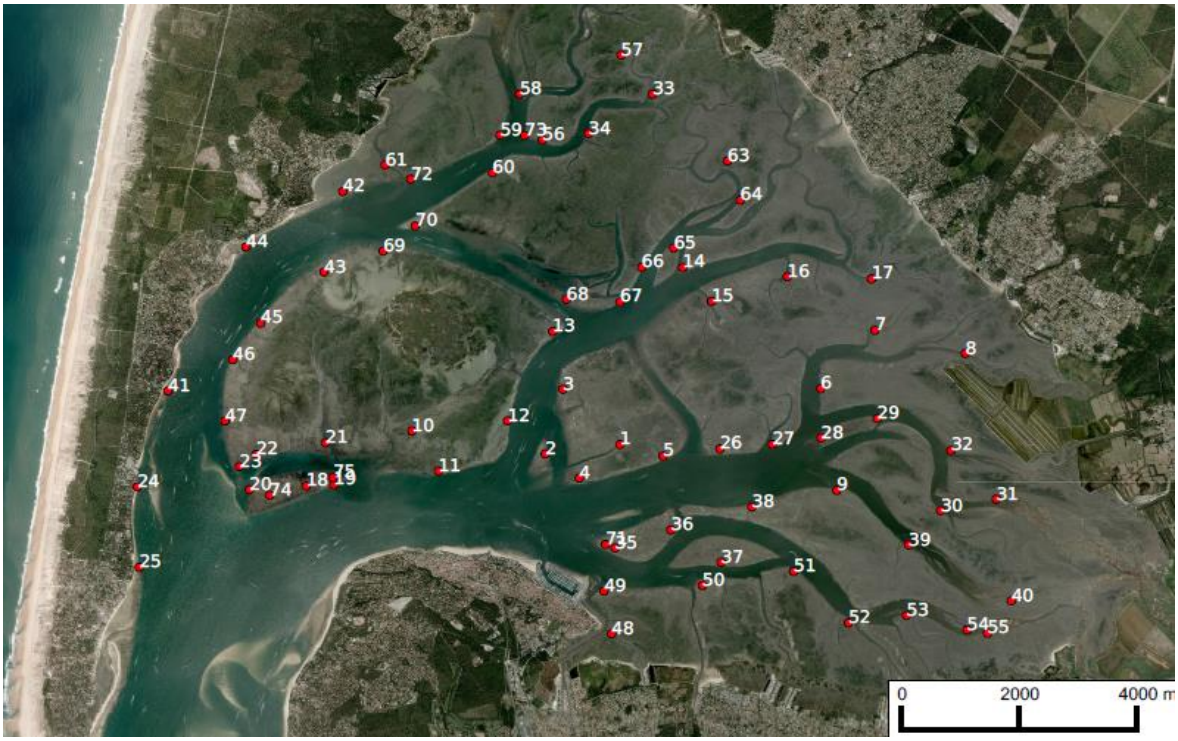
The truth is here: it is the **heterogeneity of the phyllosphere** and its **capricious behavior**.
We though have a **lot of hope for the future**, and the **advances in metagenomics** might provide what we want, so we imagined a situation in which our strategy would be relevant, and that used what is called a **GIS (Geographic Information System) coupled with metagenomics**, to provide a "**personalised treatment**".

Keep in mind that this will be a service possible in the future only and that **such orientation is not presently possible**, due to the **low amount of information** we have on the grapevine phyllosphere and metagenomics limitations.
More realistically, Softer Shock would use a classical biocontrol strain, but the goal of our project and this report is after all to use new technologies and try to adapt.

B) Geographic Information System interest in personalized treatment and safety

A geographic information system (GIS) permits the visualisation of data situated across a map or other geographical patterns

A typical example for GIS :



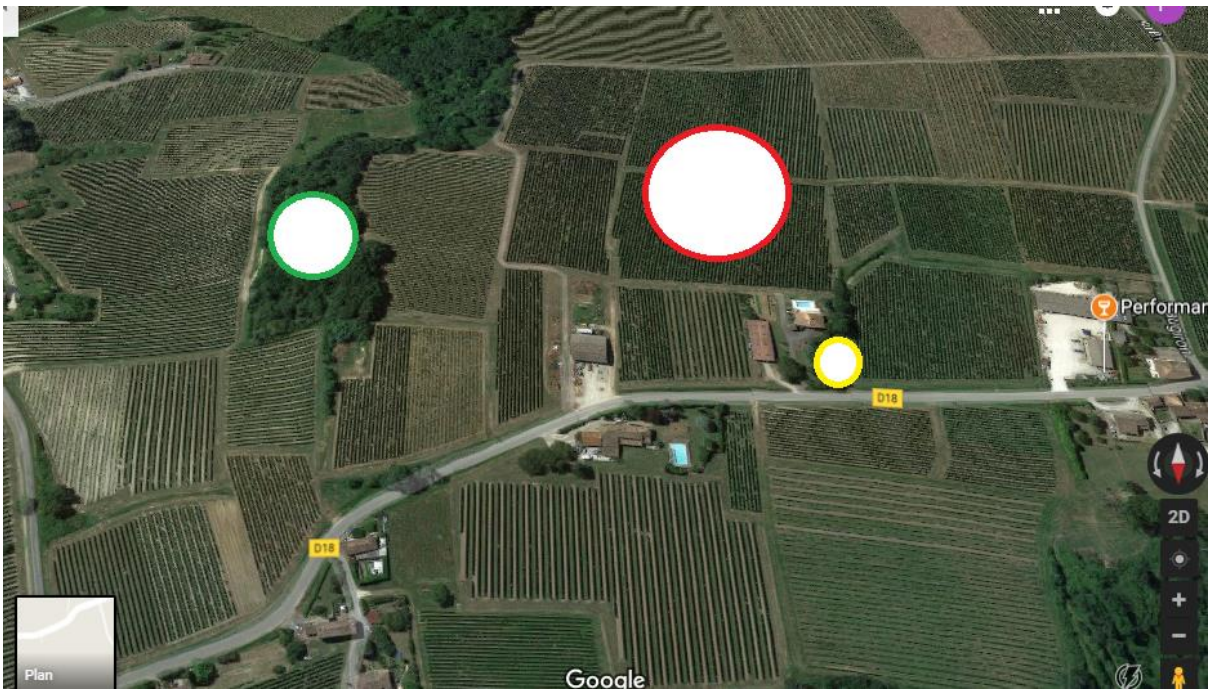
Example of GIS taken from oyster sampling in the bay of Arcachon from : Sup'Biotech fourth year Marine ecology course, Ismael Bernard.

This is taken from a practical work we had in marine biology class. This is a map of the bay of Arcachon, the dots represent a location of oyster sampling. This study aimed at correlating the location of oyster samples with metal pollution to locate the source of pollution in the bay.

A GIS is as "simple" as that, it is very important if we want to situate our vineyard into a given environment. What we could do now is imagine a GIS that is based on a map of the vineyard of our potential client.

Surrounding vineyards, we will found other environments such as other crops, forests, and of course soil or water sources.

What we can do to apply our solution, is to **study what population of organism** is found in every environment and **find organisms that are specific to the vineyard phyllosphere** only. That way, we will choose our **chassis to be as specific to the vineyard as possible**, and will reduce consequently the contamination because it will not be adapted to other ecosystems and will die in case of accidental spreading.



Simulation of a potential GIS after bacterial population study around a vineyard in Bordeaux adapted from Google Map.

This picture was taken on a vineyard near Bordeaux, each circle represents an area with potential different microbial flora. With such data, we will be able to personalise the chassis to the farmers and ensure security.

This is an example of service that Softer Shock could provide in the future if we maintain this strategy of specificity and do not "trade-off" for a more abundant strain such as *Aureobasidium Pullulans.*

It is now time to conclude the report !

Conclusion of the report :

If you came across this report and are still here to read this very sentence, **thank you**. **Thank you** for trying to understand the phyllosphere, thank you for, we hope, sensing our struggle and how we tried to integrate Softer Shock in this report. It could be said that the report ends on a negative note, giving that the strategy we tried to assess is difficult and relies on the future, but maybe in some years we will be able to adopt such methods. In a certain way, this report describes well our attitude toward our own project : we believe in it and even though we want to try a lot of different things, society, technology, time, are the variables that slow us. We love this project and we hope you sense it as well as us while reading these reports. We hope you learned things, and that you will be able to spread your knowledge on the phyllosphere and give this environment the attention it deserves.

Thank you.

The iGEM Ionis team.

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