SOFTER SHOCK

Foliar applications: nourishing and protecting from the leaves

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This report has been written for the project Softer Shock by the iGEM IONIS 2017, but aims at giving a general insight on foliar application for other IGEM teams in the future.

Summary

INTRODUCTION: What is a foliar application?

Commonly, foliar means "on the leaf", a foliar application of a fertilizer is therefore the application of the said fertilizer on leaves of the target plant.

Although not fully understood, the mechanisms of uptake from the leaves are increasingly known, but the uncertainties related to them and all the parameters influencing foliar applications make the process not optimal through the world.

In this report, the words "spray" and "application" can be considered to have the same meaning, describing the product applied on the leaves/fruits of a target plant.

First of all, why proceeding by application on the leaves of a nutrient solution or a protective solution instead of application on the roots? Many parameters can influence the choice of foliar applications:

-The bad quality of the soil that limits nutrient uptake from the roots.

-The life cycle of plants (phenology) can lead to flower and fruit formation at a time when the soil temperature in which they are settled is not favorable, therefore yielding bad results. Relying on foliar application can overcome such difficulty.

-Roots uptake may not be sufficient to meet the need of nutrients from the plant during crucial periods.

-The distribution system of the plant, using xylems and phloems, is not efficient enough to distribute the uptake of nutrients from the soil correctly to high demanding tissues.

This can happen because some nutrients are poorly distributed through phloems (like Calcium and Bromide); the flowering happens before leaf expansion (which are usually supposed to provide some nutrients to the flower); or high humidity or drought affect transports through xylems). (Fernandez & Brown 2013)

-The pest/pathogen attacking the plant focuses on the leaves or fruits and those organs must be protected. Same goes for abiotic stress such as temperature, to which leaves and fruits are very sensible. This is our main reason of using foliar application with Softer Shock.

What is commonly used in foliar application nowadays are nutrient sprays, whether they are micronutrients (trace elements) or macronutrients (nutrients necessary at an important quantity). They are applied under complexed forms:

Table 3.1. Macro-nutrient carriers normally used in foliar spray formulations.

Macronutrient	Common element compounds	References
N	Urea, ammonium sulphate, ammonium nitrate	Zhang *et al.* (2009); Fageria *et al.* (2009)
P	H_3PO_4, KH_2PO_4, $NH_4H_2PO_4$, $Ca(H_2PO_4)_2$, phosphites	Noack *et al.* (2011); Schreiner (2010); Hossain and Ryu (2009)
K	K_2SO_4, KCl, KNO_3, K_2CO_3, KH_2PO_4	Lester *et al.* (2010), Restrepo-Díaz *et al.* (2008)
Mg	$MgSO_4$, $MgCl_2$, $Mg(NO_3)_2$	Dordas (2009a), Allen (1960)
S	$MgSO_4$	Orlovius (2001), Borowski and Michalek, (2010)
Ca	$CaCl_2$, Ca-propionate, Ca-acetate	Val and Fernández (2011); Wojcik *et al.* (2010); Kraemer *et al.* (2009a,b).

Macronutrients used in agriculture, from (Fernandez & Brown 2013)

Table 3.2. Micro-nutrient carriers normally used in foliar spray formulations.

Micronutrient	Common element compounds	References
B	Boric acid ($B(OH)_3$) Borax ($Na_2B_4O_7$), Na-octoborate ($Na_2B_8O_{13}$), B-polyols	Will *et al.* (2011); Sarkar *et al.* (2007), Nyomora *et al.* (1999)
Fe	$FeSO_4$, Fe(III)-chelates, Fe-complexes (lignosulphonates, glucoheptonates, etc.)	Rodríguez-Lucena *et al.* (2010a, 2000b); Fernández *et al.* (2008b); Fernández and Ebert (2005); Moran (2004)
Mn	$MnSO_4$, Mn(II)-chelates	Moosavi and Ronaghi (2010), Dordas (2009a), Papadakis *et al.* (2007), Moran (2004)
Zn	$ZnSO_4$, Zn(II)-chelates, ZnO, Zn-organic 'complexes'	Amiri *et al.* (2008); Haslett *et al.* (2001), Moran (2004); Zhang and Brown (1999).

Micronutrients used in agriculture, from (Fernandez & Brown 2013)

It is clear that, as we will not use a nutrient spray and as we technically don't want our organism to penetrate into the leaves, most of the reasons do not concern us. We although want to describe the mechanisms of foliar uptakes before talking about microorganisms foliar application.

Moreover, we might want our proteins, such as the AFPs (Anti-Freeze Proteins), to penetrate the leaf tissues, and we can combine our organism in a spray containing nutrients for its own needs, or to help the plant even more, so it is still very relevant for us to understand the mechanisms, parameters, and regulation of foliar applications of nutrients or other non-living compounds.

Of course, foliar application of microorganisms is very common for crop protection and stimulation, this aspect will be treated later in this report (Part 4). Keep in mind that in Softer Shock we should also apply our product on fruits of the target plant. Fruits and leaves are distinct entities, but they have shared characteristics so the parameters that we will mention in this report are as valuable for spray application on fruits.

The next part hence describes every point of entry for compounds in a foliar spray, and all the barriers that will potentially oppose the penetration. If you are interested only in microorganisms spray, you can go directly to part 4.

I. Anatomy of a leaf and barriers/entry points for external compounds.

The plant organ called leaf is composed of a multiple array of compounds.

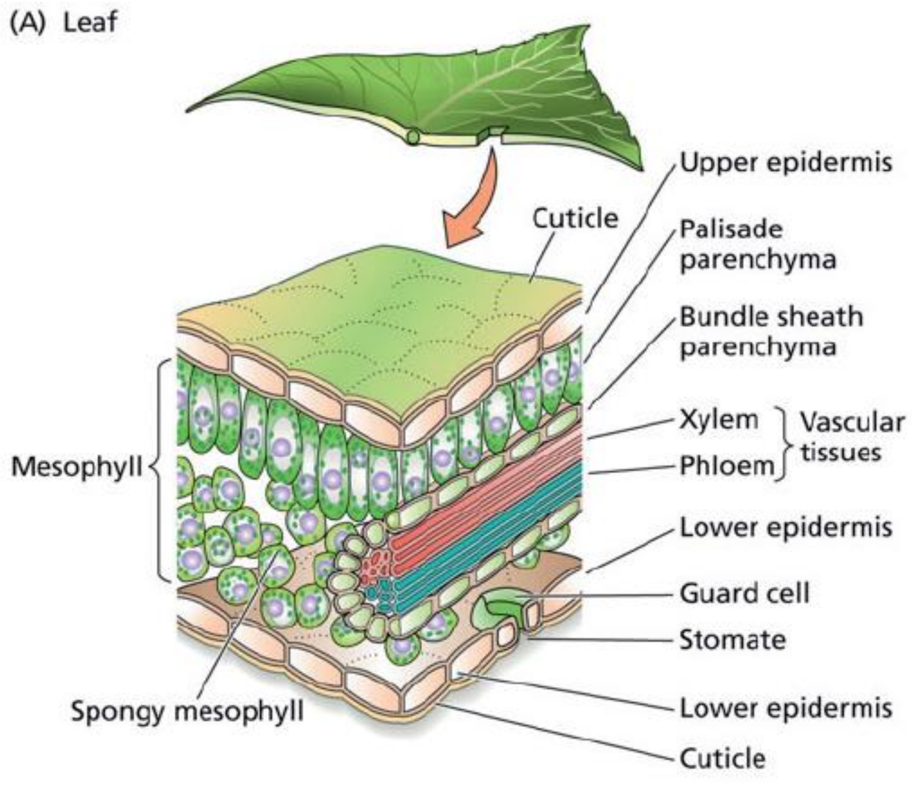


Figure 2.1. Typical structure of dicotyledonous leaf including vascular bundle in a leaf vein. (Reproduced with permission from Plant Physiology, 4th Edition, 2007, Sinauer Associates).

From Plant Physiology, 4th Edition, 2007, Sinauer Associates

A) The cuticle

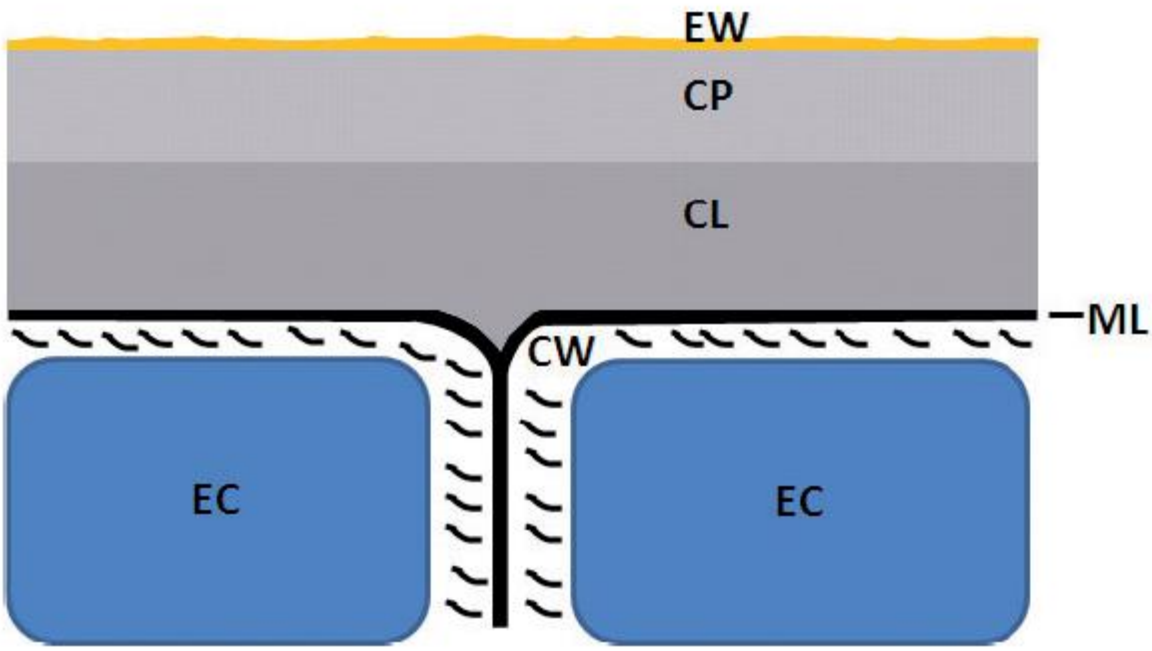
The first point of contact between the foliar application and the leaf is the cuticle, whether it is applied on the adaxial (turned toward the sky) or the abaxial (turned toward the soil) surface. (Fernandez & Brown 2013)

1) General composition and properties

The cuticle is hydrophobic. It is an extracellular matrix of waxes, mainly cutin (biopolyester of C16/C18 fatty acids). Although, cuticle composition can be supplemented by cutan (another biopolymer of n-alkenes/n-alkanes), other waxes of C20-C40 alcohols/aldehydes/alkanes, and aromatic compounds.

Hydroxycinnamic acids derivatives, flavonoids, or phenolic acids can also be found in free forms or bound to cutin or other waxes (ester/ether), as well as polysaccharides such as cellulose, hemicellulose and pectin. These compounds have an antimicrobial effect and mostly prevent the leaf from being consumed by the microorganisms at its surface. The overall wax composition of the cuticle varies according to the species, the age, the environment, and the organ. (Karabourniotis and Liakopoulos, 2005)

The cuticle can be divided in three layers: the wax layer (EW), the cuticle proper (CP), and the cuticular layer (CL). The most hydrophobic is the EW, and only the CL is mixed with polysaccharides, as it is the closest to the cells of the upper epidermis of the leaf. These layers create a gradual increasing gradient of negative charges, believed to facilitate water and cation movements, and are organised this way (Jeffree, 2006):



From (Fernandez & Brown 2013). ML = Middle Lamellae. CW : Cell wall. EC = Epithelial cell.

The cuticle waxes are believed to have a pHi of 3, even though it can vary according to the cuticle composition. It has been shown that penetration of foliar application can "play" with such property and increases its efficacy. For example, if the solution contains mainly cations, it would be more efficient if the pH of the given solution is under the pHi of the cuticle, as it will negatively charge the waxes and increase chances of attraction between the cation in solution and the cuticle. (Fernandez & Brown 2013)

The cuticle is however not a perfect uniform surface and can be crackled on one point or another (cuticular cracks), such cracks can ease nutrient and solution penetration by a lot. (Fernandez & Brown 2013).

The first layer of leaf defense is therefore hydrophobic, negatively charged, and anti-microbial. Important is the fact that this protection is absolutely to be kept intact for plant health, and that its composition/permeability varies according to species of plants, and the surrounding environment. (Fernandez & Brown 2013) Important point: fruits are also covered with a cuticle, so the problem/advantage of such biological wall are the same as for leaves.

2) Permeability measurement and penetration

Although seemingly impenetrable, substances, whether hydrophilic or hydrophobic, can penetrate the cuticle according to different models. Judging by the nature of the cuticle, it is clear that nonpolar and lipophilic substances will penetrate the wax layer more efficiently than polar hydrophilic ones.

The permeance of the cuticle to any compound can be calculated theoretically:

$$P = J / (C_i - C_o)$$

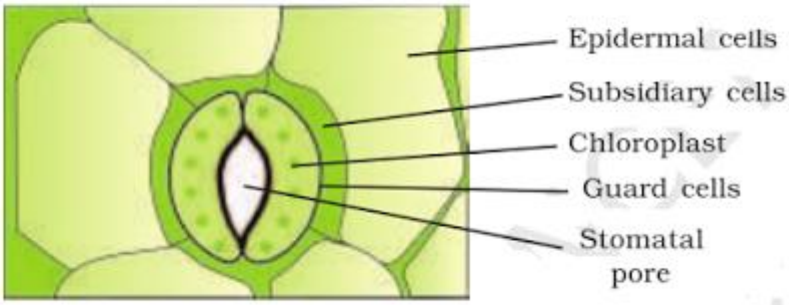
Where P is the permeance of the membrane in m/s; J is the diffusive flux, it measures the amount of substance that will flow through a unit area during a unit time interval and is expressed in $m^{-2} s^{-1}$ Ci is the concentration ($mol.m^{-3}$) at the inner side of the cuticle and Co is the concentration ($mol.m^{-3}$) in the outer side of the cuticle. This operation is called the Flick's law of diffusion. (Riederer and Friedmann 2006)

For the lipophilic compounds, the diffusion and dissolution is believed to happen just as the proteins on a SDS-PAGE gel: they infiltrate and diffuse between the gap left by the matrix of wax polymers. The cuticle is believed to be highly size-selective. (Buchholz et al., 1998)

As for the hydrophilic polar compounds, without the help of substances called adjuvants, which are going to be referred to later on, they can't penetrate the cuticle at a rate comparable to lipophilic compounds. They are believed to follow a diffusion fashion just as the lipophilic compounds, but also hypothesised to penetrate imperfections in the cuticle called aqueous pores ranging from 1 to 5 nm of diameter. These mechanisms are not yet fully understood. (Fernandez & Brown 2013). It has been proven that amino acids, organic acids and sugars from plant origin do penetrate the cuticle at a slow rate (Morris 2002).

B) The stomata

Stomata (plural of stoma) are specialized structure found in the cuticle, either on the abaxial or the adaxial face of the leaf (or fruit, all depending on the species). They are composed of two guard cells that open and close according to environmental factors (nutrients, sunlight...) or specific cellular signalisation. They play an essential role in gaseous and water exchange (evapotranspiration) between the leaf and its surrounding environment. (Eichert and Fernández, 2011)



Structure of the stoma

Their role in the uptake of nutrients from foliar applications is still discussed about, but there have been proofs that a cuticle covered by stomata is more permeant than a cuticle without (for example the cuticle of the abaxial surface absorbs more efficiently than the one of the adaxial surface). (Eichert and Goldbach, 2008)

Nutrients in solution and other polar hydrophilic compounds are believed to go through the stomatal pore wall because the latter has undercome hydrophilic modifications "such as deposited salts and particles or the formation of bacterial biofilms, where physicochemical characteristics of the leaf surface are altered by excretion of surfactants and formation of extracellular polysaccharides" (Eichert and Goldbach, 2008).

It has also been shown that uptake through the stomatal pore can be increased by the addition of a surface-active agent in the solution, lowering its surface tension. (Fernandez & Brown 2013)

The size selectivity of such mechanism of penetration is believed to include particles of 43nm and exclude particles bigger than 1µm. Due to this size selectivity, stomata have been described as being an important point of entry for microorganisms, as well as hydathodes (Vacher et al., 2016).

C) The hydathodes

Also known as "water stomata", these organs are secretory tissues that produce hydrophilic substances (guttation). They extrude water and salts in the external environment, just as the salt glands in seabirds (Arimura & Maffei, 2016).

They are considered major points of entry for microorganisms (Vacher et al., 2016), but their position at the tip of the leaves (epithem) makes them less likely to participate into solute exchange, as they are known to excrete rather than uptake.

They although differ from stomata in the way that they are always opened and their guard cells do not contain chloroplasts usually. They are very important to help the plant to force out its leaf water and maintain pressure homeostasis. They can be considered as trichomes, especially the stalked hydathodes. (Světlíková, 2015).

D) The trichomes

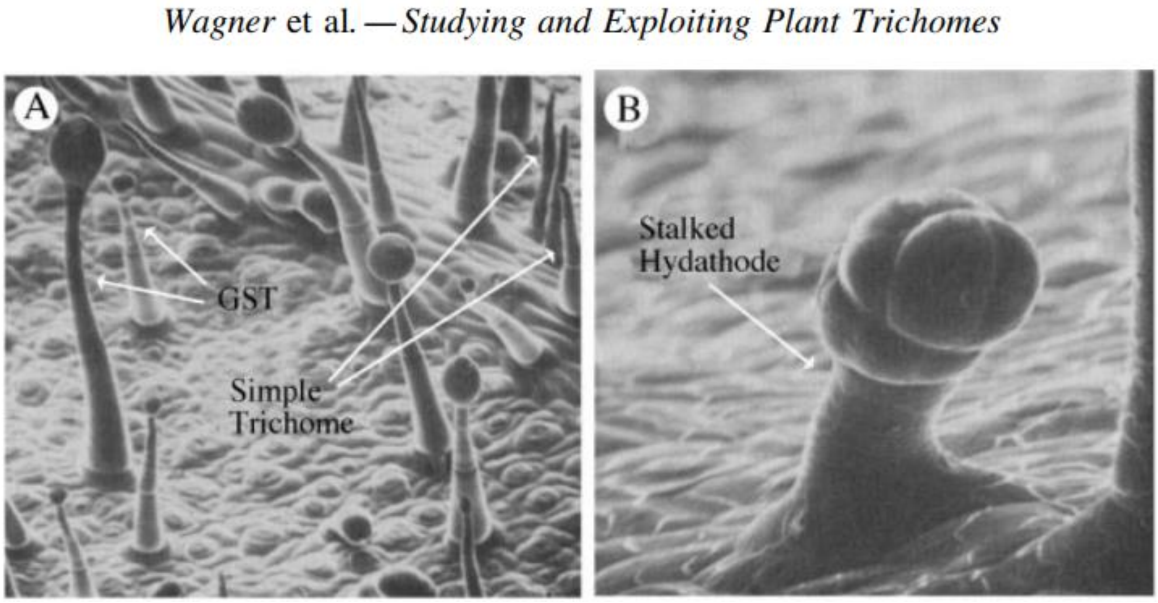
Uni or multicellular appendages that emerge from the leaf surface; those specialised structures play an important role in the leaf topography and therefore the mechanisms of absorption. Two types of trichomes are found, the glandular and non-glandular trichomes.
This table summarises their function:

TABLE 1. *Apparent functions of simple trichomes, GSTs and hydathode trichomes*

Apparent functions	Trichome type*	Reference
Reduced insect movement	ST and GST	Levin, 1973; Johnson, 1975; Kessler, 2002; Kennedy, 2003
Temperature regulation	ST and GST	Johnson, 1975; Dell and McComb, 1978; Ehleringer, 1984
Increased light reflectance (including UV)	ST and GST	Ehleringer, 1984
Decreased water loss through reflection	ST and GST	Ehlringer, 1984
Reduced mechanical abrasion	ST and GST	Uphof, 1962; Johnson, 1975
Protection of phylloplane organisms	ST and GST	Beattie and Lindow, 1999
Reduced leaf wetness	ST and GST	Brewer and Smith, 1997
Reduced photosynthesis thru reflectance	ST and GST	Ehleringer, 1984
Seed dispersal and establishment	ST and GST	Uphof, 1962; Werker, 2000
Epidermal Ca++ homeostasis	ST and GST	DeSilva *et al.*, 2001
Pollen collection and dispersal	ST and GST	Uphof, 1962; Werker, 2000
Provide insect and herbivore deterrence	GST	Johnson, 1975; Kelsey *et al.*, 1984;Wollenweber, 1984; Bennett and Wallsgrove, 1994; Berenbaum, 1995
Insect immobilization	GST	Kennedy, 2003
Fungal and bacterial toxicity/nutrition	GST	Johnson, 1975; Behnke, 1984
Pollinator attraction	GST	Werker, 2000
Ion and pollutant metal secretion	GST	Salt *et al.*, 1995 Choi *et al.*, 2001; Kupper *et al.*, 2000
Water retention and decreased desiccation in seeds	GST	Werker, 2000
Allelopathy	GST	Macias *et al.*, 1999; Werker, 2000
Assist in climbing, and seed establishment	ST	Uphof, 1962; Werker, 2000
Absorb water and nutrients	ST	Uphof, 1962; Sakai *et al.*, 1980; Werker, 2000
Guide pollinators	ST	Johnson, 1975
Water and ion secretion	HT	Johnson, 1975; Aloni *et al.*, 2003

* ST, simple trichomes; GST, glandular secreting trichomes; HT, hydathode trichomes.

Different types of trichomes, From: (Wagner et.al 2004). Note the presence of hydathodes in the table.



Trichome aspects at a microscopic level, From: (Wagner et.al 2004)

Apart from their topology influence, few specific examples of nutrient absorption by trichomes has been studied, but they are believed to play a role, probably by their usual secretory function and osmotic pressure.

E) The lenticels

Lenticels are macroscopic structures present on fruits, pedicels (stem that joins a flower to its inflorescence, a cluster of flower), and simple stems. They technically are not found on leaves but can serve as entry points for foliar spray if it is applied on other parts of the plant (which occurs a lot in the case of spray application). (Du Plooy et al., 2006)

They have not been studied greatly in the field of nutrient absorption and little is known about their properties in this domain, but they are suspected to play a role (Harker and Ferguson, 1991)).

(Left) Lenticels on apple, (Right) Microscopic aspect of a lenticel, from (V. Fernández, 2010)

Every point of entry of the foliar spray has now been described. Following will now be the parameters that might influence the penetration and adherence of the spray on these structures, coming from the plant and the surrounding environment alone (Extrinsic parameters).

II. Extrinsic parameters influencing foliar application efficiency

Firstly, considering the previous part:
-The cuticle composition influences greatly foliar spray efficiency (permeance, composition, imperfections, size selectivity, pores, species, organs, age)
-Number of stomata is positively correlated to spray uptake (size selectivity, number, opening)
- Trichomes have an impact on foliar spray efficiency (topography)
(Fernandez & Brown 2013)

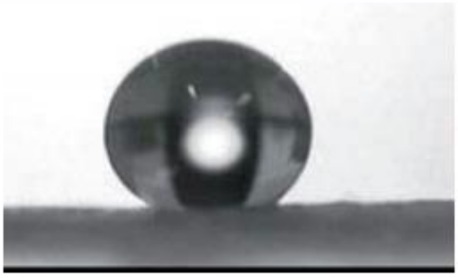
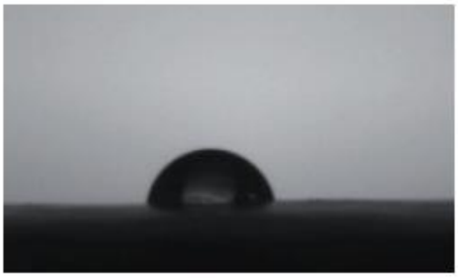
This table summarises all the extrinsic parameters to consider:

Table 4.1. The physical structure and physiology of the leaf and the plant canopy interact with the local environment affecting retention, absorption and utilization of foliar-applied fertilizers.

Leaf age, leaf surface, leaf ontogeny, leaf homogeneity and canopy development	Physical structure of the leaf affects spray retention	hairs, trichomes, surface architecture
		stomatal distribution and density
		presence of discontinuities (lenticels, cracks…)
	Chemical composition of the leaf affects penetration, distribution, absorption and 'availability' of foliar-applied nutrients	cuticle thickness, cuticle composition
		apoplast binding and complexation
	The physiological state of the leaf at the time of spraying affects nutrient assimilation and mobilization	leaf expansion and source/sink status
		leaf senescence and remobilization
Plant architecture and metabolic status	Canopy architecture and phenology have a quantitative effect on spray retention and penetration	canopy size and leaf age distribution
		new growth, presence of leaf or floral buds, presence of reproductive structures
	Plant metabolic activity and crop phenology affect uptake and remobilizations	shoot and root growth activity alters demand and source:sink dynamics
		metabolic status of plant affects availability of substrate and energy for absorption and assimilation
Short- and long-term environmental interactions	Temperature, light, humidity	immediate effects on energy and metabolites required for nutrient absorption, metabolism and transport
		long-term effects on physical and chemical properties of leaf and plant
	Plant nutrient status alters leaf structure and physiology and may alter leaf assimilation of foliar applied nutrients	
	Biotic and abiotic stress (pests, temperature, water)	

From (Fernandez & Brown 2013)

Topography governed by trichomes, as well as wettability, have an important impact on the contact angle a droplet of foliar spray application will have, and therefore how much it will interact with the cuticle and will potentially be retained (Fernández et al. 2011).

Plant organ and species	Average contact angle with pure H_2O (°)	Drop image
Adaxial side of *Eucalyptus globulus* leaf	140	
Adaxial side of *Ficus elastica* leaf	83	

Example of contact angle variation across species, From (V. Fernández, 2011)

A foliar spray supplemented by an adjuvant can have a lower contact angle and be therefore more efficient. To evaluate the topography of a structure, water, glycerol and di-iodomethane can be applied and microscopically observed. Adjuvants will be discussed about later on, in part 3 (Fernandez & Brown 2013).

Leaf age can change cuticular composition of cuticle, number of trichomes, stomata, and therefore influences greatly foliar application efficiency. The effect on age depends so much on the species and the environment that it is impossible currently to predict its effect, and empirical tests must be done.

Leaf, during their development, alternate between a “sink” and “source” physiology. Young leaves require nutrients, and old fully-developed leaves sustain the newly forming sink leaves through phloems. This “source” physiology will therefore induce the fact that the applied solution will be redistributed to young leaves from the old leaves, and therefore that a foliar spray effect will more likely affect “sink” leaves (Turgeon, 2006).

The distribution of leaves across the plant is termed canopy, and its architecture influences greatly foliar spray efficiency, since the more leaves are exposed to the spray, the more the spray will be efficient. Localisation of leaves of different ages (sink and sources) across the canopy can also influence foliar spray effect (Weinbaum, 1988).

Light intensity plays a major role in foliar spray uptake, as it has been shown that it is positively correlated with wax secondary structure development, thickness of the cuticle, and amount of cuticular waxes. Light is suspected to influence positively absorption of nutrients however, as a highly photosynthetic active tissue requires more nutrients, but that varies according to species. Light is also the master-regulator of stomatal opening (Fernandez & Brown 2013).

Temperature, through its direct action on the spray (drought, freezing), “the nutrient solution physico-chemistry; as well as its impact on leaf cuticles; and on plant metabolism, ion uptake and assimilation. (Fernandez & Brown 2013)”, influences heavily foliar spray applications. Temperature influences also leaf development and their physiology (see above) (Fernandez & Brown 2013).

High temperatures will dry out the spray more easily and decrease the absorption, but their effects on the cuticle architecture and overall solidarity might allow the spray to diffuse more easily through the biological barrier. As temperature rises, solubility increases, but viscosity decreases, as well as the POD of any compound and the surface tension.

Again, temperature effect will vary according to species and the solution applied, so empirical tests must be performed to satisfy theoretical previsions (Fernandez & Brown 2013).

As crucial as it is, leaf temperature is distinct from the temperature of ambient air, due to different factors (heat exchange, decreased wind influence), creating what is called a boundary layer or microclimate.

This variation of temperature can be up to 7°C (if the air is at 30°C, the leaf can be at up to 37°C), and varies even on the leaf itself, depending on the heat exchange variation at its surface. Sunlight is of course a major factor of this difference, as much as winds (leaf fluttering decreases surface temperature).

Care must therefore be taken when measuring the temperature of an application area to assess when to do the application, as leaves temperatures might be very different (Morris 2002).

Completing the trinity of environmental parameters (light, temperature), humidity influences foliar applications in many ways. As opposed to temperature, high relative humidity slows down the drying of the foliar spray and increases its persistence on the surface it is applied on.

An important property is the Point of Deliquescence, which is the humidity at which a salt becomes a solute, care must be taken when choosing the salts in the spray solution according to the humidity of the area it is applied on, as non-solute salts will be uptaken much less efficiently and crystalise.

At a long-term perspective, humidity influences also the composition and the permeance of the cuticle, as well as the leaf physiology and the opening/closing of stomata (Fernandez & Brown 2013).

Here is achieved the description of extrinsic parameters that influence foliar spray application. For Softer Shock, the most important parameters will be the humidity and temperature because they have an important impact on the persistence of the spray. Weather analysis of the area of application before any treatment is done will be very important.

Topography also is primordial, but only through empirical tests will we be able to measure its significance. Intrinsic parameters will now be treated in the next part (Fernandez & Brown 2013).

III. Intrinsic parameters influencing foliar application efficiency

Many parameters concerning the spray directly need to be assessed, they are as important as the extrinsic parameters seen above.

A) Concentration of any compound in the spray

As a matter of fact, and as mentioned, the diffusion pathway undertaken by compounds either lipophilic or hydrophilic, follow the Flick's law which is linked to concentration. As a result, spray with higher concentration of a compound will be uptaken more efficiently by the target it is applied on (Fernandez & Brown 2013).

However, it would be folly to over-concentrate any compound if the uptake of this compound is wished. High concentration may lead to severe damages and potential toxicity, either for the plant, or its microbial flora, or even its consumers, which is to be avoided.

Such concentration ranges vary depending on the species, the age of the tissue, its nutritional status and the weather conditions (Fernandez & Brown 2013). Furthermore, over-concentrating a compound in a foliar spray might lead to saturation of targeted site and have the direct unwished effect of decreasing its uptake (Fernandez & Brown 2013), except if the initial goal is to make the surface totally saturated to limit uptake.
The later method, however, is dangerous for the plant and environment as mentioned above.

B) Solubility and point of deliquescence (POD)

It is very important for a salt to be soluble or suspended in water before any application, otherwise application will be unpaired. Saturation concentration of a compound, the concentration at which any additional compound to the solution will not increase its concentration, needs also to be taken in account. The POD must also be taken in account for any salt as crystallization is not to be wished (Schönherr, 2001).

C) Molecular weight and size

As discussed above, size of a particle and its molecular weight will decide whether or not a compound can penetrate the cuticle through the matrix , aqueous pores, or through stomata.

Range of aqueous pore size : 0,3 to 2,4 nm radius depending on the species
Range of stomatal pore size : let particles of 43nm diameter enter and rejects 1µm diameter particle (Beyer et al., 2005; Luque et al., 1995; Popp et al., 2005; Schönherr, 2006).

Some proteins and microorganisms will therefore be able to penetrate the leaf through stomata and hydathodes, these interactions are described in the report “Working with the plant : Perspectives for chassis selection in accord with the phyllosphere ”

D) Solution pH

As mentioned above, the cuticle waxes have a pH of 3 and any value different than this will lead to either higher uptake of cation (pH >3) or anion (pH<3). pH must be taken into account very seriously because of its effect on nutrient uptake (Schönherr and Huber, 1977).

E) Presence of adjuvants

Surface active agents, or adjuvants are additional formulants put in a solution to modify its properties. They can on one hand enhance the penetration and uptake of the application (activator) or modify simply properties without affecting the application uptake (utility) (Penner, 2000).

Here is a table that summarises the properties given to adjuvants:

Table 3.3. Example of adjuvants available on the market classified according to their purported mode of action.

Adjuvant name on label	Proposed mode of action
surfactant	lowering surface tension
wetting agent	equivalent to "surfactant"
detergent	equivalent to "surfactant"
spreader	equivalent to "surfactant"
sticker	increasing solution retention; rainfastness
retention aid	increasing solution retention; rainfastness
buffering agent	pH buffering
neutraliser	pH buffering
acidifier	lowering pH
penetrator	increasing the rate of foliar penetration (e.g. by 'solubilizing' cuticular components)
synergist	increasing the rate of foliar penetration
activator	increasing the rate of foliar penetration
compatibility agent	improving formulation compatibility
humectant	retarding solution drying by lowering the formulation's point of deliquescene (POD) on the leaf
drift retardant	better spray targeting and deposition on foliage
bounce and shatter minimizer	better spray targeting and deposition on foliage

Different types and actions of adjuvants, from (Fernandez & Brown 2013)

Plenty of adjuvants can be found commercially, some are made of only one compound, some are a mixture of different compounds that act together. These products are really important and must not be forgotten because they can compensate the weaknesses of a foliar spray. They also must be evaluated in term of toxicity for the plant, its microbial flora, the environment, and the potential consumers/farmers. (Fernandez & Brown 2013)

The effect of a given adjuvant on a foliar application will always be difficult to predict, and as mentioned before, the best way to test out this efficiency will be empirical tests and experiments. (Fernandez et al., 2008a; Liu, 2004)

One example of adjuvants that lower the surface tension of a liquid and enhance the contact surface of a solid against this liquid are the surfactants. These are large molecules that have a hydrophobic and a hydrophilic property. At a concentration called Critical Micelle Concentration (CMC), surfactants molecules associate into larger units called micelles.

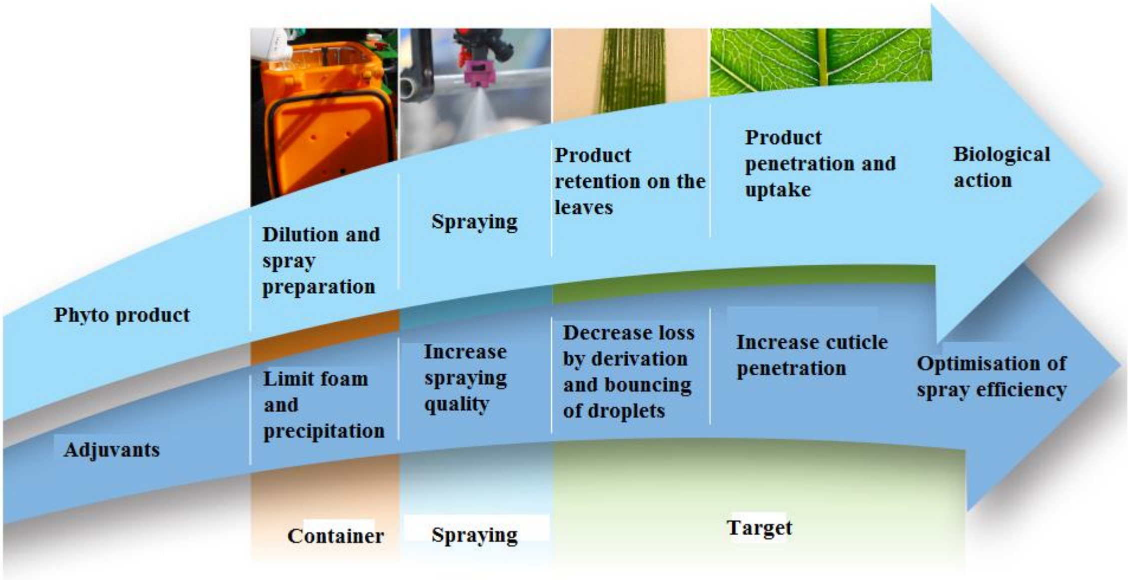
Each surfactant has different CMC and behaves differently and can:

-Increase the effective contact area of deposits (Fernandez & Brown 2013)
-Dissolve or disrupt epicuticular waxes (DANGER) and affect permeability of plasma membrane
-Solubilize agrochemicals in deposits
-Prevent or delaying crystal formation in deposits
-Retain moisture in deposits
-Promote stomatal infiltration

Surfactants can be either non-ionic (like the Silwet L-77); ionic, or zwitterionic (anionic and cationic at the same time). Non-ionic surfactants are believed to interact less with the other substances in the spray as compared to ionic or zwitterionic ones. (Fernandez & Brown 2013)

This example of adjuvant shows how complex and various just one adjuvant group can be, and how many parameters need to be taken in account when choosing the right adjuvant for a foliar spray.

In France, there exists an organism called "Association Française des Adjuvants" that aims to vulgarise adjuvants actions and justify their use, the website is the following : http://afa-adjuvants.com/



Potential roles of adjuvants in foliar sprays, translated from http://afa-adjuvants.com/?q=node/4

For Softer Shock, the most important parameters to take in account will be the solution pH because we don't want to damage the cuticular waxes and perturbate the plant ecosystem; the concentration of the compounds (organisms as much as medium) for the same reason and for safety reasons; and adjuvants of course to maximise the efficiency of the application (surface of contact and adherence) and limit contamination of surrounding environment by droplet derivation.

Now that all the parameters influencing foliar spray efficiency have been described, it is time to focus on a specific type of application, which is microorganism spray.
In the next part this specific type will be described, along with other parameters implying the use of living organisms, their interests, and how they are applied on crops.

IV. Microorganisms sprays: definition, methods of use, interests and limitations

This part focuses mainly on why and how micro-organisms are used on plants and what can influence the application itself. The interactions of microorganisms with the plant and its microbial flora and how they develop on leaves will be treated more precisely in the report "Working with the plant: Perspectives for chassis selection in accordance with the phyllosphere"

A) Why are microorganisms used on crops?

Nowadays, care is given increasingly to the protection of the environment and the use of so-called natural products for different applications.

Of these applications, crop protection is of course one of the most important. Stricken by different pathogens and pests, crops like grapevines (*Powdery Mildew, Botrytis cinerea, Paralobesia viteana*…) suffer a lot and massive quantity of production is lost each year.

In a recent report, the INRA, French institute of agricultural scientific research, has shown that up to 60% of a grapevine plot can be lost to pathogens such as *Erysiphe necator* (INRA 2015). To counter such assaults, different options are available to farmers, and using microorganisms is one of them.

Plant are surrounded by a microbial flora, whether aerial (phyllosphere) or root-associated (rhizosphere). These organisms fulfill roles critical for plants, and using microorganisms in sprays will more likely be oriented toward a collaboration strategy or a biomimicking strategy instead of simply applying a fungicide or other pest-controlling synthetic agents.

During interviews with INRA specialists, it appeared that the use of micro-organisms for what is called biocontrol and bio-stimulation was increasingly seeing applications (the sector grows by 10% each year (Berg 2009).

Understanding those bio-sprays is therefore crucial for our project and for other iGEM teams that wish to use organisms on crops.

Upon their arrival on the plant, the organisms firstly need to colonize it. This step is primordial if any further interaction is wished. The organism will begin by recognising the plant, then adhere on it, and colonisation will begin.

Such interaction is promoted and induced by molecules like plant polysaccharides that have been shown to induce biofilm formation of Bacillus Subtilis and root colonization (Beauregard et al, 2013).

During and after this colonisation step, the organism will also interact with other organisms of the plan microbial flora, so care must be taken to minimize any negative outcomes out of this interaction. For more information about the plant microbial flora, you can see our report called “Working with the plant: Perspectives for chassis selection in accordance with the phyllosphere”.

The abbreviations used for the organisms used are PGPAs (Plant Growth Promoting Agents) and BCA (Biological Control Agents). They can be classified into four categories:

-Biological fertilizers
-Plant strengtheners
-Phytostimulators
-Biopesticides

We can consider the Softer Shock organism as being a PGPA Plant strengthener for example.

When unmodified microorganisms are used on crop as fertilizers or stimulants, they are called Effective Microorganisms (EM). However, this term engulfs as well other uses than just agriculture, as EM are for examples used is wastewaters treatment.

“I must emphasize at this point that EM does not contain any special set of microbes. Neither does it have any genetically engineered organisms. Thus, EM has only a combination of specially selected microorganisms capable of producing multiple benefits. All these microbes are present in nature.” - Dr. Teruo Higa 1999

Microorganisms used in plant pest control and stimulation have been shown to have beneficial impacts on both plant growth promotion and pathogen regulation.

This graph summarises these interactions :

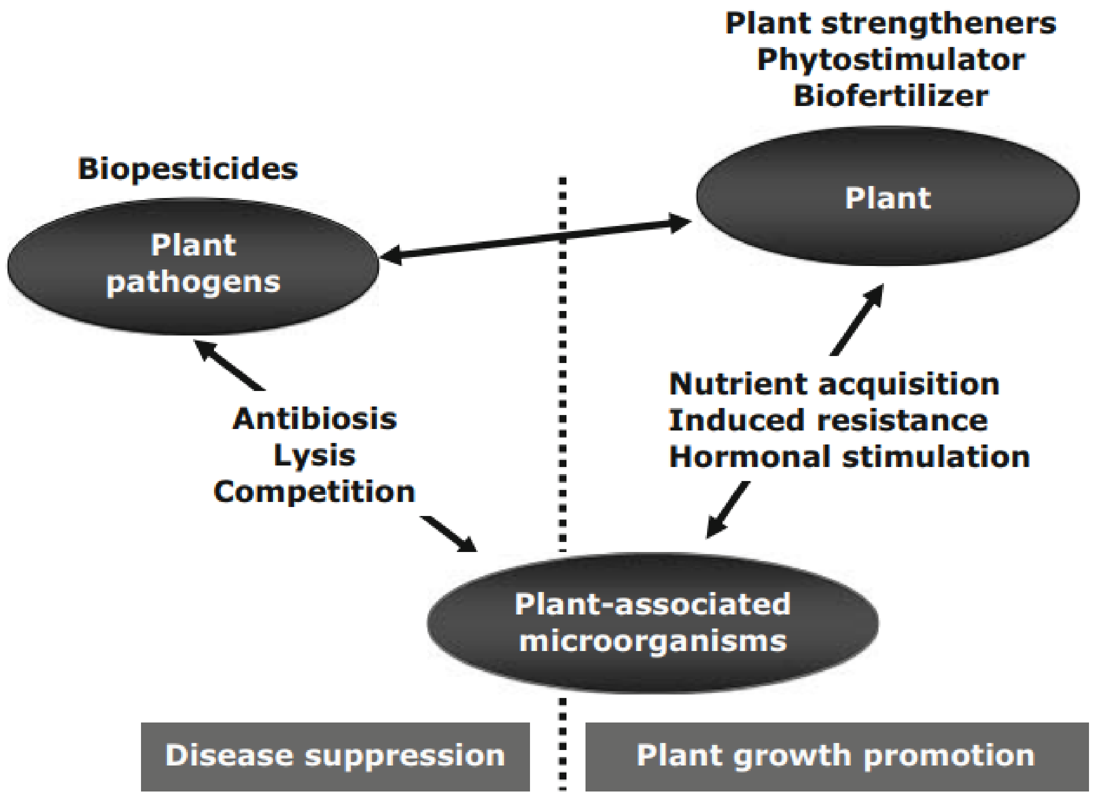


Fig. 1 Plant–microbe interactions promoting plant growth and health: mode of action and potential use in biotechnological applications

From Berg 2009.

All these beneficial attributes show the importance to consider strongly the use of microorganisms for crop protection and stimulation. Other advantages given to the use of organisms instead of synthetic agents are technically:

-If chosen correctly, the organisms have less environmental impact and are less dangerous for human health.
-The organisms show a more targeted activity than synthetic agents usually do
-Small quantity are required
-The organisms multiply themselves but are controlled by both the plant and its microbial flora
-The organisms decompose quicker than conventional synthetic agents.
(Berg, 2009)

There are other advantages, but all of this is very theoretical and it is clear that if any organisms need to be used on crops for protection and stimulation, just like Softer Shock, numerous tests will be required to show cost-efficiency and environmental impact/safety.
However, this gives an insight of why using micro-organisms and work with the plant is crucial, and why we chose to apply our organism directly on the crops instead of applying only the proteins of interest. Indeed, if plant protection against temperature effects is our main objective, why ignoring the fact that microorganisms can have many other beneficial impacts on plants? If we manage to engineer a product that protects and stimulate, it could lead to something even greater and will show that synthetic biology can also cooperate with nature, but a lot still needs to be done.

Here are some examples of species used in biocontrol and bio-stimulation:

Table 1 Representatives of microbial inoculants

Microorganisms	Name of the product	Plants, pathogens, or pathosystems	Company
Ampelomyces quisqualis M-10	AQ10 Biofungicide	Powdery mildew on apples, cucurbits, grapes, ornamentals, strawberries, and tomatoes	Ecogen
Azospirillum spp.	Biopromoter	Paddy, millets, oilseeds, fruits, vegetables, sugarcane, banana	Manidharma Biotech
Bacillus subtilis FZB24	FZB24 li, TB, WG RhizoPlus	Potatoes, vegetables, ornamentals, strawberries, bulbs, turf, and woods	AbiTep
Bacillus subtilis strain GB03	Kodiak	Growth promotion; *Rhizoctonia* and *Fusarium* spp.	(Gustafson); Bayer CropScience
Bacillus pumilus GB34	YiedShield	Soil-borne fungal pathogens	(Gustafson); Bayer CropScience
Bacillus subtilis QST716	Serenade	Tobacco, tomato, lettuce, spinach	AgraQuest
Bacillus subtilis GB03, other *B. subtilis*, *B. lichenformis*, and *B. megaterium*	Companion	*Rhizoctonia*, *Pythium*, *Fusarium*, and *Phytophthora*	Growth Products
Bradyrhizobium japonicum	Soil implant+	Soy bean	Nitragin
Coniothyrium minitans	Contans WG, Intercept WG	*Sclerotinia sclerotiorum*, *S. minor*	Prophyta Biologischer Pflanzenschutz
Delftia acidovorans	BioBoost	Canola	Brett-Young Seeds Limited
Paecilomyces lilacinus	Bioact WG	Nematodes	Prophyta Biologischer Pflanzenschutz
Phlebiopsis gigantea	Rotex	*Heterobasidium annosum*	E~nema Biologischer Pflanzenschutz
Pseudomonas chlororaphis	Cedomon	Leaf stripe, net blotch, *Fusarium* sp., sot blotch, leaf spot, etc. on barley and oats	BioAgri AB
Pseudomonas fluorescens A506	BlightBan A506	Frost damage, *Erwinia amylovora*, and russet-inducing bacteria on almond, apple, peach, pear, etc.	NuFarm
Pseudomonas trivialis 3Re-27	Salavida	Lettuce	Sourcon Padena
Pseudomonas spp.	Proradix	*Rhizoctonia solani*	Sourcon Padena
Serratia plymuthcia HRO-C48	RhizoStar	Strawberries, oilseed rape	Prophyta Biologischer Pflanzenschutz
Streptomyces griseoviridis K61	Mycostop	*Phomopsis* spp., *Botrytis* spp., *Pythium* spp., *Phythophora* spp.	Kemira Agro Oy
Trichoderma harzianum T22	RootShield, PlantShield T22, Planter box	*Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp.	Bioworks

Different species used in crop protection and stimulation, From (Berg 2009)

It has been reported that around 90% of all the products in biopesticides market are based on the species *Bacillus thuringiensis*, a gram-positive bacterium well adapted to plant ecosystems. (Satinder et al. 2006).

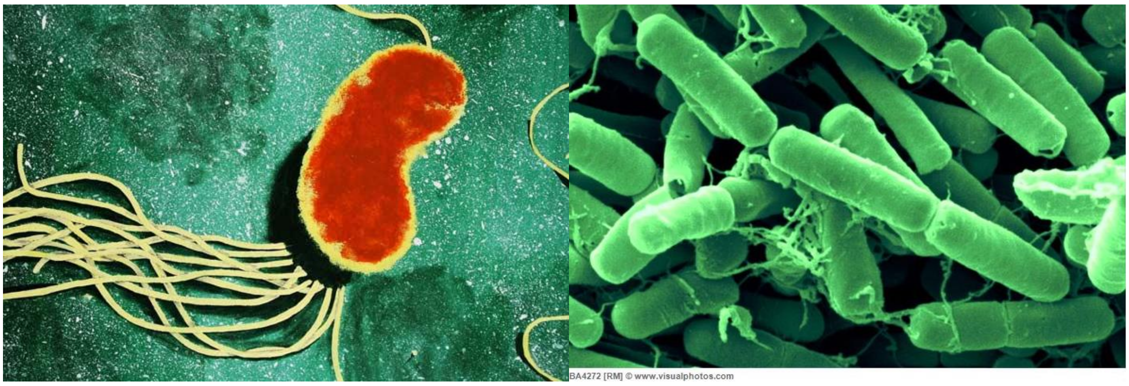
Dipel WP from Valent Biosciences Corporation, as well as Aquabac II XT from AFA Environment Inc are examples of products with this species (Satinder et al. 2006).

Now that all the benefits of using microorganisms as treatment for pest control and stimulation have been described, methods used to spread the organisms on the crops must be seen.

B) How are they applied?

For this part, it is important to differentiate spore-forming microorganisms from non-spore forming ones, as this characteristic alone will influence how the application and storage is done.

Here will be described the possible ways to apply microorganisms on crops, and some examples will be given. *Bacillus thuringiensis* is a spore-forming gram-positive bacterium. *Pseudomonas fluorescens* is a gram-negative non-spore-forming bacterium for example.



(Left) *Pseudomonas fluorescens* (Right) *Bacillus thuringiensis*

To apply micro-organisms on targeted crops, the former must be mixed with carriers and adjuvants. Usually the organisms are in what is called a “dormant state” before the mixing for better storage.

For spore-forming bacteria and fungi, the dormant state is simply called the “spore”, while non-spore-forming bacteria can also have a dormant state that is reversible (Sachidanandham et al, 2009). When mixed with the carrier and adjuvants, the organisms hence enter in the active state and are operational for application, even though process of such revitalisation must be studied in detail for each organism (bacterial spores, fungal spores, dormant cells).

Most important before any application of microorganisms at an agricultural (therefore consequent) scale, is the mass production of the organisms before use. This is crucial to obtain a decent quantity of organisms in the least expensive way.

Fermentation is either performed in liquid state or in solid state (Daigle et al., 1998) and permits to obtain the cells in major quantity, and of course spores for the formulation of the product. The organisms will although need to be harvested from the fermenter before use, this can be done by vacuum filtration, centrifugation, or spray drying depending on what is wished for the organisms after. (Satinder et al 2006)

Two physically distinct ways to spread microorganisms on crops exist, the liquid and dry formulations. In each, the microorganism used is termed the active ingredient.

a) Liquid formulations

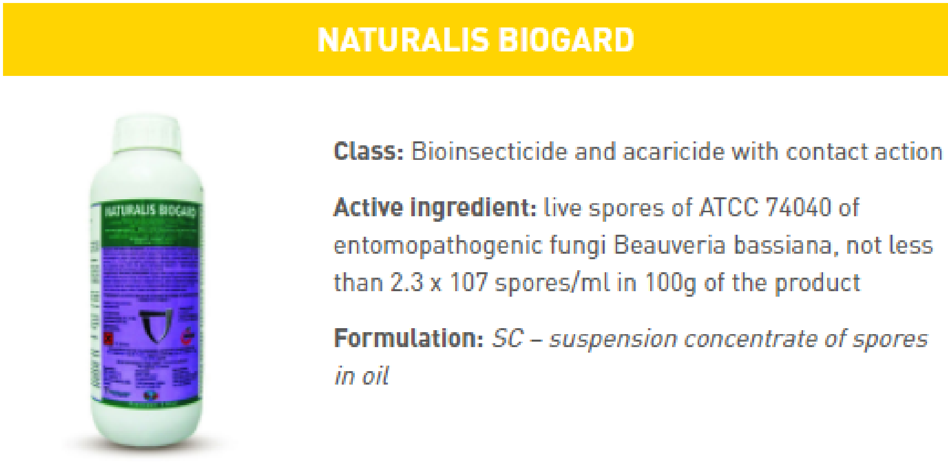
Either water/oil/biopolymer based (or combination), the liquid formulations can be categorized into different methods of application:

<u>The suspension concentrates (SCs) :</u>

“They are suspensions of particulates in liquids, with 10– 40% microorganism, 1–3% suspender ingredient, 1–5% dispersant, 3–8% surfactant, and 35–65% carrier liquid (oil or water)” (Satinder et al 2006).

To each compound its functionality. The carrier is primordial for spreading, dispersant is used to prevent agglomerations of particles and make them reversible, surfactants (usually non-ionic) act as wetting agents that facilitate spreading (see part (2)). Suspender ingredient as well as dispersant are other adjuvants that prevent foaming and enhance spreading and stability (Satinder et al 2006, Gašić & Tanović 2009).

These formulations required to be agitated before use (Gašić & Tanović 2009).

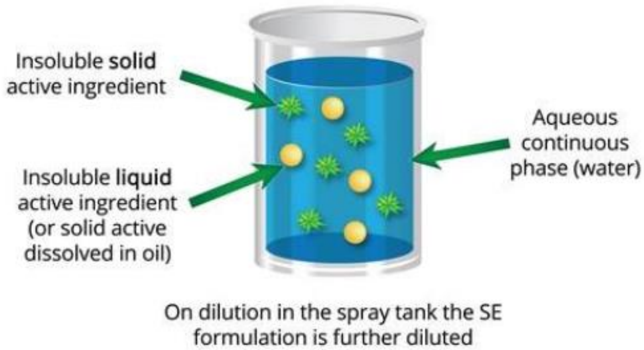


Example of a SC from the company Agromarket (http://www.agromarket.rs/eng/plant-protection/13/Biopesticides*)*

SC in oil are called oil dispersions, the oil used are most of the time from vegetal origins as they are biodegradable and less dangerous for both the manipulator and the plant (Gašić & Tanović 2009).

<u>Suspoemulsions (SEs):</u>

Liquid droplets dispersed in another immiscible liquid, they are considered a mixture between emulsion and suspension concentrate. Although they have some advantages, such as no sedimentation and reduced evaporation, they also raise environmental concerns (quick spray drift) and their use are limited. They are also difficult to elaborate (Satinder et al 2006, Gašić & Tanović 2009). However, they can permit the combination of different products with various properties (water and oil for example).



Principle of suspoemulsions, from https://www.crodacropcare.com/en-gb/products-and-applications/suspoemulsion

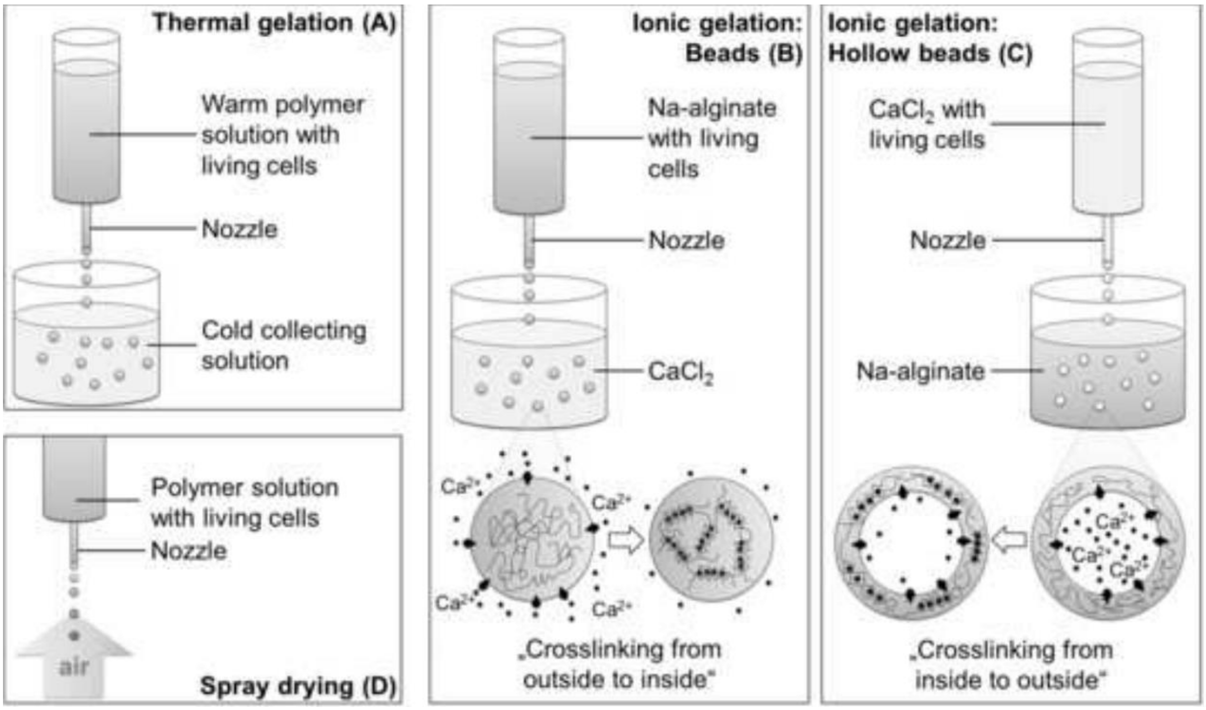
Encapsulation:

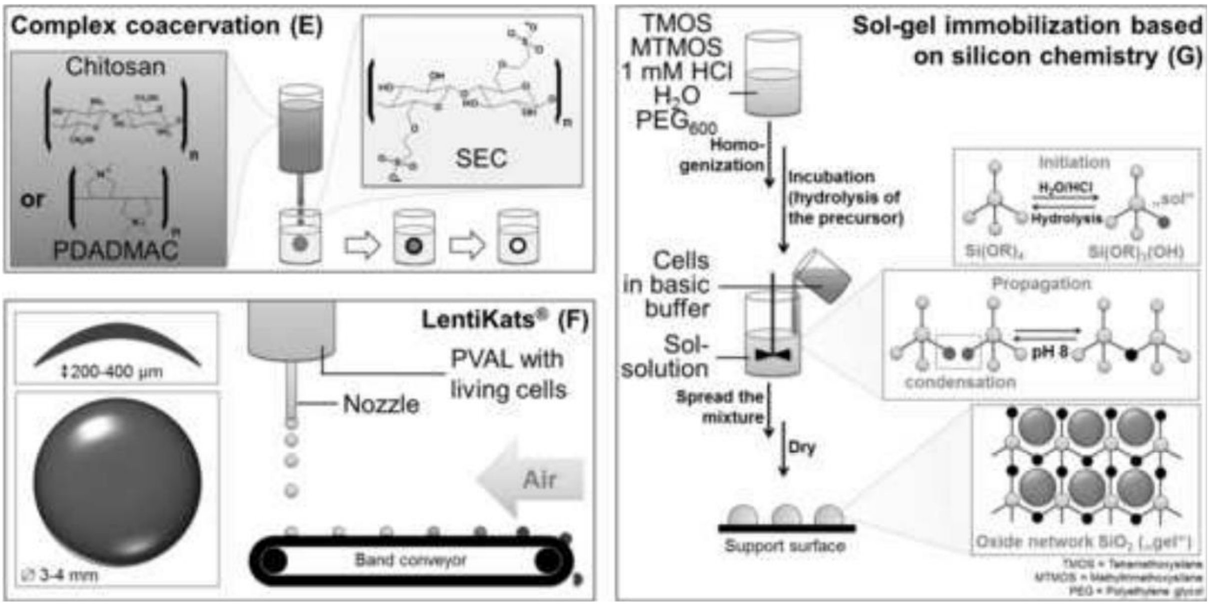
As described in the next part, microorganisms undercome a lot of pressure from the environment they are applied on (temperature, UV...). Engineered to protect the organisms against these parameters that could potentially limit drastically the efficiency of the application, encapsulation strategy is seeing more and more use recently.

They are composed of liquid in which the microorganisms are contained in microcapsules of polymers such as gelatin, starch, cellulose, or other dead cells previously emptied (ghost encapsulation) (Satinder et al 2006). The capsules can also be water-based (hydro capsules).

The protection of the organisms in such capsules is very advantageous for long-term treatments as the durability of the organisms is hence increased, but they are considered costly and hard to produce.

These disadvantages are nevertheless balanced by the fact that the organisms will stay longer on the crops, therefore requiring fewer number of applications and material. There are several ways to encapsulate microorganisms:





Different methods used in microencapsulation of microorganisms, from (Vemmer et al., 2013)

No more detail will be given on these methods in this report, but readers are encouraged to look at Vemmer's review on the subject (Vemmer, M., Patel, A.V., Review of encapsulation methods suitable for microbial biological control agents, Biological Control (2013))

The cells are, after the application, released from the capsule by dividing and growing out of the matrix, and the capsule itself will degrade, but all these processes are slow enough to guarantee long sustain of the application (Vemmer et al., 2013).

Other substances can be added to the capsule to act synergistically with the micro-organisms (Vemmer et al., 2013). This is very promising for Softer Shock because we could add other antifreezes or solar-protecting compounds such as talc to make an even more efficient product.

b) Dry formulations

<u>Dusts:</u>

Formulated by the sorption (ab or ad) of the active ingredient into 50-100µm particles of solids such as chalk, talk and clay, they contain around 10% of microorganisms and their adherence is influenced by their size and floatability. Applied with a plane (crop dusting) or manually, they are slowly being removed due to their negative impact on health and environment (notably the drift of the product from its original target).

Granules:

Granules are bigger than dusts particles in size (100-1000µm) and are mostly applied on the roots of crops for protection. They are made of different material (kaolin (clay), attapulgite (clay), silica, starch, dry fertilizers and ground plant residues), where organisms either adhere on them with or without a sticker, or are absorbed in them directly. The last possibility is close to encapsulation. To prepare such granules, the ingredients are firstly made into a paste and then extruded with the help of a granulation die (Satinder et al 2006, Gašić & Tanović 2009). Concentration of active ingredients (microorganisms) ranges from 5% to 20% in granules.
Wettable granules (WGs) are granules that can be dispersed in water, making their application easier and safer. Vectobac WG, from the Valent Biosciences Corporation, is an example of product made from *Bacillus thuringiensis* granules (Satinder et al 2006).
(https://publichealth.valentbiosciences.com/products/vectobac)

Briquettes:

Very close to Wettable Granules, these blocks size ranges from 100µm to 250µm. They have the advantage of totally neglecting any drift and can be very persistent ‘2 months). They are floatable and made from polymers like polyvinyl (Satinder et al 2006).

Briquettes of Bacillus thuringiensis from the company Arrow-Magnolia (Bactimos briquettes)

<u>Wettable Powders (WPs) :</u>

These are the wettable version of dusts, the final product is often made of “50–80% technical powder, 15–45% filler, 1–10% dispersant and 3–5% surfactant by weight to achieve a desired potency formulation (measured in International Units” (Satinder et al 2006).

The filler is hydrophilic, often silica, to prevent friability. The dispersant is an adjuvant used to maintain the powder in water as long as possible and prevent precipitation. (Satinder et al 2006)

They are advantageous in a way that they are less risky for health than regular powder as they are suspended in water, and they have a long shelf-life. The particles are very small (5µm) and the incorporation of microorganisms is made by sorption as well as the one with dusts (Gašić & Tanović 2009).

Now that all the possible formulations for microorganisms sprays have been treated, here is a table summarising all the advantages and drawbacks of each:

General advantages and disadvantages of different formulations

Dry solid formulations	Liquid suspensions
Advantages	
Are ready-to-use types like dusts, granules, briquettes and applicable with simple equipments	*Emulsion* concentrates and suspension concentrates hold high a.i., bulk storage not necessary
Granules show less drift and applicable to hidden foliage too	*ULV* concentrates can be used without mixing
WPs are easy to transport, store and apply when required; low risks of operator safety	*Encapsulated* suspensions increase residual toxicity and decrease user hazard
Cost effective—transportation costs are low	*Development* process is without harsh conditions of drying—higher recovery
Do not need high quantity of preservatives	*Used* in agriculture, gardens and forests
	Less expensive to apply
Disadvantages	
Dusts are subject to drifts and pose user hazards	*Subject* to deterioration on long storage
Some WPs could clog sprayers	*a.i.* may settle out of emulsions and suspensions, at times
Formulation involves harsh spray drying steps—loss of a.i.	*Sometimes*, require complex spray equipments, e.g., in forestry
Use restricted to gardens, agriculture and water streams	
More expensive to apply	

From (Satinder et al 2006) ULV = Ultra Low Volume, used in forestry.

For now, liquid formulations will be more suited for Softer Shock, as the drift risks are limited, the overall treatment would be cheaper, and methods will be gentler to our microorganisms.

Furthermore, Softer Shock aims at treating harsh climate conditions, which are either drought or spring frost.

These are situated from April to August (in Europe at least), so a "ready-to-use" product is less likely to as interesting as if the product was designed to be applied the whole year. This of course still needs to be discussed.

But the formulation is far from being what will influence our final choice for Softer Shock.

Indeed, many other parameters need to be considered, just like what was described previously (intrinsic and extrinsic parameters influencing foliar sprays). Other factors influencing the development of our microorganisms on the plant will be treated in the next part.

C) Parameters to take in account

To complete what was said previously on the influence of many parameters in foliar applications, here are parameters needed to be taken in account for microorganisms applications.

Some are much alike that what was previously described, but using organisms as plant stimulant instead of using basic synthetic agents adds difficulties:

-Temperature: as much as the drying of the formulation (Fernandez & Brown 2013), degradation of our microorganism by heat needs to be predicted. The range of temperature into which our organism can develop efficiently needs absolutely to be assessed, as it can affect hugely the efficiency of our product. If a protein is to be expressed by the organism, like the toxins produced by *Bacillus Thuringiensis* or the proteins expressed by the organism in Softer Shock, their activity can also be damaged by temperatures (Satinder et al 2006). Always remember that leaf temperature can be very distinct from the ambient air temperature, as said above. (Morris 2002)

-Canopy architecture is even more crucial for microorganisms sprays, as it has been demonstrated that, apart from dictating the efficiency of the application, microorganisms tend to last much longer on shaded foliage as compared to unshaded (20 days against 2 days) (Satinder et al 2006). This is crucial for Softer Shock as we want our organism, especially in response to heat, to act as a solar reflectant.

-Leaf physiology and growth are very important as well, as fast-growing plants will tend to dilute the organism solution and limit its efficiency (Satinder et al 2006).

-Impact on environment: One of the major criteria, if not the most important one in Softer Shock. This impact is measured first by selecting a species of microorganisms described as being in the lowest risk factor group (Berg 2009). As our microorganism will be modified, the risks emerging from it will be higher than "just" potential toxicity. The evaluation of the impact of Softer Shock on the environment, human health, and how we aim at reducing it will be assessed in two other reports called "Biosafety: Killswitch and contamination-limiting diffusion" and "Risk assessment: Toxicity & ecotoxicity studies".

-Sunlight and UV radiations: In *Bacillus thuringiensis* application, UV radiations have been considered to be a major factor in the long-term effect and stability of application. UV radiations can be responsible to alteration of protein actions, damage in the cell. It has been reported that the most dangerous UV radiations to spores, proteins and cells of *Bacillus Thuringiensis* are between 250 and 400 nm. By protecting the organisms with oil, water, adjuvant, or in capsules against those radiations, their effects could be lowered (Satinder et al 2006)

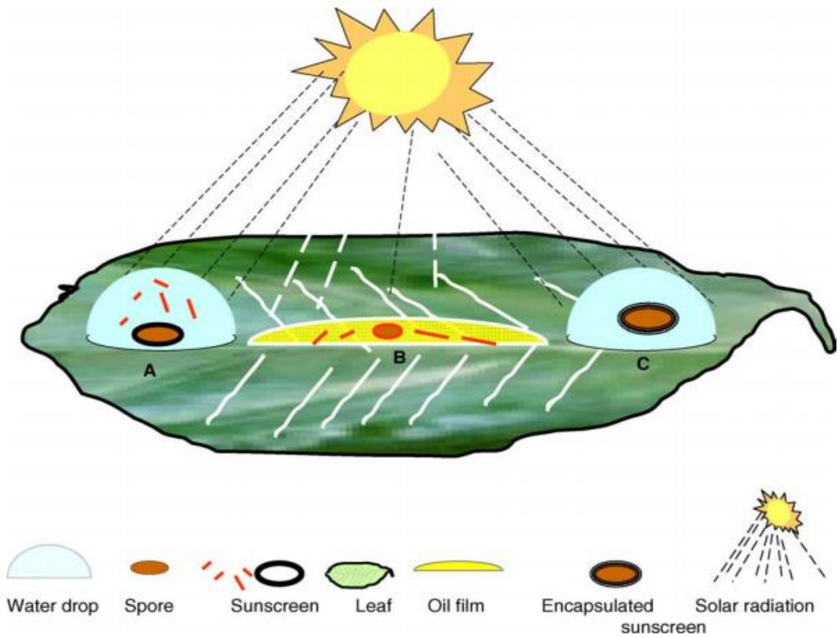


Fig. 1. Water and oil-based formulations on interaction with leaf (hydrophobic) surfaces. (A) Water soluble sunscreen; water droplet forms a large contact angle on the hydrophobic surface. As the droplet dries the water-soluble sunscreen concentrates around the spore [6,119]. Water based formulations will cause minimum drifts owing to density effects during field spray. (B) Oil soluble sunscreen; oil droplet forms a thin film and small contact angle on the hydrophobic surface. As the oil droplet spreads over the surface, the oil-soluble sunscreen spreads with the oil exposing the spore [6]. (C) Water medium and sunscreen is encapsulated to give a long term UV radiation protection (suggested approach).

Methods to protect Bacillus thuringiensis spores against UV radiations, from (Satinder et al 2006)

-Rainfalls: It is very important to consider that, at around a certain volume of rainfall, an applied microorganism can be washed off from the plant and the efficiency of the treatment can be reduced severely. Furthermore, rainfall can promote contamination of the environment by infiltration in the soil and carrying of the organisms of the desired application spot. A sticking adjuvant can be added to the application to reduce such loss. Encapsulation, sugars, and oils can be used to such extent (Satinder et al 2006). The use of bacterial ghosts as encapsulation technique, using an envelope of a naturally sticking bacteria, can help as well (Satinder et al 2006). With the temperature, UV light, humidity (see above), rainfalls complete the list of weather parameters and confirm that an efficient application of microorganisms can be accomplished mainly if the weather conditions allow so.

-pH: The pH of the formulation plays a major role in the activity and development of the microorganism. As said previously, care must be taken not to perturbate the cuticular waxes, of which the pHi is of 3 (Fernandez & Brown 2013), to not affect the plant's health. pH can be regulated by an adjuvant that acts as a pH buffer. pH of the application can be used for better conservation of the product against other microorganisms that can contaminate it, as it can slow their growth. Therefore, the optimal pH of the development of the chosen microorganism must be considered, as well as its pH range (Satinder et al 2006).

-Other products used on the crops at the same time of the application can also interfere with the microorganism and the performance of the formulation. Products like cation and anions used for foliar fertilisation, as seen previously, can change the pH and affects greatly the organism (Satinder et al 2006).

-Topography of the leaf as well as its potential entry points (in the case of microorganisms, the only entry points that can be seriously considered are stomata and cuticular cracks). The penetration of the organism in the leaf can potentially cause problems such as immune reaction and stress from the plant. Organisms that do not possess any enzymes weaponry against plants (cutinase, cellulase, xylanases...) and are less likely to cause immune responses and stress must be considered in priority.

-Conservation of the product against the effects of time and contamination is also an important criterion, more in the economical point of view. The formulation technique must be chosen with care to be well-adapted to the application wished (Berg 2009).

-Interaction with the microbial flora of the plant and the plant itself is very important as well.
This part is so crucial that we dedicated a report on it, called "Working with the plant: Perspectives for chassis selection in accord with the phyllosphere ".
We would like to maximize the harmony between our organism and the phyllosphere of the target plant, as much as its rhizosphere to a lesser extent, as our microorganism surely will be in contact with the later after the application (some of the spray will surely fall down on the soil).

-Even though we insisted a lot on this point previously, adjuvants are crucial for the success of a microorganism formulation:

Different types of adjuvants/additives used in microbial formulations

Adjuvants/additives	Function(s)	Example(s)
Dispersant	Dispersion of formulation into dispersant medium	Amylose; Aluminium silicate; Sodium starch glycolate;
Surfactants and wetters	Enhance the emulsifying, dispersing, spreading, sticking or wetting properties of the biopesticide (includes spray modifiers)	Ethoxylates (Tween/Triton series); polyethylene glycol
Stickers and spreaders	Adhesion of pesticides onto the foliage protecting from rain wash-off and spreading evenly for maximum coverage	Gelatin; gums; molasses; skimmed milk; proprietary like Nufilm and chevron; vegetable gels; vegetable oils; waxes; water-soluble polymers
Drift control agents/anti-evaporants/ humectant	Reduce spray drift, which most often results when fine (<50 µm diameter) spray droplets are carried away from the target area by breezes, including those caused by the vehicle carrying the spray equipment and control of foam while mixing	Polyacrylamides, polysaccharides, and certain types of gums, sorbitol, sucrose, molasses, polyglycol, molasses, glycerol
Thickening agents	Modify the viscosity of spray solutions and are used to reduce drift, particularly for aerial applications	Water swellable polymers producing a "particulate solution," hydroxyethyl celluloses, and/or polysaccharide gums
pH Buffers	Adjust or buffer pH; improve the dispersion or solubilization in the formulation, control its ionic state and increase adjuvant compatibility	Sodium phosphate; Potassium phosphate
Defoaming and antifoam agents	Reduce surface tension, physically burst the air bubbles, and/or otherwise weaken the foam structure	Dimethopolysiloxane-based; silica; alcohol and oils
UV radiation screens	Protect from the deleterious effect(s) of sunlight by forming a protective layer on the formulations	Congo Red; folic acid; lignin; molasses; *p*-aminobenzoic acid; alkyl phenols
Phagostimulants	Stimulate feeding of formulations by pests	Corn meal; sucrose; wheat germ; corn germ; soya flour; casein, edible oil, glutamate, molasses
Synergists	Multiple modes of action; generally complements various formulation components	Sorbitol; sorbic acid; sodium phosphate; stilbene; Tinopal; silicate; protease inhibitors, oleic acid, linoleic acid
Anti-microbial agents	Suppresses the growth of other microorganisms, retaining formulation purity	Sorbic acid; propionic acid; crystal violet
Carriers	Aid in delivery of formulation to target	Alginate; carrageenan; peat, acrylate and acrylamide supersorbents, diatomaceous earth
Binders	For binding the particulates in granules together	Gums; molasses; PVP, resins
Suspending agents	Keep the formulation in suspension	Sorbitol; soya polysaccharides; starch glycolates; sucrose
Attractants	Act as baits to attract target pests	Pheromones, cucurbitacin and various alkaloids; plastisol (PVC and cotton seed oil)
Multi-purpose	Perform various functions at the same time	Molasses; starch, lignin

From (Satinder et al 2006).

Our choice of adjuvant for Softer Shock will be discussed at the end of the report.

-As described above, production of the microorganism through fermentation must be cheap and efficient to provide many cells and spores ready for the application (Satinder et al 2006).

We can just quote here: "Commercial biopesticides should be economical to produce, have persistent storage stability, high residual activity, be easy to handle, mix and apply, and provide consistently effective control of target pests" (Gašić & Tanović 2009)

Note that Softer Shock is not a biopesticide, but more of a bio-protectant, or anti-abiotic stress biocontrol agent (as opposed to biopesticides which are anti-biotic stress, biotic referring being define as "what emerges from living organisms" (http://www.dictionary.com/browse/biotic).

The description of a good biopesticide given by this quotation is however totally applicable to our product.

-Finally, motility of the organism is encouraged to participate to the cross-talk between plants and organisms which was mentioned (Berg 2009).

D) Final composition of the Softer Shock spray

Here we will try to define a concrete formulation for our spray. Keep in mind that this composition and the strategies that we will employ only result from our experience and the help of professionals, so this formulation could be seen as a trial rather than a final decision.

-Our first choice will be to use encapsulation for our chassis. The method will be the ionic gelation, relying on calcium ions and alginate interaction, and already used for several biocontrol agents belonging to bacterial and fungal phylum (Vemmer et al., 2013). Concentration of organisms in foliar sprays seem to range from 10^4 CFU (colony forming units)/ml to 10^{10} CFU/mL (for the Dipel) and his highly variable of the species used and formulation (Satinder et al., 2006), we can predict a mean of 10^8 CFU/ml for our product. This was the concentration used for the field test of Frostban, a bacteria spray engineered to counter frost damages in the 80's (Supkoff et al., 1987). You can learn more about this product by looking at the case study we did in our wiki.

-As each capsule can contain up to 5 active ingredients, we will of course integrate our synthetic amino acid (see Biosafety report) with the organism during the process of encapsulation. As mentioned in the Biosafety report, the amino acid we will use will most likely be the L-4,4'-biphenylalanine, of which the optimal concentration is 0,1 mM.

-Once the organism encapsulated with the L-4,4'-biphenylalanine, we will add adjuvants to the solution. We will use a vegetable oil as a spray sticker, as such adjuvant has been showed to be usually biodegradable and harmless (Satinder et al., 2006). Vegetable oil adjuvants like Kwickin, Protec Plus and Synertrol are concentrated from 200ml/100L of spray to 2L/100L of spray. We are going to use the concentration of 500mL/100L of spray. As for the spray drifting control adjuvant, we will chose hydroxyethyl cellulose at a concentration of 1%, following the guidelines of Cellosize. Adding such compounds to the spray will most likely stabilize the pH around 6-7. If the final pH is not great for the application, we can always stabilize it with acids or bases.

-The spray will otherwise be in a water solution.

Conclusion:

Compound name	Concentration in water
Softer Shock organism (encapsulated, dormant state)	10^8 CFU/mL
Vegetable oil (sticker adjuvant)	500mL/100L
Hydroxyethyl Cellulose (drifting control adjuvant)	1L/100L
L-4,4'-biphenylalanine	0,1 mM

The final Softer Shock spray formulation.

E) How are we going to apply our organism on grapevines?

This part is for us crucial and we inserted it in the Biosafety report that you can find on our wiki. It is all about Tunnel Sprayers to provide efficiency and better safety!

Conclusion of the report::

As this part ends, readers can now see how complex the engineering of an efficient foliar organism spray is, and how many parameters can influence this engineering. All the points above are nevertheless not described very precisely. Readers are therefore encouraged to use this report as a starting point for their iGEM applications and to explore more by themselves.

However, after all this talk about microorganisms, if you are interested to know what was our chassis selection strategy for our project, you can read the report "Working with the plant: Perspectives for chassis selection in accordance with the phyllosphere".
We sincerely hope this report was useful and permits to understand how the project works and what is our vision about it.

Thank you.

The iGEM Ionis team

Useful websites :

https://www.emnz.com/ EMNZ is a company using Effective organisms as crop stimulants.
For other companies, you can just look at the table situated above called "Different species used in crop protection and stimulation".
http://bacdive.dsmz.de/ This is an interesting database about a lot of known organisms and the risk they represent. You can even order them!

We can't thank enough all the reviews and articles used for this report, which were very useful.

Reference:

Gen-ichiro Arimura, Massimo Maffei, Plant Specialized Metabolism: Genomics, Biochemistry, and Biological Functions, CRC press, 2016

Berg, "Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture", Appl Microbiol Biotechnol (2009) 84:11–18

Beauregard et al, "Bacillus subtilis biofilm induction by plant polysaccharides", PNAS Microbiology, available at www.pnas.org/cgi/doi/10.1073/pnas.1218984110

Beyer, M., S. Lau, and M. Knoche. 2005. Studies on water transport through the sweet cherry fruit surface: IX. Comparing permeability in water uptake and transpiration. Planta. 220:474-485.

Buchholz, A., P. Baur, and J. Schönherr. 1998. Differences among plant species in cuticular permeabilities and solute mobilities are not caused by differential size selectivities. Planta. 206:322-328

Daigle et al., "Solid-state fermentation plus extrusion to make biopesticide granules", Biotechnology Techniques, Vol 12, No 10, October 1998, pp. 715–719.

Du Plooy, G.W., C.F. Van Der Merwe, and L. Korsten. 2006. Lenticel discolouration in mango (Mangifera indica L.) fruit - a cytological study of mesophyll cells from affected tissue. Journal of Horticultural Science & Biotechnology. 81:869-873.

Will, S., T. Eichert, V. Fernandez, J. Moehring, T. Mueller, and V. Roemheld. 2011. Absorption and mobility of foliar-applied boron in soybean as affected by plant boron status and application as a polyol complex. Plant and Soil. 344:283-293

Eichert, T. and Goldbach, H. E. (2008), Equivalent pore radii of hydrophilic foliar uptake routes in stomatous and astomatous leaf surfaces – further evidence for al pathway. Physiologia Plantarum, 132: 491–502.

V. Fernández, T. Sotiropoulos and P. Brown, "Foliar Fertilization: Scientific Principles and Field Practices", First edition, IFA, 2013

Fernandez-Escobar, R., J.M. Garcia-Novelo, and H. Restrepo-Diaz. 2011. Mobilization of nitrogen in the olive bearing shoots after foliar application of urea. Scientia Horticulturae. 127:452-454.

Restrepo-Diaz, H., M. Benlloch, and R. Fernandez-Escobar. 2008a. Plant water stress and K+ starvation reduce absorption of foliar applied K+ by olive leaves. Scientia Horticulturae. 116:409-413

Harker, F.R., and I.B. Ferguson. 1991. Effects of surfactants on calcium penetration of cuticles isolated from apple fruit. Scientia Horticulturae. 46:225-233

Jeffree, C.E. 2006. The fine structure of the plant cuticle. In Biology of the Plant Cuticle. Vol. 23. M.M.C. Riederer, editor. 11-125

Karabourniotis, G., and G. Liakopoulos. 2005. Phenolic compounds in plant cuticles: Physiological and ecophysiological aspects. Advances in Plant Physiology. 8:33-47.

Liu, Z.Q. 2004. Effects of surfactants on foliar uptake of herbicides - a complex scenario. Colloids and Surfaces B-Biointerfaces. 35:149-153

Luque, P., S. Bruque, and A. Heredia. 1995. Water permeability of isolated cuticular membranes - a structural-analysis. Archives of Biochemistry and Biophysics. 317:417-422.

Papavizas et al, "Liquid fermentation technology for experimental production of biocontrol fungi", The American Phytopathology Society, Vol 74, N°10, 1984.

Penner, D. 2000. Activator adjuvants. Weed Technology. 14:785-791

Plant Physiology, 4th Edition, 2007, Sinauer Associates.

Popp, C., M. Burghardt, A. Friedmann, and M. Riederer. 2005. Characterization of hydrophilic and lipophilic pathways of Hedera helix L. Cuticular membranes: Permeation of water and uncharged organic compounds. Journal of Experimental Botany. 56:2797-2806

Slavica Gašić and Brankica Tanović, "Biopesticide Formulations, Possibility of Application and Future Trends", Pestic. Phytomed. (Belgrade), 28(2), 2013, 97–102

Dr. Teruo Higa, “Application of Effective Microorganism for Sustainable Crop Production”, HWRI Organic Solutions, 1999

MorrisCE. 2002. Phyllosphere. eLS. doi: 10.1038/npg.els.0000400

Riederer, M., and A. Friedmann. 2006. Transport of lipophilic non-electrolytes across the cuticle. In Biology of the Plant Cuticle. Vol. 23. M. Riederer and C. Muller, editors. Blackwell Publishing, 9600 Garsington Rd, Oxford, Oxen, UK. 250-279.

Sachidanandham et al, “A dormancy state in non spore-forming bacteria”,Appl Microbiol Biotechnol (2009) 81:927–941

Satinder et al., “Recent advances in downstream processing and formulations of Bacillus thuringiensis based biopesticides”, Process Biochemistry 41 (2006) 323–342.

Schönherr, J., and M. Luber. 2001. Cuticular penetration of potassium salts: Effects of humidity, anions, and temperature. Plant and Soil. 236:117-122.

Schönherr, J., and R. Huber. 1977. Plant cuticles are polyelectrolytes with isoelectric points around 3. Plant Physiology. 59:145-150

Schönherr, J. 2006. Characterization of aqueous pores in plant cuticles and permeation of ionic solutes. Journal of Experimental Botany. 57:2471-2491

Nathalie SMITS, Marc FERMAUD, Lionel DELBAC, “Projet SMaCH – DAMAGE : Grapevine Yield Loss Pertes de récolte dues aux bioagresseurs en viticulture”, INRA, 2015, available at https://inra-dam-front-resources-cdn.brainsonic.com/ressources/afile/300733-5a1da-resource-qmpv-smach-smits.html

Supkoff et al., “Monitoring of the winter 1987 field release of genetically engineered bacteria in Contra Costa County ” , State of California Department of Food and Agriculture 1987.

Světlíková P, Hájek T, Těšitel J. Hydathode trichomes actively secreting water from leaves play a key role in the physiology and evolution of root-parasitic rhinanthoid Orobanchaceae. *Annals of Botany*. 2015;116(1):61-68. doi:10.1093/aob/mcv065.

Turgeon, R. 2006. Phloem loading: How leaves gain their independence. Bioscience. 56:15-2

Vacher et al., "The Phyllosphere: Microbial Jungle at the Plant–Climate Interface", Annu. Rev. Ecol. Evol. Syst. 2016. 47:1–24

Vemmer, M., Patel, A.V., Review of encapsulation methods suitable for microbial biological control agents, Biological Control (2013)

Wagner, G.J., Wang, E. and Shepherd, R.W. (2004) New approaches for studying and exploiting an old protuberance, the plant trichome. Annals of Botany, 93, 3-11. doi:10.1093/aob/mch011

Weinbaum, S. 1988. Foliar nutrition in fruit trees. In Plant Growth and Leaf Applied Chemicals. P.M. Neumann, editor. CRC Press, Boca Raton. 81-100