Promoter:

Pr. Thonart Philippe

Walloon Center of Industrial Biology

University of Liege / Faculty of Gembloux Agro-Bio Tech

Co-promoter:

Dr. Ongena Marc

Walloon Center of Industrial Biology

University of Liege / Faculty of Gembloux Agro-Bio Tech

Other members of the jury:

Pr. Sindic Marianne	University of Liege / Faculty of Gembloux Agro-Bio Tech
Dr. Deleu Magali	University of Liege / Faculty of Gembloux Agro-Bio Tech
Pr. Dommes Jacques	University of Liege / Faculty of Gembloux Agro-Bio Tech
Dr. Dorey Stephan	University of Reims
Pr. Höfte Monica	University of Gent
Pr. Lognay Georges	University of Liege / Faculty of Gembloux Agro-Bio Tech
Pr. Portetelle Daniel	University of Liege / Faculty of Gembloux Agro-Bio Tech

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UNIVERSITE DE LIEGE – GEMBLOUX AGRO-BIO TECH

Study of the induced systemic resistance of plants: molecular aspects of the interaction between plant cells and amphiphilic elicitors produced by non-pathogenic rhizobacteria

Guillaume HENRY

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Promoters:
Pr. Thonart Philippe
Dr. Ongena Marc

Walloon Center of Industrial Biology
University of Liege / Faculty of Gembloux Agro-Bio Tech

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Summary / Résumé

Henry Guillaume: "Study of the induced systemic resistance of plants: molecular aspects of the interaction between plant cells and amphiphilic elicitors produced by non-pathogenic rhizobacteria" (Ph.D. Thesis). Belgium, University of Liège - Gembloux Agro-Bio Tech, 161 pp., 12 Tabl., 49 Fig.
Summary: Some non pathogenic rhizobacteria could locally interact with plants, leading to the stimulation of a primed protection state in the host plant. Upon subsequent pathogen attack, this priming state allows an accelerated activation of defense responses extending to all organs of the plant. Fundamental as well as applied research about this induced systemic resistance (ISR) has been tremendously boosted in the past decades, driven by its evident potential for biological control of plant diseases in agriculture. However very little information is available about molecular mechanisms governing the recognition by plant cells of ISR-specific elicitors. Various compounds retaining the ability to elicit ISR have been isolated, among which the recently emerged class of biosurfactants. We conducted the present thesis work with the scope to highlight how such amphiphilic compounds are perceived at the plant cell surface to stimulate the systemic plant immune system. In the first part, the strain *B. amyloliquefaciens* was selected out of other isolates for its technological traits and previously characterized ISR activity. We demonstrated that surfactin is the most competent cyclic lipopeptide (cLP) family produced by this strain to confer ISR on the selected plant model, tobacco. In order to improve our knowledge about the mechanism governing the perception of this biosurfactant at the plant cell surface, we have combined various approaches such as structure/activity (stimulation of the defense-related oxidative burst) relationship, insertion kinetics within membranes of tobacco cultured cells and thermodynamic determination of binding parameters on various model membranes via isothermal titration calorimetry. Our data indicate that surfactin perception relies on a lipid-driven process which is quite uncommon regarding the typical proteic receptor-mediated recognition of molecular patterns governing the plant basal immunity. We then demonstrate that cLPs induce some enrichment or depletion into specific lipid platforms of different proteins. We hypothesize that it may be: (1) the direct consequence of the generation of insertion sites for some proteins in the entire plasma membrane due to specific cLP segregation at lipid phase interfaces; or (2) result from the indirect adjustment of lipid-modifying enzymes activities through an induced rearrangement of the plasma membrane lipid organization. It reinforces the current hypothesis of a role for lipid microdomains-driven lateral compartmentalization in plant cell signaling. The lipid bilayer of target cells could now be considered as the focal point of cLPs perception from where start cellular responses. As they display an original mechanism of action, the class of amphiphilic ISR elicitors to which they belong become more attractive than ever for further development of innovative biopesticides.

Henry Guillaume : « Etude de l'immunité systémique induite des plantes : aspects moléculaires de l'interaction entre cellules végétales et éliciteurs amphiphiles produits par des rhizobactéries non pathogènes ». (Thèse de Doctorat en anglais). Belgique, Université de Liège - Gembloux Agro-Bio Tech, 161 p., 12 Tabl., 49 Fig.
Résumé: Certaines rhizobactéries non pathogènes peuvent interagir localement avec les plantes, menant à la stimulation d'un état de protection « anticipé ». Lors d'une attaque ultérieure par un agent pathogène, cet état de défense anticipé permet une activation plus rapide des réponses de défense s'étendant à l'ensemble des organes de la plante. La recherche appliquée et fondamentale sur cette résistance systémique induite (ISR) a connu un essor important ces dernières années étant donné son potentiel dans la lutte biologique des maladies des plantes en agriculture. Peu d'informations sont cependant disponibles concernant les mécanismes moléculaires gouvernant la reconnaissance par les cellules des plantes des éliciteurs de l'ISR. Différents composés capables d'induire l'ISR ont été isolés, parmi lesquels ceux constituant la récente classe des biosurfactants. Nous avons réalisé ce travail de thèse dans le but d'éclaircir la manière dont les composés amphiphiles sont perçus à la surface des plantes pour stimuler le système immunitaire systémique des plantes. Dans la première partie, la souche *B. amyloliquefaciens* a été sélectionnée pour ses caractéristiques technologiques et son potentiel à induire l'ISR précédemment mis en évidence. Nous avons démontré que la surfactine est la plus active des familles de lipopeptides cycliques (LPc) produites par cette souche sur le modèle végétal sélectionné, le tabac. Afin d'augmenter notre connaissance des mécanismes de perception de ce biosurfactant à la surface des cellules de plantes, nous avons combiné différentes approches comme l'étude de la relation structure/activité, la cinétique d'insertion dans les membranes de cellules de tabac en culture cellulaire et la détermination thermodynamique des paramètres de liaison sur différents modèles

membranaires au moyen de la titration calorimétrique isotherme. Nous résultats montrent que la perception de LPc tels que les surfactines repose sur un processus dépendant des lipides, ce qui est singulier au regard de la reconnaissance typiquement dépendante de récepteurs protéiques des motifs moléculaires gouvernant l'immunité basale des plantes. Nous avons ensuite démontré que les LPc induisent l'enrichissement et la déplétion de différentes protéines au sein de plateformes lipidiques spécifiques. Nous émettons l'hypothèse que cette réorganisation protéique peut être (1) la conséquence directe de la génération de sites d'insertion pour certaines protéines dans la membrane plasmique due à une ségrégation spécifique des LPc aux interfaces de phases lipidiques ou (2) résulter d'un ajustement indirect de l'activité d'enzymes dépendant des lipides via l'induction du réarrangement de l'organisation de la membrane plasmique. Cela renforce l'hypothèse actuelle du rôle de la compartimentalisation latérale de microdomaines lipidiques dans les voies de signalisation des plantes. La bicouche lipidique des cellules cibles peut dorénavant être considérée comme le point focal de la perception des LPc d'où partent les réponses cellulaires. Etant donné leur mécanisme d'action original, la classe des éliciteurs amphiphiles de l'ISR à laquelle appartiennent les LPc devient plus attractive que jamais pour le développement de biopesticies innovants.

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Contents

Introduction

Chapter 1. Stimulation of the plant immune system: a promising strategy to cope with the future world's food supply

1.1. Context

One of the major challenges of the 21th century is to warrant world's food supply. Old and recent advances in agricultural productivity have not yet resulted in a world free of hunger and malnutrition. The world population is expected to grow from nearly 7 billion in 2011 to 9 billion by 2045 (http://www.census.gov/, September 2012 update), mainly in developing countries. This increased population density, coupled with urbanization, changes in dietary habits and increasing use of grains for livestock feed, will require grain production to more than double. However, land suitable for agricultural production is limited and most of the soils with high productivity potential are already under cultivation. In addition, the availability of water is restricted, and in some regions land resources are depleted with the consequence that cultivated area is shrinking. In this context; higher crop yields are required. Crop management systems based upon genetically improved (high-yielding) cultivars, enhanced soil fertility via chemical fertilization, pest control via synthetic pesticides, and irrigation were hallmarks of the Green Revolution and allowed world food production to double in the past 35 years. As 20–40% of the agricultural production worldwide is lost due to pests and diseases (Oerke *et al.*, 2004), reducing such pre-harvest and post-harvest losses is currently one of the most investigated strategy to increase yields. Pesticide use to date has increased 50-fold since 1950 and currently there are thousands of synthetic pesticide products made up of more than 1000 different chemicals and combinations thereof (Karabelas *et al.*, 2009). Due to human health risks and environment detrimental consequences associated with their use, there is consensus emerging about the need for integrated pest management (IPM), a general effort to reduce losses to pests without harmful side effects. One of the strategies for IPM is the biological control of pest by protecting, enhancing, and releasing pests natural enemies and by judiciously and in last resort combine it with chemicals. The challenge of microbiologists and plant-pathologists is thus to mitigate crop loss by new healthy and environmentally-safe means as a complement to chemical applications and molecular breeding technologies. In this aim, dealing with the plant immune system is one of the most promising axes of research. We will show in the next sections

that biological stimulation of the plant immunity provides means for triggering plant defenses and thereby resistance to pathogens in a nontransgenic manner.

1.2. The immune system of plants

As they are constantly exposed to pathogens but lack mobile defender cells and an adaptive immune system, plant defenses rely on the innate immunity of each cell and on systemic signals emanating from infection sites (Dangl *et al.*, 2001, Ausubel, 2005). They have evolved a vast array of passive and active defense mechanisms that are manifested in the pest-colonized organ. Defense signals could be systemically emitted to activate a plethora of defense responses in the non-colonized organs of a plant locally infected by a microbe, infested by an herbivore or even stimulated by a chemical compound. Defense signals could also be primed for rapid activation after a localized perception of non pathogenic fungi or bacterial strains.

The presence of infectious agents is detected through the recognition of microbial signals. More globally, signals that are perceived by plant cells and induce defense responses are named elicitors. They may be categorized in two classes: general (or non-specific) elicitors, which do not significantly differ in their effect on different cultivars within a plant species and may therefore be involved in general resistance; and specific elicitors, which are formed by specialized pathogen races or strains and function only in plant cultivars carrying the corresponding disease resistance gene (Montesano *et al.*, 2003).

General elicitors are designated pathogen-associated molecular patterns (PAMPs) when isolated from infectious agents but they may also correspond to endogen plant-host derived signals resulting from the action of the pathogen agent, called damage-associated molecular patterns (DAMPs), to signals from non pathogenic microorganisms referred here as microbe-associated molecular patterns (MAMPs) and to chemicals. The perception of general elicitors triggers a broad array of reactions, which culminate in the activation of the basal resistance or PAMP-triggered immunity (PTI) (Nicaise *et al.*, 2009) (Figure 1A). This defensive reaction may be strong enough to halt infection before the invading microbe becomes established. However, some successful pathogenic microorganisms may overcome basal resistance by delivering virulence effector proteins or DNA into host cells. These elicitors inhibit signalization pathways or the synthesis of defense compounds by the host plant and thus suppress this first type of immunity. Such signals are the specific elicitors and are likely the cause for susceptibility of many crops to virulent microbial pathogens.

In response, plants have evolved a second line of defense through specific disease resistance (*R*) genes, the so-called effector-triggered-immunity (ETI, Figure 1B) (Pelletier *et al*; Jones *et al.*, 2006). The major class of *R* genes encodes a nucleotide binding site (NBS) and a block of leucine-

rich repeats (LRR) (McHale *et al.*, 2006). Generally, NBS-LRR proteins do not contain predicted transmembrane segments or signal peptides, suggesting they are soluble cytoplasmic proteins. NBS-LRR sequences subclassification is based on the N-terminal domain, which contains either a leucine zipper (LZ) motif (Lokossou *et al.*, 2009) or a Toll and interleukin-1 receptors homology region (Picard *et al.*; Yang *et al.*, 2010). ETI is generally associated with hypersensitive response (HR) and programmed cell death at the penetration site (Jones *et al.*, 2006). The recognized effector is termed an avirulence (Avr) protein. Because the effector-R protein relationship is highly specific, this *R* gene-mediated resistance appears to be similar to adaptative immunity in mammals. However, as *R* gene-mediated resistance is expressed through similar defense responses as those that are active in basal resistance, but on a much greater scale (Boller *et al.*, 2009), ETI is considered as another form of plant innate immunity. Therefore, PTI and ETI are considered as primary and secondary innate immunity respectively. Induction of primary innate immunity is now considered as a key component of biocontrol of pest in the Integrated Pest Management.

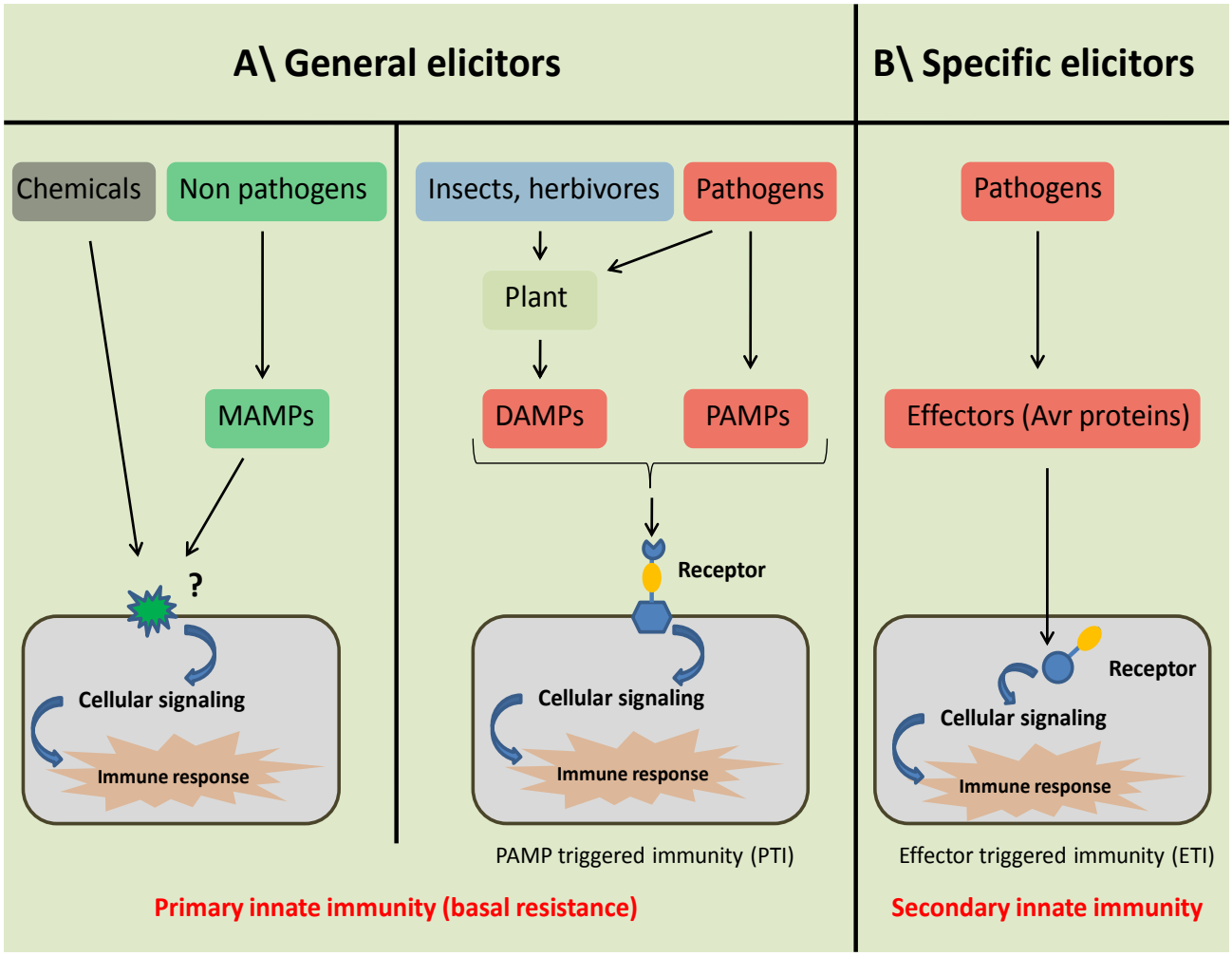


Figure 1: Elicitors may be categorized in two classes: A\ General (or non-specific) elicitors do not significantly differ in their effect on different cultivars within a plant species and are involved in primary innate immunity. They include chemicals, microbes-associated-molecular-patterns (MAMPS) from non pathogenic microorganisms, damage-associated-molecular-patterns (DAMPS) from plant surfaces resulting from the action of the invading agent and pathogen-associated-molecular-patterns (PAMPs) from pathogenic microorganisms. Even if perception of elicitors is often described as being receptor-mediated, only few binding sites have been characterized to date. B\ Specific elicitors (or effectors) are formed by specialized pathogens and function only in plant cultivars carrying the corresponding disease resistance gene. Effectors typically lead to the secondary innate immunity after an intracellular receptor-mediated perception.

1.3. The principles of systemic immunity

When the primary innate immunity is established in the tissue surrounding the site of initial infection, it is called localized acquired resistance (LAR; Figure 2A) (Kombrink *et al.*, 2001). However, via emission of molecular signals, defense mechanisms can also be induced in distal organs of a plant that is locally infected by a pathogen. Such systemic resistance reaction renders the host less susceptible to subsequent challenge by a pathogen or a parasite in distal tissues. This long-lasting phenomenon was termed systemic acquired resistance (SAR, Figure 2B) (Iriti *et al.*, 2010) and has been extensively reviewed in the last years (Durrant *et al.*, 2004; Spoel *et al.*, 2012).

Recently, major advances have been made in identifying metabolites that are candidate systemic signals in plant defense against pathogens. Methyl salicylate, jasmonates, azelaic acid and a diterpenoid have been proposed as mobile signals involved in the activation of SAR which confers enhanced resistance against a broad spectrum of pathogens (Shah, 2009). Conceptually, SAR has been associated with the perception of elicitors from avirulent pathogens but a similar systemic defense may also be lighted on by DAMPs or by other compounds of biological but not microbial origins and by chemicals.

Another form of induced resistance may be triggered by MAMPS isolated from beneficial non-pathogenic microorganisms, and is referred as induced systemic resistance (ISR; figure 2B) (Mishra *et al.*, 2009). Mutually beneficial interactions between plants and microbes improve plant nutrition, help the plant to overcome biotic or abiotic stresses, or both. In many cases, plant-microbe associations enhance the defense capacity of the plant (Barea *et al.*, 2005). The best characterized organisms inducing ISR are the so-called plant-growth-promoting rhizobacteria (PGPR) among which several species of *Pseudomonas* and *Bacillus* (Lugtenberg *et al.*, 2009, Van Loon *et al.*, 1998). ISR is phenotypically similar to SAR and is also effective against a broad range of diseases caused by viruses, bacteria and fungi (Vallad *et al.*, 2004), being therefore a promising tool to control crop pest.

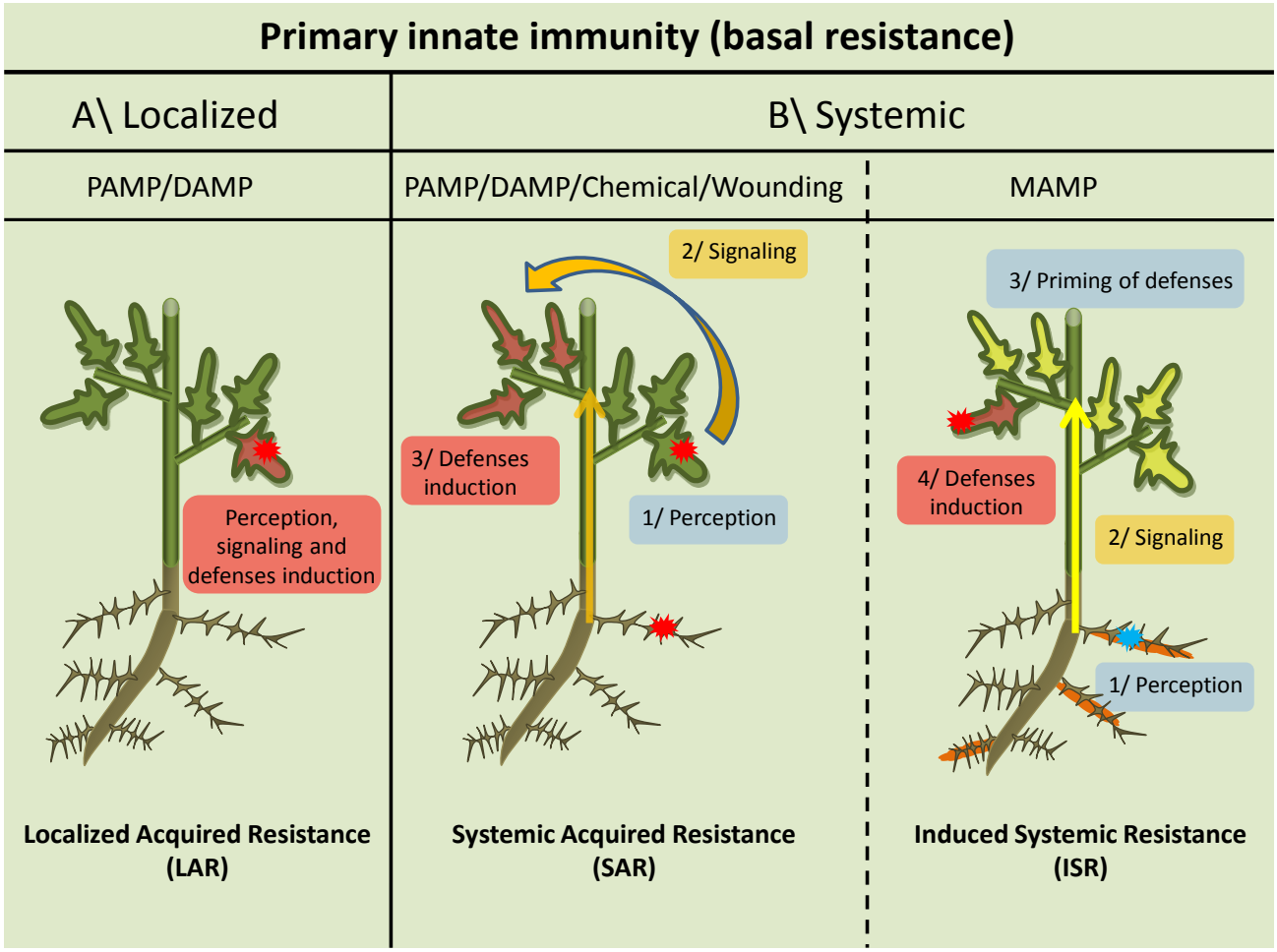


Figure 2 : The primary innate immunity could be localized (A) or systemic (B). Systemic acquired resistance corresponds to an enhanced state of defense responses after perception of pathogens or a range of compounds and is invariably associated with accumulation of salicylic acid and pathogenesis-related proteins in resistant tissues. Besides, induced systemic resistance is typically stimulated after perception of signal from beneficial microorganisms. Perception of such MAMPs firstly leads to the establishment of an enhanced defense potential, the priming state, allowing faster defense responses induction upon subsequent pathogen attack.

Over the last 20 years, research on SAR and ISR has considerably improved our understanding of the molecular basis of systemic resistance. Both two main types of systemic resistance SAR and ISR can be both globally viewed as a three-step process involving sequentially i) the perception by plant cells of elicitors produced by the inducing agent that initiates the phenomenon, ii) signal transduction that is needed to propagate the induced state systemically through the plant and iii) expression of defense mechanisms *sensu stricto* that limit or inhibit further pathogen penetration into the host tissues. It appeared that, from a molecular point of view, ISR differs from SAR. It may explain why SAR is typically effective across a wide array of plant species, whereas there is some specificity in the ability of PGPR strains to elicit ISR in certain plant genotypes (Yan *et al.*, 2002, VanWees *et al.*, 1997). Globally, local and systemic defense responses triggered by microorganisms are controlled by signaling pathways that crosscommunicate (Persello-Cartieaux *et al.*, 2003). SAR

stimulated following infection by necrosis-inducing pathogens is dependent on salicylic acid signaling (Park *et al.*, 2008) while ISR triggered by beneficial rhizobacteria typically relies on the jasmonic acid and ethylene signaling pathways (Pieterse *et al.*, 2002). However, both SAR and ISR phenomenon converge downstream since they are controlled by the same transcriptional regulator NRP1 (see section 3.3). Moreover, a recent study provides new insights into a SA-dependent ISR response induced by non-pathogenic rhizobacteria (van de Mortel *et al.*, 2012).

1.4. Practical applications of compounds interfering with the plant immunity

Recent progresses in the understanding of the principles of plant systemic immunity has been the driving force to set up field and greenhouse crop protection experiments based on this phenomenon. The continuous discovery of new PAMPs and MAMPs contributes to enlarge the reservoir of very efficient structural patterns for boosting plant immunity. The most active of these compounds may be produced biotechnologically and purified to the required level for commercialization. Alternatively they may serve as molecular basis for the development of new structural derivatives with higher activity and/or lower susceptibility to degradation and/or lower lateral toxicity. Also, some chemicals are strong inducers of a SAR-like response. Compounds such as 2,6-dichloro isonicotinic acid, benzothiadiazole and its derivative acibenzolar-S-methyl, or β-amino butyric acid, are nowadays successfully employed to control diseases of various crop plants (Vallad *et al.*, 2004). In another approach, interfering with the molecular dialogue between microbial patterns and their cognate plasma membrane sensing systems may be the basis of novel genetic-based strategies to engineer durable plant disease resistance. For instance, enhancement of the potential of plants to recognize a broader range of PAMPs and therefore resist to a broader range of pathogens has been successfully achieved via heterologeous expression or overexpression of their receptors in some plants (Gust *et al.*, 2007). Enhancing the expression of key regulators of systemic resistance controlling immunity-associated genes is also a promising strategy to boost the defense reaction in its entirety (Makandar *et al.*, 2006). One more way to improve disease resistance is the overexpression in plants of antibodies fused with antimicrobial peptides that will recognize specific pathogen surface components (Li *et al.*, 2008).

1.5. The place of ISR in biopesticide development

Biopesticides can be broadly defined as living organisms, or natural products derived from these organisms, that are used to suppress pest populations. These organisms include plants (genetically modified crops), insects, nematodes and microorganisms. Their overall contribution to plant health

management is currently relatively small, representing ~2.5% of total agricultural sales. However, the growth rate of the biopesticide market is estimated to be currently around 15% against 1,3% for synthetic pesticides, to reach 3 billion $ by 2014 (www.soci.org, update November 2012). One of the advantages of microbial biopesticides compared to most of the other phytosanitary products is the multiplicity of their ways of action. They are globally based on competition for nutrients and space, direct antagonism of plant pathogen growth and host plant immunization. Based on the promising results obtained with beneficial ISR-inducing microorganisms, the development of microbial formulations was promoted for application in conventional agriculture. Microorganism-based products (bacteria, fungi, virus, and yeasts) represent ~30% of total sales and new products are regularly brought to the market. The ISR inducers, by priming the immune system of plants, could confer protection against a broad array of pathogens, without affecting the fitness of the host (and thus crop yield), resistant pathogen selection, and health or environment toxicity.

ISR-based biocontrol can thus be used in situations where no control is currently available, where conventional pesticides cannot be used owing to residue concerns, or in the rapidly growing sector of organic farming. They can also be applied together with chemicals, either in rotation to reduce the possible development of pathogen resistance or in an integrated pest management strategy with the goal of minimizing the use of synthetic insecticides. Even if use of biopesticides will not become a stand-alone method for pest control, it is now clear that they will be further integrated into pest management systems. Microbial products are currently successfully used in field crops and greenhouses to reduce diseases on various cereals, legumes, fruits, flowers and ornamentals caused either by soil-borne, foliar or post-harvest pathogens (Vallad *et al.*, 2004).

Chapter 2. Elicitors of plant systemic resistance: diversity and modes of perception

2.1. Direct perception of pathogens via PAMPs

PAMPs represent structures that are essential for microbial life and that are typically harboured by invading pathogens. These include cell surface constituents but may also be secreted enzymes or proteins normally located in the cytoplasm. A broad array of structurally diverse PAMPs has been described originating from fungal, oomycete and bacterial pathogens. Most of these PAMPs are oligosaccharides, glycopeptides, and peptides. Some of these patterns such as Pep-13, xylanase and cold-shock protein are only perceived by a narrow range of plant species belonging to only one plant family (Felix *et al.*, 2003, Ron *et al.*, 2004). A representative example is EF-Tu in the family of *Brassicaceae* (Kunze *et al.*, 2004). By contrast, other PAMPs such as chitin, LPS and flagellin trigger defense responses in many host species even if there is some degree of specificity and perception efficacy for a plant family/species as in the case of flagellin (Chinchilla *et al.*, 2006).

PAMPs are perceived at the plant cell surface by high-affinity membrane-anchored pattern recognition receptors (PRRs) typically consisting in an extracellular ligand-binding domain with leucine-rich repeats (LRR), a single transmembrane domain and an intracellular serine/threonine kinase-signaling domain. They are referred to as receptor-like kinases (RLK). Receptor-like proteins (RLPs) are similarly structured, but lack the cytoplasmic kinase domain. In *Arabidopsis*, 610 RLKs and 56 RLPs have been identified (Shiu *et al.*, 2001, Fritz-Laylin *et al.*, 2005). A large number of genes encoding RLKs and RLPs are transcriptionally induced upon PAMP treatment, illustrating the large diversity of such perception systems and their crucial role in defense (Zipfel *et al.*, 2004, Zipfel *et al.*, 2006). Meanwhile, only a small number of PRR have been characterized so far compared to the diversity of PAMPs identified (Table 1).

Table 1. PAMPs identified to date as potential elicitors of plant immunity responses (DR: Defense Responses, EE: Early Events; LAR: Localized Acquired Resistance; SAR: Systemic Acquired Resistance; ni: non identified). In some cases, their binding site at the plant cell surface have been identified and characterized.

Elicitor	Origin	Associated receptor	Response type	Reference
Enzymes and proteinaceous elicitors				
Superoxide dismutase	*Xanthomonas campestris, E. coli*	?	DR	(Watt *et al.*, 2006)
Xylanase	*Trichoderma viride*	LeEix1/2	DR	(Ron *et al.*, 2004)
Transglutaminase	*Phytophthora sp.*	ni	DR	(Brunner *et al.*, 2002)
Cold shock protein	*Staphylococcus aureus*	?	EE	(Felix *et al.*, 2003)
Cellulose-binding lectin	*Phytophthora parasitica*	ni	DR	(Gaulin *et al.*, 2006)
Cryptogein	*Phytophthora sp.*	ni	SAR	(Osman *et al.*, 2001, Keller *et al.*, 1999)
Megaspermin	*Phytophthora sp.*	ni	SAR	(Baillieul *et al.*, 2003)
Oligandrin	*Pythium oligandrum*	ni	LAR	(Picard *et al.*, 2000)
PebC1	*Botrytis cinerea*	ni	SAR	(Zhang *et al.*, 2010b)
Glycoprotein 15 Kd	*Phytophthora*	ni	SAR	(Mishra *et al.*, 2009)
PeaT1	*Alternaria tenuissima*	ni	SAR	(Zhang *et al.*, 2010b).
P1390	*Phytophthora boehmeriae*	ni	SAR	(Wang *et al.*, 2003).
Elongation factor EF-Tu	*E. coli*	EFR	LAR	(Kunze *et al.*, 2004)
Sulfated peptide Ax21	*Xanthomonas oryzae*	XA21	DR	(Lee *et al.*, 2009)

Necrosis-inducing protein 1 (NPP1)	*Phytophthora parasitica*	ni	DR	(Fellbrich *et al.*, 2002)
Pen elicitor	*Penicillium chrysogenum*	ni	SAR	(Thuerig *et al.*, 2006)
Flagellin	*Pseudomonas syringae*	FLS2	SAR	(Chinchilla *et al.*, 2006)
PaNie	*Pythium aphanidermatum*	ni	DR	(Veit *et al.*, 2001)
Harpin	*Erwinia amylovora, Pseudomonas syringae*	Non proteinaceous	SAR	(Bi *et al.*, 2005, Reboutier *et al.*, 2007)
Poly(oligo)saccharides				
Exopolysaccharides	*Pantoea agglomerans*	ni	DR	(Ortmann *et al.*, 2006)
Heptaglucoside	*Phytophthora*	GBP	SAR	(Umemoto *et al.*, 1997, Fliegmann *et al.*, 2004)
Peptidoglycan	*Xanthomonas campestris*	ni	LAR	(Erbs *et al.*, 2008, Gust *et al.*, 2007)
Chitin	Fungi	CEBiP	LAR	(Kaku *et al.*, 2006, Ben-Shalom *et al.*, 2002, Wang *et al.*, 2008)
Glycolipids				
Nodulation factor	*Sinorhizobium meliloti*	NFBS	DR	(Baier *et al.*, 1999, Gressent *et al.*, 1999)
Lipopolysaccharides	*Burkholderia cepacia, Pectobacterium atrosepticum, Pseudomonas corrugata*	ni	SAR	(Coventry *et al.*, 2001, Desender *et al.*, 2006, Gerber *et al.*, 2004)

Cerebrosides	*Magnaporthe grisea, Schizophyllum commune*	ni	SAR	(Koga *et al.*, 1998, Deepak *et al.*, 2003, Umemura *et al.*, 2000)
Lipids				
Lipoteichoic acid	*Staphylococcus aureus*	ni	DR	(Zeidler *et al.*, 2004)
Ergosterol	Ascomycetes, basidiomycetes	ni	SAR	(Granado *et al.*, 1995, Lochman *et al.*, 2006, Laquitaine *et al.*, 2006),
Arachidonic acid	Oomycetes	ni	DR	(Boller, 1995)

2.2. Indirect perception of pathogens via DAMPs

In a more indirect way, plants can also detect the presence of pathogens through the perception of endogenous compounds that have been released from structural barriers or from other macromolecules by lytic enzymes produced by the invader or by the host itself (Table 2). Such DAMPs typically appear in the apoplast and may thus, like PAMPs, play the role of signal for danger to induce innate immunity. For instance, oligogalacturonides are released by microbial enzymes and putatively recognized by the receptor WAK1 (D'Ovidio *et al.*, 2004). Emission of these endogenous signals allows disrupted or injured cells to communicate their damage to the tissue or systemically to all organs. Systemin is formed in damaged tomato leaves and is further perceived as primary signal for systemic defense induction (Ryan *et al.*, 2003). Similarly, the 23-residue peptide AtPep1 is released from precursor proteins in response to wounding and triggers an innate immune response in *Arabidopsis* via recognition by the PEPR1 receptor (Yamaguchi *et al.*, 2006).

Table 2. DAMPs released from structural barriers or from other plant macromolecules by lytic enzymes produced by the invader or by the host itself, identified as potential elicitors of plant immunity responses. (ni: non identified)

Elicitors	Origin	Receptor	Reference
Oligosaccharides			
Oligogalacturonides	Arabidopsis cell wall	WAK1	(D'Ovidio *et al.*, 2004)
Lipids			
Cutin monomers	Cucumber cutin	ni	(Kauss *et al.*, 1999)
Peptides			
Hydroxyproline glycopeptides, HypSys	Secreted protein	ni	(Pearce *et al.*, 2002)
Rapid alkalinization inducing factor, RALF	Extracellular protein	ni	(Pearce *et al.*, 2001)
Systemin	Cytoplasmic prosystemin	ni	(Ryan *et al.*, 2003)
AtPEP1	C-terminal of *PROPEP1* product	PEPR1	(Yamaguchi *et al.*, 2006)

2.3. Perception of non pathogenic microorganisms via MAMPs

2.3.1. MAMPS elicitors from beneficial rhizobacteria

Compared to PAMPs from pathogens, less information is available on the determinants from non pathogenic rhizobacteria that trigger ISR (Table 3). However, driven by the obvious potential for biological control of plant diseases in agriculture, fundamental as well as applied research on ISR has been tremendously boosted in last decades. Various compounds retaining plant defense eliciting properties were isolated from multiple genera such as *Pseudomonas*, *Serratia* and *Bacillus* (Ongena *et al.*, 2008a).

2.3.1.1. Cell surface components

It has been demonstrated that flagellin from the plant beneficial rhizobacterium *Pseudomonas putida* strain WCS358 can act as elicitor of systemic resistance in *Arabidopsis* against *P. syringae* (Meziane *et al.*, 2005). However, additional experiments with other bacterial isolates and on multiple pathosystems are required to accurately evaluate to what extend flagellins may be considered as general determinants of the rhizobacteria-mediated ISR. Lipopolysaccharides (LPS) are cell surface components of Gram-negative bacteria associated with the outer membrane of the cell envelope. These compounds have also been occasionally reported as PAMPs. They are tripartite amphipathic molecules comprising a lipid A moiety which is embedded in the outer leaflet of the phospholipid/protein bilayer, a core oligosaccharide and a O-antigen side chain. Involvement of LPS in the elicitation of ISR by beneficial bacteria has been reported for *P. fluorescens* (Leeman *et al.*, 1996, Duijff *et al.*, 1997, Tang *et al.*, 2005, Vanpeer *et al.*, 1992) and *P. putida* strains (Meziane *et al.*, 2005) but also for *Burkholderia cepacia* in the tobacco/*Phytophthora nicotianeae* pathosystem and *Rhizobium elti* G12 on cyst nematode-infected potato (Reitz *et al.*, 2002). It was evidenced by testing purified LPS, heat-killed cells, crude cell envelope extracts or mutants with modified LPS. In many cases, mutants that lack the O-antigen side chain are not inducers, suggesting a crucial role of this sub-structure. Therefore the observed degree of specificity should be related to the composition of pseudomonad LPS that are almost strain-specific regarding the structure of the O-side chain and their eliciting activity seems to be dependent on the isolate studied.

2.3.1.2. Siderophores and salicylic acid

To ensure their growth in iron-limited environments, microorganisms have evolved powerful Fe^{3+}-acquisition systems based on the excretion of high-affinity iron-chelating molecules termed siderophores (Loper *et al.*, 1991). Pyoverdines are siderophores typically synthesized by fluorescent

Pseudomonas (Budzikiewicz, 2004). Based on experiments involving pyoverdin-non-producing mutants or addition of pure pyoverdines, these compounds were also demonstrated as potential ISR elicitors (Hofte *et al.*, 2007, De Vleesschauwer *et al.*, 2009). For instance, WCS358 can elicit ISR in several plants such as *Arabidopsis*, bean, tomato and *Eucalyptus* through its siderophore (Bakker *et al.*, 2003, Meziane *et al.*, 2005, Ran *et al.*, 2005). Salicylic acid (SA) is produced by some of the rhizobacteria that induce systemic resistance under iron-limited conditions. Its role in the ISR elicitation process was demonstrated in the case of *Pseudomonas aeruginosa* KMPCH (De Meyer *et al.*, 1997, De Meyer *et al.*, 1999). Nevertheless, several reports showed that SA production by other strains was not associated with ISR (Leeman *et al.*, 1996, Press *et al.*, 1997). SA is also an intermediate in the biosynthesis of other siderophores such as pyochelin in *Pseudomonas aeruginosa* (Serino *et al.*, 1997) and a role for pyochelin was proposed in ISR triggered in tomato by *P. aeruginosa 7NSK2* (Audenaert *et al.*, 2002b).

2.3.1.3. Antibiotics, biosurfactants and other compounds

Some *Pseudomonas* products known for their antibiotic activities such as pyocyanine and 2,4-diacetylphloroglucinol (DAPG) may act as elicitors of systemic resistance (Iavicoli *et al.*, 2003, Siddiqui *et al.*, 2003). The phenazine-type molecule pyocyanine was proposed to act synergistically with pyochelin to trigger ISR in tomato treated with *P. aeruginosa 7NSK2* (Audenaert *et al.*, 2002b). DAPG is another antibiotic produced by *P. fluorescens* CHA0 that also retains some ability to stimulate defense-related reactions in the host plant as it is an essential component of the ISR-mediated disease reduction by this strain in *Arabidopsis* and tomato plants infected respectively by *Peronospora parasitica* and the nematode *Meloidogyne javanica* (Iavicoli *et al.*, 2003, Siddiqui *et al.*, 2003).

In our laboratory, searching for molecular determinants of *P. putida* BTP1 responsible for ISR elicitation led to the isolation of an excreted compound consisting of a tri-N-alkylated benzylamine derivative (NABD, Figure 3A) (Ongena *et al.*, 2005c).

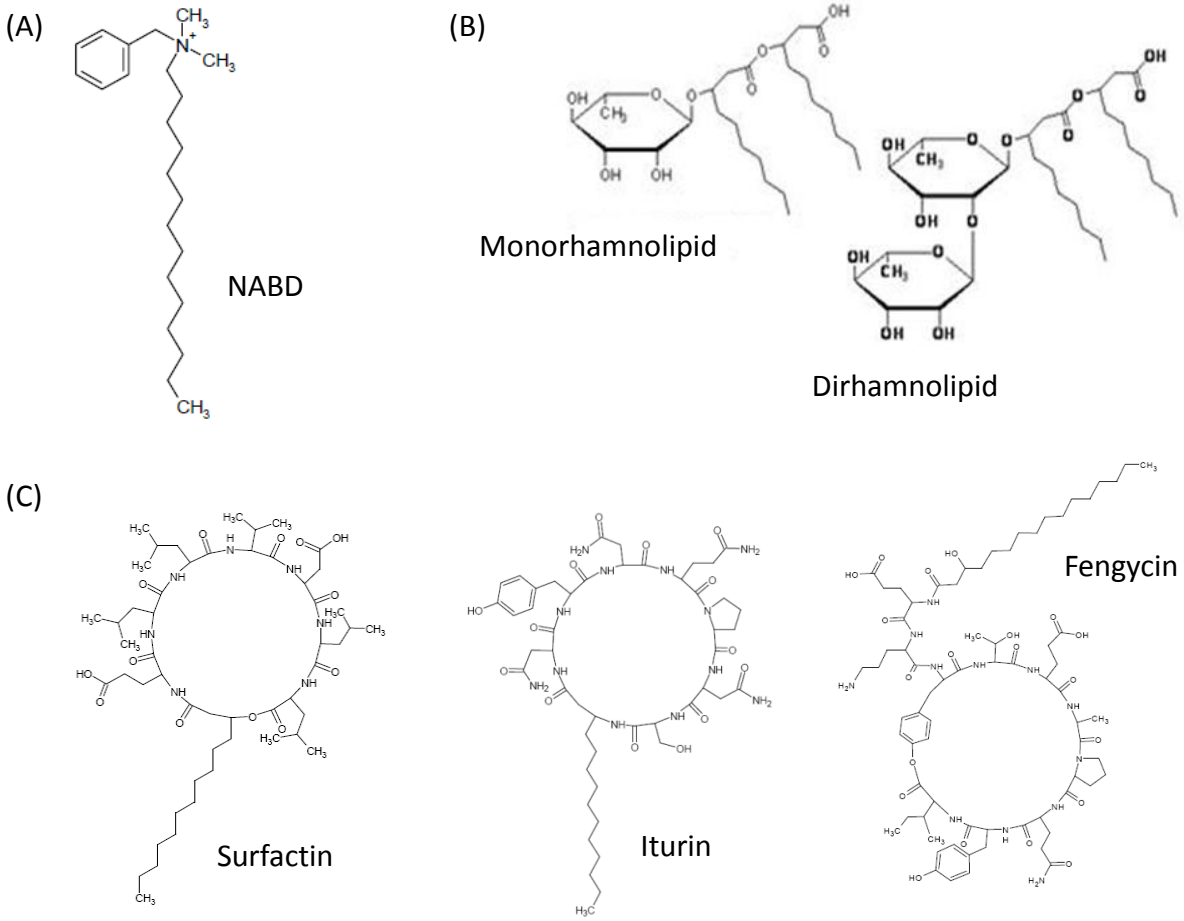


Figure 3 : Structures of some representative biosurfactant ISR elicitors. A. The benzylamine derivative from *Pseudomonas putida* BTP1 identified as elicitor of ISR (N,N-dimethyl-N-tetradecyl-N-benzylammonium). B. The two major rhamnolipids produced by *Pseudomonas putida* strains. C. Representative members of the three lipopeptide families synthesized by some *Bacillus* species.

The elicitor properties were mainly established on the basis of treatment of bean roots with the pure compound NABD that mimicked the protective effect of the producing strain and by showing that a BTP1 derivative affected in NABD synthesis was also impaired in its efficacy to stimulate ISR.

Another class of compounds that recently emerged as ISR elicitors are biosurfactants such as rhamnolipids and cyclic lipopeptides (Figures 3B and C) (Vatsa *et al.*, 2010). The potential of cLPs as plant resistance inducers was demonstrated in 2007 for two different molecules synthesized by *Pseudomonas* and *Bacillus*. Tran and collaborators showed that massetolide A produced by *Pseudomonas fluorescens* retains ISR-eliciting activity in tomato plants for the control of *Phytophthora infestans*, the causal agent of late blight (Tran *et al.*, 2007). Pure fengycin and surfactin from *Bacillus amyloliquefaciens* provided a significant induced protective effect similar to the one induced by living cells of the producing strain. In a complementary approach, experiments conducted on bean and tomato showed that overexpression of both surfactin and fengycin

biosynthetic genes in the naturally poor producer *B. subtilis* strain 168 was associated with a significant increase in the potential of the derivatives to induce resistance (Ongena *et al.*, 2007). Until this discovery, volatile organic compounds and more particularly 2,3-butendiol were the sole determinants for elicitation identified from *Bacillus* spp. (Ryu *et al.*, 2004).

Some other molecules from beneficial rhizobacteria retain plant defense eliciting activity. These last years, exopolysaccharides (Ipper *et al.*, 2008), quorum sensing signal molecules (N-acyl-L-homoserine lactone) (Schuhegger *et al.*, 2006), acetoin from *Bacillus subtilis* FB17 (Rudrappa *et al.*, 2010) and 2-aminobenzoic acid from *Bacillus sp*. BS107 (Yang *et al.*, 2011) were identified as main ISR determinants of their respective producing strains, again illustrating the diversity in structure and nature of that kind of MAMPs.

2.3.2. MAMPs elicitors from beneficial fungi and yeasts

MAMPs involved in systemic resistance triggered by beneficial fungi are not so well characterized compared to rhizobacteria. Djonovic´ and collaborators (Djonovic *et al.*, 2006) demonstrated that the hydrophobin-like elicitor Sm1 of the beneficial soil-borne fungus *Trichoderma virens* induces systemic resistance in maize. Maize plants grown with SM1-deletion strains or SM1-overexpressing strains displayed decreased or enhanced levels of systemic disease protection, respectively, demonstrating its role in triggering host defense. Peptaibols are linear peptide antibiotics produced by *Trichoderma* and other fungal genera. In the biocontrol agent and inducer of plant defense responses *Trichoderma virens*, enzymes forming peptaibols are encoded by *tex1* and disruption of these genes led to a significantly reduced systemic resistance response in cucumber plants against the leaf pathogen *Pseudomonas syringae pv. Lachrymans* as compared with plants grown in presence of the wild-type (Viterbo *et al.*, 2007). Two synthetic 18-amino-acid peptaibol isoforms induce systemic protection when applied to cucumber seedlings suggesting that these peptides are critical in the chemical communication between *Trichoderma* and plants as triggers of defense responses. However, the peptaibol alamethicin induced a form of active cell death in *Arabidopsis thaliana* cell cultures and caused lesions in leaves of plants after a few days showing that these molecules may also retain some phytotoxicity on certain plant species (Rippa *et al.*, 2010). It has also recently been demonstrated that some other secondary metabolites of plant beneficial *Trichoderma* spp. such as harzianolide and pentyl-pyranone may have a role in activation of plant defense responses (Vinale *et al.*, 2008).

Table 3. MAMPs from beneficial micro-organisms associated to plants identified to date as potential elicitors of different plant immunity responses (DR: Defense Responses, ISR: Induced Systemic Resistance). In most cases, their binding site at the plant cell surface is unknown and only few receptors have been characterized.

Elicitor	Other bioactivities	Origin	Perception by plant cells	Response type	Reference
From fungi					
Proteins/enzymes					
Sm1 protein		*Trichoderma atroviride*	?	ISR	(Djonovic *et al.*, 2007)
Endo-(1-4)-i-xylanase		*Trichoderma viride*		DR	(Lotan *et al.*, 1990)
Peptides					
Peptaibols		*Trichoderma pseudokoningii*, *Trichoderma virens*	?	ISR	(Viterbo *et al.*, 2007, Luo *et al.*, 2010)
Other secondary metabolites					
Harzianolide	antibiotic	*Trichoderma harzianum*		ISR	(Vinale *et al.*, 2008)
Harzianopyridone		*Trichoderma harzianum*		ISR	(Vinale *et al.*, 2008)
6-n-pentyl-6H-pyran-2-one		*Trichoderma atroviride*	?	ISR	(Vinale *et al.*, 2008)

From yeasts					
N-glycosylated peptide		*Yeast extract*	ni	DR	(Albus *et al.*, 2001, Meyer *et al.*, 2001)
From rhizobacteria					
Proteins					
Flagellin	Cell surface component	*Pseudomonas putida, P. fluorescens*	ReceptorFLS2	ISR	(Meziane *et al.*, 2005)
Modified peptides					
Pyoverdines	Siderophore	*Pseudomonas fluorescens, P. putida*	Induction of iron cytosolic depletion?	ISR	(Hofte *et al.*, 2007, De Vleesschauwer *et al.*, 2009)
Lipid-containing compounds					
Massetolide A	Biosurfactant, antibiotic	*Pseudomonas fluorescens*	Interaction with lipids in plasma membrane?	ISR	(Tran *et al.*, 2007)
Surfactine, Fengycine	Biosurfactant, antibiotic	*Bacillus subtilis, amyloliquefaciens*	Interaction with lipids in plasma membrane?	ISR	(Ongena *et al.*, 2007, Jourdan *et al.*, 2009)
N-AcylBenzylamineDerivative	nd	*Pseudomonas putida*	ni	ISR	(Ongena *et al.*, 2005c, Ongena *et al.*, 2008b)
N-Acyl Homoserine lactone	Quorum-sensing signal	*Serratia liquefaciens*	ni	ISR	(Schuhegger *et al.*, 2006)

rhamnolipids	Biosurfactant, antibiotic	*Pseudomonas aeruginosa*	ni	ISR	(Varnier *et al.*, 2009)
Aromatic compounds					
Salicylic acid	Siderophore	*Pseudomonas fluorescens, P.putida*	Signal for systemic resistance?	ISR	(Leeman *et al.*, 1996, Audenaert *et al.*, 2002a, DeMeyer *et al.*, 1997), (Bigirimana *et al.*, 2002)
Pyochelin	Siderophore	*Pseudomonas aeruginosa*	?	ISR	(Audenaert *et al.*, 2002a)
2,4-Diacetylphloroglucinol	Antibiotic	*Pseudomonas fluorescens*	Interference with pathogen virulence factor?	ISR	(Iavicoli *et al.*, 2003, Siddiqui *et al.*, 2003)
Pyocyanine	Antibiotic	*Pseudomonas aeruginosa*	Redox-based mechanism?	ISR	(De Vleesschauwer *et al.*, 2006)
Volatiles					
Butandiol	Antibiotic, plant growth promotion	*Bacillus subtilis, B. amyloliquefaciens*	Modification of membrane potential?		(Heil *et al.*, 2010, Cho *et al.*, 2008)
Polysaccharides					
Exopolysaccharides	Cell surface component	*Burkholderia gladioli, Serratia sp.*	?	ISR	(Cho *et al.*, 2008, Ipper *et al.*, 2008)
Lipopolysaccharides	Cell surface component	*Pseudomonas, Burkholderia, Rhizobium*	Low affinity receptor?	ISR	(Vanpeer *et al.*, 1991, Duijff *et al.*, 1998, Reitz *et al.*, 2002) (Meziane *et al.*, 2005, Coventry *et al.*, 2001)

Chapter 3. Molecular characteristics of ISR

3.1. Perception of ISR elicitors: still searching for receptors

It has been speculated that patterns from pathogens and beneficial microbes are recognized in a similar way, ultimately resulting in an enhanced defensive capacity of the plant. Intriguingly, no specific proteinaceous binding sites have been identified for MAMPs perception while a few plasma membrane-located receptors for PAMPs have been characterized.

The ISR-elicitors LPS and flagella have previously been demonstrated to act as PAMPs elicitors and to function likewise as elicitors of innate immunity in animals (Nurnberger *et al.*, 2004). In animals, the action of bacterial LPS is mediated by Toll-like receptors (Godowski *et al.*, 1998); however receptors to LPS in plants have not been identified yet (Newman *et al.*, 2007). The mechanism by which flagella activate basal resistance responses as PAMP elicitor is better understood. Conserved peptides within the major flagellar protein, flagellin, are perceived by the Toll-like receptor-like kinase FLS2 in *Arabidopsis* (Gomez-Gomez *et al.*, 2001) and a presumably similar receptor in tomato (Robatzek *et al.*, 2007). Recognition of different parts in the LPS and flagella structures may therefore allow plant cells to discriminate between symbiotic and infectious Gram-negative bacteria. It suggests that a somewhat specialized perception system is involved at the plant cell wall level. However, this has yet to be demonstrated.

The strain-specific effect of pyoverdins in ISR may be explained as far as the peptide chain is involved in the perception process by plant cells because the structure of the chromophore part is conserved. Actually, there is no partial sequence shared by three active pyoverdins from WCS358, WCS374 and CHA0. Testing a wider range of heterogeneous pyoverdins on the same plant is required to evaluate whether some amino acid sequences may represent epitopes perceived by specific receptors in the membrane of root cells. An alternative to this direct recognition of pyoverdins by the plant is the indirect perception of rhizobacterially induced alterations in the plant's immediate environment i.e. the rhizosphere. Indeed, given the scarcity of bioavailable iron and the high affinity of pyoverdin for the ferric ion, pyoverdin-producing rhizobacteria are thought to interfere with the iron acquisition by other soil organisms, including the host plant (Vansuyt *et al.*, 2007). A model implying pyoverdin-induced iron stress on the roots as a primary event in the activation of rhizobacteria mediated resistance has been proposed (De Vleesschauwer *et al.*, 2009).

Structural similarities are neither obvious in other bacterial products identified so far as ISR determinants like NABD, SA, DAPG, pyocyanin or volatile 2,3-butanediol. Results obtained by comparing the activity of pure benzylamine with that of NABD in ISR assays with bean and

cucumber suggest that the aromatic amino part of the molecule is important for its biological activity (Ongena *et al.*, 2008b). SA and 2,4-diacetylphloroglucinol also contain an aromatic phenolic group and thus such phenyl-derived moieties could constitute a general motif widely recognized by specific plant cell receptors. Additional experiments are required to appreciate the relative importance of such structural traits by testing multiple naturally co-produced or chemically synthesized derivatives.

3.2. Early induced events

When a plant recognizes some beneficial microorganisms or their elicitors, a rapid induction of cellular responses is stimulated. Early responses thought to represent initial signaling steps in the elicitation of PAMPs elicitors have been extensively characterized by working with the oomycete elicitor cryptogein in tobacco cells as plant model (Garcia-Brugger *et al.*, 2006).

Numerous effects, including NO production, mitogen-activated protein kinase (MAPK) activation, cell death, and gene expression, were confirmed in whole plants. These early reactions, especially production of reactive oxygen species (ROS), medium alkalinization, and Ca^{2+} influx, appear typical of microbial effectors triggering reactions that lead to a hypersensitive response. Notably, ROS have been associated with stress adaptation as well as programmed cell death (Gechev *et al.*, 2006) and are known to act as signals mediating both defense gene activation and the execution of the hypersensitive reaction in response to pathogens (Dangl *et al.*, 2006).

Early defense reactions in the context of ISR are by far less characterized than reactions to other biotic stresses; but are characteristically not associated with extensive transcriptional reprogramming or with cell death (van Loon *et al.*, 2008b, Verhagen *et al.*, 2004). Responses such as extracellular pH alkalinization, oxidative burst and calcium fluxes appeared in both SAR and ISR phenomenon. Kinetic and quantitative differences depend on either the type of resistance pathway involved or on the origin of the elicitor (van Loon *et al.*, 2008).

3.3. From priming to defensive mechanisms

3.3.1. Signaling pathways and priming

Following elicitor perception, plants are usually primed for faster and stronger activation of cellular defense responses upon attack of a pathogen (especially necrotizing) (Conrath, 2011). However, priming profiles in SAR and ISR responses are different. In absence of pathogen, ISR, by contrast to SAR, is typically not associated with major changes in the expression of defense genes (Pieterse *et al.*, 2002), probably because this would lead to heavy investments in resources and reduced

fitness of the host (Heil, 2002). Although priming has been known for a long time, it is still poorly understood. An elevated accumulation of some inactive MAPK in addition to specific chromatin modifications of defense gene promoters and accumulation of SA and azaleic acid (AZA) are currently considered as potentially crucial steps of the priming mechanism (Conrath, 2011). Meanwhile, priming of defense is regulated by different pathways, depending on the inducing agent and the challenging pathogen.

Similarly, many components of the transduction pathway of ISR are still unknown and those characterized differ in many aspects from the pathogen-induced SAR (Figure 4).

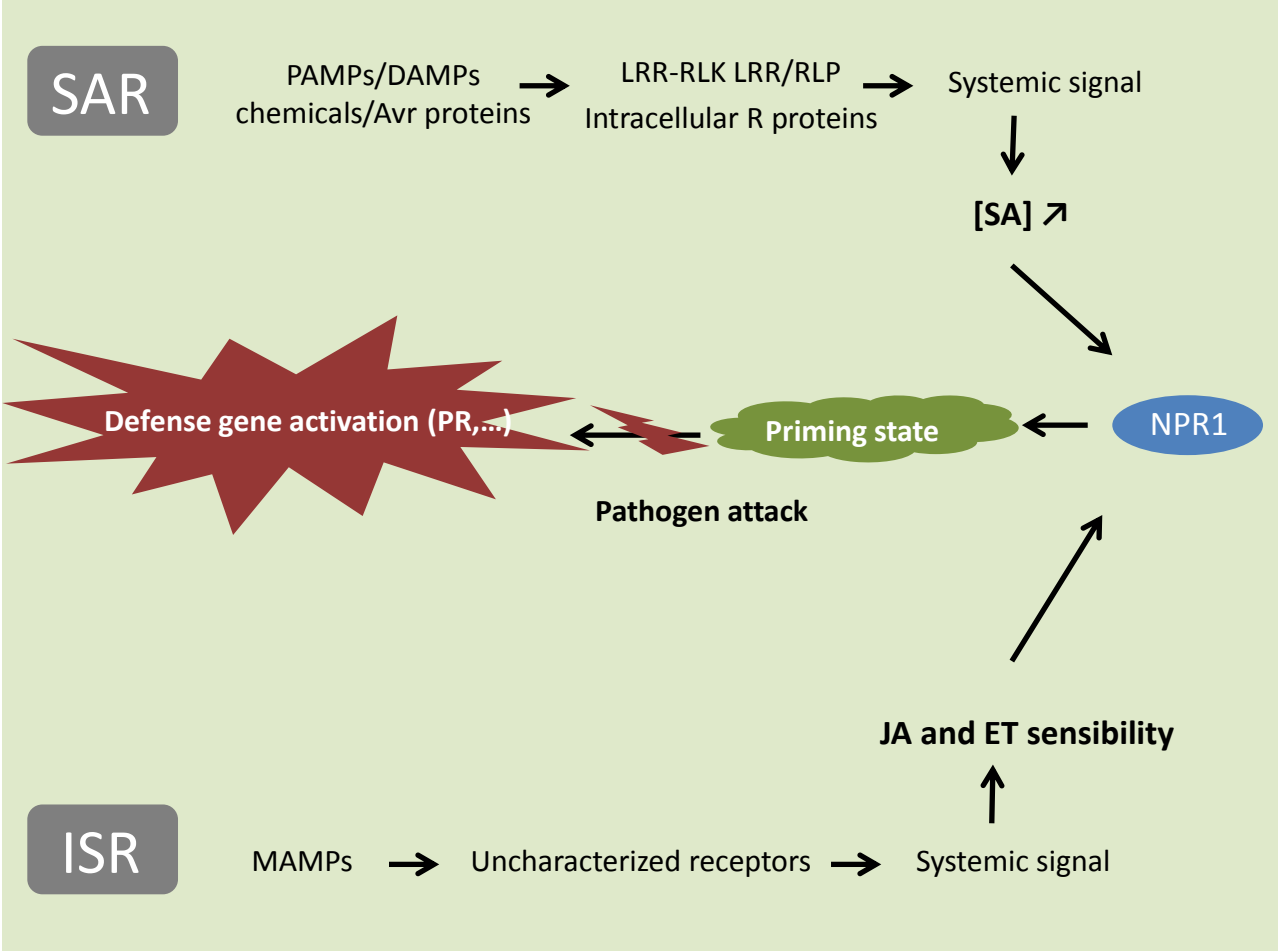


Figure 4 : Transduction pathways of SAR and ISR. SA always plays a central role in the signaling cascade of SAR while ISR is typically independent of SA but requires jasmonate (JA) and ethylene (ET) signaling in the plant.

SAR-related sequence of events from early reactions to the induction of defense genes expression (reviewed by Durrant *et al.*, 2004) involves a cross-talk between different signaling pathways. SA always plays a central role in the signaling cascade. However, SAR is not always correlated with systemic accumulation of SA (Cameron *et al.*, 1999) and a recent publication revealed in *Arabidopsis* that cell priming during SAR involves accumulation of the secondary metabolite azelaic acid (Jung *et al.*, 2009). Upon localized infection, azelaic acid may be transported throughout the plant and primes the plant to accumulate higher SA levels upon challenge infection. This locally produced SA causes changes in redox status that provokes conformational modifications in the protein NPR1 (*Non-expressor of Pathogenesis-Related* Genes 1). This positive regulator then interacts with TGA transcriptional factors leading to activation of a large set of defense genes including those that encode for pathogenesis-related proteins (PRs) (Van Loon *et al.*, 1998).

Usually, rhizobacteria-mediated ISR is independent of SA but requires jasmonate (JA) and ethylene (ET) signaling in the plant (Pieterse *et al.*, 1998). However, no alterations in the production of either JA or ET could be detected in plants, suggesting that the induced resistance state is based on an enhanced sensitivity to these plant hormones rather than on an increase in their production (Pieterse *et al.*, 2000). In ISR, the regulatory protein NPR1 is also typically activated by a still unknown systemic signalization. But in contrast to SAR, ISR (before pathogen attack) is not commonly associated with an accumulation of PR proteins, even if some changes at the transcriptional level have been reported in reaction to resistance-inducing rhizobacteria (van Loon *et al.*, 2008). Finally, SAR and ISR are very complex pathways that could cross-communicate in addition to other regulatory mechanisms to form a regulatory network able to respond specifically in function of the nature of the attacker (De Vos *et al.*, 2005).

3.3.2. Main defense mechanisms

Subsequent infection of primed plants leads to the activation of a broad array of defense mechanisms that will restrict pathogen ingress.

3.3.2.1. Accumulation of hydrolytic enzymes

Some increases in specific enzyme activities such as phenylalanine ammonia-lyase, peroxidase, polyphenoloxidase, ß-1,3-glucanase, chitinase, cellulose as well as induction of specific PR proteins transcriptional level have been reported in leaves of plants colonized by PGPR at the root level (Van Loon *et al.*, 2006). Some of these enzymes are hydrolytic and can inhibit fungal pathogen growth through cell wall degradation.

3.3.2.2. Cell wall reinforcement

In several plant species such as tomato, cucumber or pea, microscopy studies have provided direct evidence for a rapid accumulation of callose at or beyond the infection sites to slow down or stop penetration of the pathogen in the host tissues. It was suggested that phenolics derived from the phenylpropanoid metabolism can also contribute to the creation of a fungitoxic environment around the sites of penetration of the pathogen in addition to their role as buiding blocks for lignin polymerization (Benhamou *et al.*, 1996, Benhamou *et al.*, 1998). Wall appositions containing callose, pectin, cellulose and phenolics were also related to pathogen restriction in *Pseudomonas*-treated carnation roots (Benhamou *et al.*, 1999). An accumulation of peroxidases and enzymes involved in lignification in cucumber roots by treatment with plant growth-promoting rhizobacteria was also reported (Chen *et al.*, 2000).

3.3.2.3. Accumulation of phytoalexins

Another prominent feature of SAR is the stimulation of the synthesis of low-molecular-weight antifungal compounds called phytoalexins (Hammerschmidt, 1999). However, reports about this phenomenon and its role in rhizobacteria-mediated ISR are still limited. Phytoalexins have for exemple been reported in carnation (Van Peer *et al.*, 1991) and bean (Zdor, 1992). ISR in cucumber treated with the non-pathogenic *P. putida* strain BTP1 also depends on the accumulation of antifungal phenolic compounds in a pattern similar to the one described for phytoalexins. Such modification of the phenolic pattern was observed both as a local response in bacteria-inoculated root cells and systemically within non-bacterized root parts and in leaves of cucumber plants (Ongena *et al.*, 1999). This simultaneous involvement of multiple defense molecules may be necessary to confer a sufficient resistance level to cucumber against invasion by a particular pathogen. The plant could also take advantage of this multiplicity to protect itself against various pathogen species.

Chapter 4. *Bacillus* cyclic lipopeptides: their role in biocontrol

Cyclic lipopeptides with antibiotic and biosurfactant properties are produced by a number of soil bacteria, including *Pseudomonas spp* (Bender *et al.*, 1999; Nielsen *et al.*, 2002), and members of the *Bacillus* genus which are among the beneficial bacteria mostly exploited as biopesticides to control plant diseases (Fravel, 2005; figure 3C).

Given their biological origin, these surfactants have some advantages compared to those produced chemically such as a higher specificity and a lower toxicity. They therefore represent a very promising alternative for environmental, industrial and pharmaceutical applications when used as emulsifiers, wetting/foaming agents or detergents. Interestingly, in addition to these physicochemical properties, the amphiphilic nature of bacterial cLPs also serves the expression of some other specific activities such as promotion of bacterial mobility and attachment to root surfaces, quorum sensing, cytotoxicity toward a broad range of (micro)organisms and immuno-stimulation in plant (Mulligan, 2005, Ron *et al.*, 2001; Figure 5). All these additional traits that will be reviewed in this chapter are very important for the fitness of producing strains in the rhizosphere and for their efficacy as biocontrol agent.

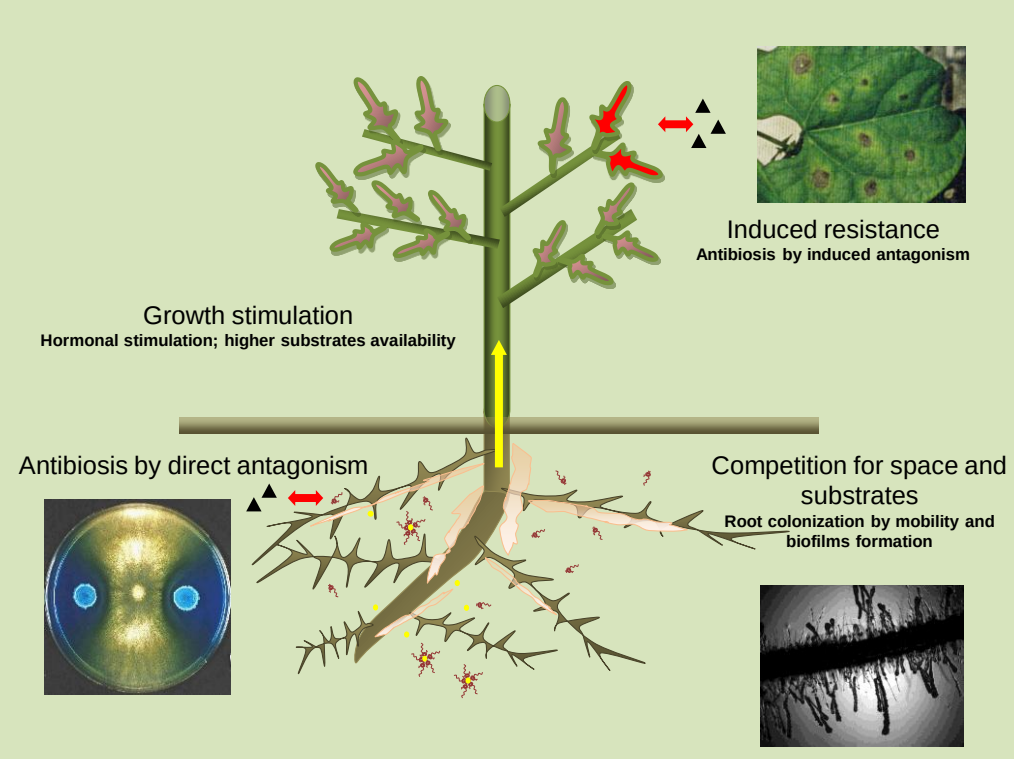


Figure 5 : Biocontrol-related activities of cLPs-producing strains. cLPs confer an efficient root colonization which enables to compete with other rhizosphere microorganisms. Their amphiphilic nature leads to solubilization of lipophilic substrates for subsequent use by the host plant. cLPs also allow the systemic induction of a primed resistance state which could lead to antagonism against a broad array of potential pathogens.

4.1. A complex biosynthetic system

cLPs are synthesized by non ribosomal peptide synthetases (NRPS) or hybrid polyketide synthases/non ribosomal peptide synthetases (PKS–NRPS). NRPS, unlike ribosomes, are independent of mRNA which confer them the ability to generate peptides with cyclic or branched structures and/or containing non-proteinogenic amino acids. This results in very diverse families of compounds exhibiting a broad range of biological activities. NRPS are megaenzymes organized in iterative functional units called modules that catalyze the different reactions leading to peptide formation (Stein, 2005; Finking *et al.*, 2004). Each module is subdivided into several catalytic domains responsible for specific biochemical reaction. The basic set of domains within a module can be extended by substrate modifying domains, including domains for substrate epimerization (E-domain), hydroxylation, methylation and heterocyclic ring formation. These last domains are either inserted at specific locations into the module or act as independent catalytic units. A thioesterase domain (Te domain) is usually present in the last module to ensure the cleavage of the thioester bond between the nascent peptide and the last peptidyl carrier protein (PCP) domain. In several cases, this thioesterase is responsible for the cyclization of the peptide.

4.2. A huge structural diversity

Such biosynthetic systems lead to a remarkable heterogeneity among the cLPs products which vary in the type and sequence of amino acid residues, the nature of the peptide cyclization and in the nature, length and branching of the fatty acid chain (Figure 6). In *Bacillus*, the three main cLPs families are surfactin, iturin and fengycin and each type encompass structural variants depending on the genetic background of the strain considered. The variants differ in their peptide sequence due to some amino acid substitutions. Within each variant group, there are several homologues that are co-produced and differ in the length and isomery of the fatty acid chain. Surfactin are heptapeptides interlinked with a β–hydroxy fatty acid to form a cyclic lactone ring structure. The group of iturin encompasses seven variants including bacillomycins and mycosubtilin. All are heptapeptides linked to a β-amino fatty acid chain with a length from C 14 to C 17. The third family comprises fengycin A and B, also called plipastatins. These molecules are lipodecapeptides with an internal lactone ring in the peptidic moiety and with a β-hydroxy fatty acid chain (C 14 –C 18) that can be saturated or unsaturated.

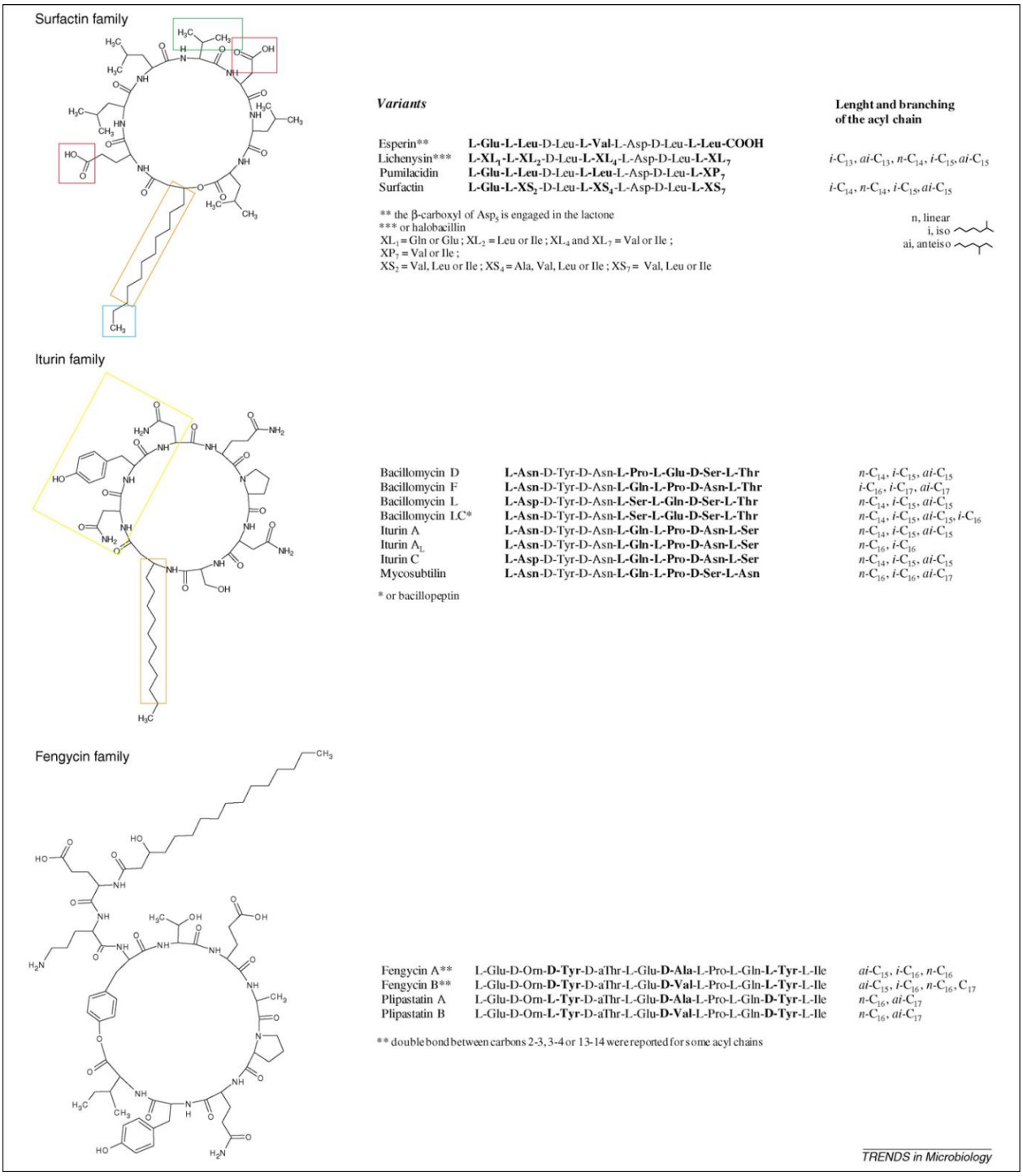


Figure 6 : Structures of the representative members of the three cyclic lipopeptide families synthesized by *Bacillus* species. Boxed structural groups are those supposed to be important for biological activity in addition to the cyclic nature of the molecule (Bonmatin *et al.*, 2003a, Peypoux *et al.*, 1999, Dufour *et al.*, 2005). No obvious data are available to date for fengycin in this context. Boxed blue, type of branching (linear, iso, anteiso); boxed orange, acyl chain length; boxed red, ionisable or polar groups; boxed green, hydrophobicity of residue in position 4; boxed yellow, L-Asx(1)–D-Tyr(2)–D-Asn(3) sequence.

4.3. Multiple functions in plant-*Bacillus*-pathogen interactions

4.3.1. Involvement in root colonization

The ability of *Bacillus* to efficiently colonize surfaces of plant roots is a prerequisite for phytostimulation. The first step of translocation on surfaces like roots, referred as spreading, could be achieved through several ways. The probably most studied is swarming motility which involves differentiation of vegetative cells into hyperflagellated 'swarmer cells' that undergo rapid and coordinated population migration across solid surfaces (Fraser *et al.*, 1999). This swarming process allows an easier colony spreading but also an improved antimicrobial resistance. The second step for rhizosphere competence is linked to the capability to form sessile, highly structured and antimicrobial resistant multicellular communities. Microbial populations such as plant-associated bacteria evolve and behave as structured communities called biofilms (on solid surfaces) or pellicles (at air/liquid interfaces) that could adhere to root and on soil particle surfaces (Danhorn *et al.*, 2007). Such microcolonies are sites for bacteria to communicate with each other (quorum sensing) and to act in a coordinated manner. When confronted with nutrient deprivation, *B*acillus cells could, among others, sporulate at highly ordered and surface associated cell clots within the aerial structures projecting from the surfaces of *B*acillus biofilms and pellicles, the "fruiting-bodies" (Branda *et al.*, 2001). Several studies showed that cLPs could be involved at different levels in the complex network linking motility and biofilms/pellicles/fruiting-bodies formation. So the cLPs surfactin and mycosubtilin have been shown to be implicated in a flagella-independent surface motility of *B. subtilis* (Kinsinger *et al.*, 2003, Leclère *et al.*, 2006). Surfactin is thought to act by the aggregation of the cells into dendrites and by the coordination of their advance throughout the swarm front (Julkowska *et al.*, 2005). This surfactant-induced spreading is most likely due to a reduction of frictions between cells and surface in combination with a surface tension-driven flow. A low surface tension which could be reached with strong surfactants such as surfactin and mycosubtilin was demonstrated to be sufficient to facilitate microbial colonization (Leclère *et al.*, 2006). This could explain why surfactin production is necessary but not sufficient for swarming, in which other factors like genes *swrABC* and *efp* are additionally involved (Kearns *et al.*, 2004). Nonetheless, results obtained with *B. subtilis* A1/3 have shown that surfactin but not other lipopeptides produced by the strain was required for the formation of biofilms and pellicles indicating that surfactin may still serve specific developmental functions (Hofemeister *et al.*, 2004). More conclusively in the view of biocontrol, the production of surfactin was demonstrated to be essential for biofilm formation and colonization of *Arabidopsis* roots by the strain *B. subtilis* 6051 and that biocontrol exhibited against *P. syringae* is linked to the formation of this antibiotic at the

root surface (Bais *et al.*, 2004). Finally, genes that mediate production of surfactin (*srfAA* and *sfp*) were shown to be required for the erection of fruiting-bodies (Branda *et al.*, 2001), at least in part by their ability to lower the surface tension of water. Interestingly, the regulation of surfactin biosynthesis is under the control of a complex network that governs cellular differentiation, including quorum sensing, confirming a correlation between *Bacillus* colonization and this cLP (Hamoen *et al.*, 2003).

4.3.2. Involvement in the direct antagonism with phytopathogens

4.3.2.1. Antibiotic activities via cell lysis

4.3.2.1.1. Against fungi

Fungitoxicity is likely one of the best known bioactivities of bacterial cLPs. It has been demonstrated for almost all the cLPs tested so far to that purpose. Among those produced by *Bacillus*, fengycin retains the most antifungal potential followed by iturin. In some instances, the fungitoxic activity was clearly related to the permeabilization of spore/conidia therefore inhibiting germination or alternatively to hyphal cell perturbation. As revealed by transmission electron miscroscopy techniques, both phenomena most probably result from membrane damaging (Chitarra *et al.*, 2003, Romero *et al.*, 2007a, Etchegaray *et al.*, 2008). Iturin A has also a potent fungicidal action towards growing cells of *S. cerevisiae* (Latoud *et al.*, 1987b). It is worth to note that cells incubated with iturin A showed severe modifications in the lipid composition of their membranes, suggesting that iturin A activates phospholipases activities or disturb the distribution of lipid components in the cytoplasmic membranes, rending phospholipids more accessible to phospholipases.

In the case of soil-borne diseases, iturin A produced by *B. subtilis* RB14 was involved in damping-off of tomato (a seedling disease) caused by *Rhizoctonia solani* (Asaka *et al.*, 1996). Overexpression of mycosubtilin in *B. subtilis* ATCC 6633 also led to a significant reduction of seedling infection by *Pythium aphanidermatum* (Leclère *et al.*, 2005). As examples in control of phyllosphere diseases, a contribution of both iturin and fengycin is characterized in the antagonism of *B. subtilis* toward *Podosphaera fusca* infecting melon leaves (Romero *et al.*, 2007b). This was demonstrated by identifying iturin and fengycin as the main antibiotic products excreted by the strains, by showing the strong inhibitory effect of these cLPs on *P. fusca* conidia germination, and by recovering cLPs from bacterial-treated leaves and using cLP-deficient transformants. In the protection of post harvest diseases, the strain *Bacillus subtilis* strain GA1, which efficiently produces cLPs from the three families and notably a wide variety of fengycin, protected wounded

apple fruits against gray mold disease caused by *Botrytis cinerea*. The role of fengycin was demonstrated by the very effective disease control provided by treatment of fruits with cLPs-enriched extracts and by *in situ* detection of fengycin in inhibitory amounts (Toure *et al.*, 2004).
However, no marked fungitoxic effects have been reported to date for surfactin except on the plant pathogen *Magnaporthe grisea*, with morphological changes and inhibition of growth (Tendulkar *et al.*, 2007). Interestingly, the antifungal effect of fengycin was reduced after commercial surfactin was added (Tao *et al.*, 2011). By contrast, synergistic antifungal activities have been reported between iturin and surfactin (Hiraoka *et al.*, 1992, Magetdana *et al.*, 1994, Souto *et al.*, 2004). cLPs with antifungal properties from other *Bacillus* species were more recently characterized (Batrakov *et al.*, 2003; Zhang *et al.*, 2010a). A surfactin derivative called WH1fungin produced by *Bacillus amyloliquefaciens* WH1 was shown to play an anti-fungal role by two models: high concentration to elicit pores on cell membrane and low concentration to induce apoptosis (Qi *et al.*, 2010). Moreover, it can inhibit the glucan synthase resulting in a decreased synthesis of callose on fungal cell wall (Qi *et al.*, 2010). Two new cLPs maribasins A and B from *Bacillus marinas* also exhibit broad-spectrum activity against phytopathogens (Zhang *et al.*, 2010a).

4.3.2.1.2. Against bacteria

cLPs also display some antibacterial activities. Within *Bacillus* cLPs, fengycin has only been shown active against *E. Coli* in milk with a MIC of 31.25 µg.mL^{-1} (Huang *et al.*, 2008). The sensitivity of *E. Coli* to surfactin is higher with a MIC of 15.625 µg.mL^{-1}, but surfactin mainly displays antimycoplasma activities and are active against several human and animal pathogenic mycoplasmas (Vollenbroich *et al.*, 1997b). Electron microscopic studies provided evidence that surfactin affects the envelopes of contaminating mycoplasma. Obviously, it disrupts the plasma membrane, which is its primary site of activity, leading to complete disintegration of the membrane systems at higher concentrations, and finally causing the mycoplasmas to burst (Vollenbroich *et al.*, 1997b). Finally, surfactin bursts more than 90% of protoplasts prepared from *Bacillus megaterium* KM at a concentration of 14 µg/mL (Tsukagoshi *et al.*, 1970).
A lipid extract of iturin and surfactin (5 mg/mL) also displays antibacterial activity against *Xanthomonas campestris* and *Xanthomonas axonopodis* (Etchegaray *et al.*, 2008).

4.3.2.1.3. Against virus

Surfactin was shown to inactivate various enveloped viruses by acting directly on the lipid envelope, leading to disintegration of the virus particles (Vollenbroich *et al.*, 1997a; Huang *et al.*, 2006). For viruses susceptible to its action, surfactin was active from 25 µM.

4.3.2.1.4. Against insects

A few studies have revealed some insecticide activity of cLPs from *B. subtilis*. Surfactin but not iturin was described for its antagonistic effect against fruit fly *Drosophila melanogaster* (Assie *et al.*, 2002) and cLPs contained in a crude extract were efficient at inhibiting the development of larvae of the mosquito *Culex quinquefasciatus* (Das *et al.*, 2008). Surfactin also exhibits activity at quite high doses (approx. 200 µM) against pupae of mosquitoes (Geetha *et al.*, 2010).

4.3.2.2. Antibiotic activity via Interference with cellular functions

Like other antimicrobial peptides, cLPs are not only membrane disruptive but can also directly or indirectly act on intracellular targets and inhibit some crucial functions such as enzymatic activity.

Surfactin could interfere with numerous stages of the cellular processes in animals. It displays anti-inflammatory activity through binding and neutralizing lipopolysaccharide (Hwang *et al.*, 2005, Takahashi *et al.*, 2006) and stimulation/inhibition of phospholipases A2 (IC50: 8,5 µM) (Kim *et al.*, 1998) and antitumor activity (IC50 from 15 to 85 µM) (Cao *et al.*, 2009, Kim *et al.*, 2007). Latter activity is based on apoptosis induction via cellular calcium augmentation, induction of kinases and related up/down regulation of protein factors associated with apoptosis (Wang *et al.*, 2007, Cao *et al.*, 2009). Another study also demonstrated that anti-proliferative activity of surfactin on human colon carcinoma cell line was related to induction of apoptosis and cell cycle arrest (Kim *et al.*, 2007). Again, the anti-cancer activity of surfactin is hypothetically explained by its effect on up-stream signaling notably on extracellular related kinases. It was proposed that surfactin may be acting as a molecule disturbing a biochemical phenomenon that occurs at a specific site (e.g. lipid raft) of the membrane (Kim *et al.*, 2007). In addition, surfactin can also inhibit platelet aggregation (Kim *et al.*, 2006), inhibit AMPc phosphodiesterase and alkaline phosphatase activities (Bortolato *et al.*, 1997).

In the recent report on inhibition of toxic fumonisin B1 synthesis in *Fusarium verticillioides*, it has been suggested that some products deriving from phospholipase A2 activity may be somewhere involved in the down-regulation of toxin genes transcriptions observed upon treatment with fengycin (Hu *et al.*, 2009). Such antitoxic activity has also recently been demonstrated for iturin in the inhibition of aflatoxin producing *Aspergillus* isolates (Cho *et al.*, 2009). The plipastatins are

cLPs structurally very similar to fengycin isolated from *B. subtilis* and *B. cereus* and were also shown to inhibit the porcine pancreatic phospholipase A_2 as well as phospholipases C and D (Umezawa *et al.*, 1986). As a direct or indirect activity on phospholipases was also suggested about the antifungal activity of iturin toward *Saccharomyces cerevisiae* (Latoud *et al.*, 1987a), it seems that such enzymes sensitive to the physics of their surrounding lipid substrates are common targets of cLPs.

4.3.3. Involvement in plant systemic resistance elicitation

Another well established way for beneficial *Bacillus* isolates to provide plant protective effect is the stimulation of the plant immune system. The list of *Bacillus* strains reported as plant resistance inducers has grown rapidly over the last decade and includes members of the *B. pumilus*, *B. mycoides*, *B. subtilis*, *B. amyloliquefaciens*, *B. pasteurii*, *B. thuringiensis* or *B. cereus* species (Bent, 2005, Kloepper *et al.*, 2004). By contrast, very few things are known about the molecular aspects of the *Bacillus*-mediated immunity and until 2004, volatile organic compounds were the sole determinants for elicitation identified from *Bacillus* spp. (Ryu *et al.*, 2004). However, results from our laboratory demonstrated these last years that surfactin and fengycin also retain such eliciting activity. In bean, pure fengycin and surfactin provided a significant induced protective effect similar to the one induced by living cells of the producing strain. Experiments conducted on bean and tomato showed that overexpression of both surfactin and fengycin biosynthetic genes in the naturally poor producer *B. subtilis* strain 168 was associated with a significant increase in the potential of the derivatives to induce resistance (Ongena *et al.*, 2007). These first works have shed light on the potential of cyclic lipopeptides, and to a greater extent to amphiphilic compounds as promising biocontrol compounds. By being able to produce high amounts of cLPs, *B. amyloliquefaciens* S499 is become our laboratory strain to investigate their ISR potential.

Chapter 5. Plasma membranes of organisms interacting with lipopeptides

The different antimicrobial activities of cLPs suggest selectivity for some membrane composition depending on the target organism. In this chapter, we provide some information about the theoretical composition in lipids and sterols of biological plasma membranes and the influence of this composition on the membrane structure. This theoretical information comes mainly from the website of American Oil Chemists' Society lipid library (www.lipidlibrary.AOCS.org; updated September 2012 version).

5.1. Typical lipid composition of plasma membranes

Most plasma membranes consist of approximately 50% lipids and 50% proteins by weight, with the carbohydrate portions of glycolipids and glycoproteins constituting 5 to 10% of the membrane mass. Since proteins are much larger than lipids, this percentage corresponds to about one protein molecule per every 50 to 100 molecules of lipid (Cooper, 2000). Compared to other membranes, plasma membranes are typically enriched in sphingolipids and sterols, which are packed at a higher density than glycerophospholipids and resist mechanical stress. Dissimilar phospholipids and sterols compositions exist between plasma membranes of different cell types which could affect both their structure and organization leading to different plasma membrane functions.

Glycerophospholipids typically contain four major phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol and diphosphatidylglycerol (PG and cardiolipin respectively) and sphingolipids. Together, they account for more than half of the lipid content in most membranes. The proportion of each of these phospholipids greatly varies between different cell types (Cooper, 2000). The head groups of PS, PI, PG and cardiolipin are negatively charged at physiological pH, so their predominance results in a net negative charge of the plasma membrane. Phospholipids are asymmetrically distributed between the two halves of the membrane bilayer. The length and the degree of unsaturation of their fatty acid chains have a profound effect on membrane characteristics as unsaturated lipids create a kink that prevents the fatty acids from tightly packing together, thereby increasing fluidity.

The entire membrane is held together via non-covalent interaction of phospholipid hydrophobic tails. However the structure is quite fluid and not fixed rigidly in place. Under physiological

conditions, plasma membrane phospholipids are in the liquid crystalline state or liquid disordered (Ld), meaning that they are free to diffuse and exhibit rapid lateral diffusion along the layer in which they are present (Cooper, 2000).

Sphingolipids are a class of extremely diverse glycerophospholipids located mainly in the outer leaflet of eukaryotic plasma membranes. They mainly have a structural function but may also represent adhesion sites for proteins from the extracellular environment or play a key role in signal transduction. The fatty acids of sphingolipids are very different from those of other glycerophospholipids, being mainly composed of very-long chains, odd- and even-numbered, saturated or monoenoic. The packing of saturated acyl chains of sphingolipids with sterols is thermodynamically favored over that with unsaturated acyl chains. This preferential interaction of sphingolipids with cholesterol in comparison with glycerophospholipids is believed to lead to phase separation in the membrane, giving rise to sphingolipid-rich regions in liquid-ordered (Lo) phase surrounded by glycerophospholipid-rich domains in Ld phase. Sphingolipids have freer hydroxyl groups thereby contributing to the stability of these phases via hydrogen bond formation. Moreover, selective affinities between sphingolipids and membrane proteins trigger their compartmentalization and thus segregate different biochemical functions.

As amphiphilic molecules, sterols are able to intercalate between phospholipids, spanning about half the membrane bilayer. The interaction with other lipids is mainly via van der Waals and hydrophobic forces with a contribution from hydrogen bonding of the sterol hydroxyl group with the polar head group and interfacial regions of sphingolipids. The main effect of sterols is to restrict the movement of fatty acyl chains and thus to increase the degree of order (cohesion and packing) in membranes, leading to formation of a liquid-ordered (Lo) phase. In contrast, it renders bilayers composed of more saturated lipids, which would otherwise be in a solid gel state (So), more fluid. Similarly to sphingolipids, sterols have a key role in the lateral organization and free volume distribution of membranes, both factors contributing to more intimate protein-sterol interactions that may regulate multiple protein activities.

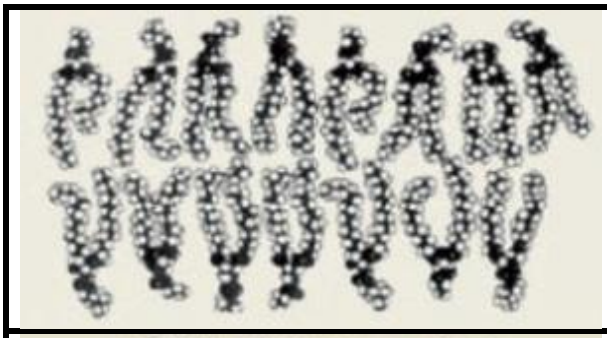
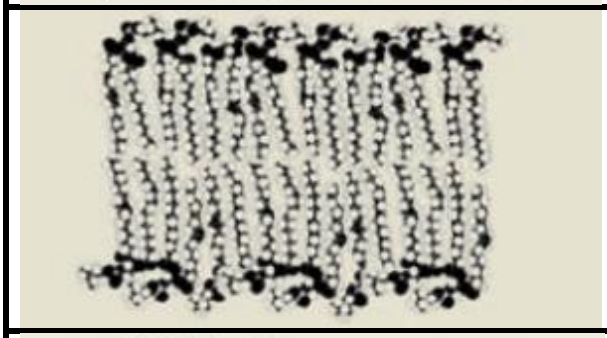
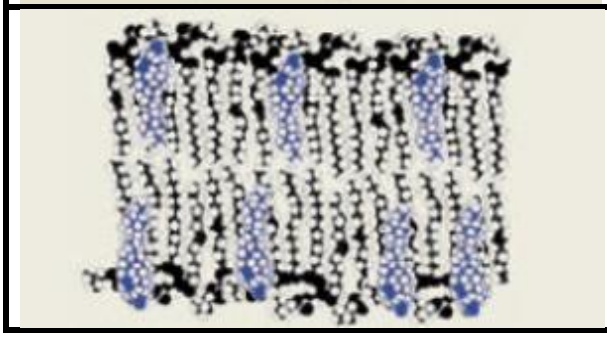
Many different sterols may be present in plant species (over 200 have been characterized). Their amounts and relative proportions depend on the plant species. Cholesterol is usually a minor component of plant sterols, but it can be in major proportions for some families of higher plants (*Solanaceae, Liliaceae, Scrophylariacea*).

5.2. Lipid phases in membranes

5.2.1. The lamellar states in biomembranes

The lipids of biological membranes can thus exist in multiple possible phase states depending on the proportion of each of their constituent. The lamellar states relevant to biomembranes are shown in table 4. Non-bilayer lipid phases, such as hexagonal and cubic phases (not shown), may relate to transient biomembrane events, such as fusion, fission and pore formation. The adopted phase depends on lipid structure: long, saturated hydrocarbon chains are found in sphingolipids, so sphingolipids-rich mixtures tend to adopt solid-like phases; unsaturated hydrocarbon chains are found in most membrane glycerophospholipids, so these tend to be enriched in liquid-disordered (Ld) phases. Sterols by themselves do not form bilayer phases, but together with a bilayer-forming lipid, a liquid-ordered (Lo) phase can form. This remarkable phase has the high order of a solid but the high translational mobility of a liquid. Membrane proteins modify and perhaps control the phase behaviors that are generated from the lipid–lipid interactions.

Table 4 : Lamellar phase states that may be present in biomembranes. (Illustrations from Gerrit *et al.*, 2009)

	**Liquid crystalline, liquid disordered (Ld)**
	**Solid gel (So)**
	**Liquid ordered (Lo), 'raft'**

5.2.2. The lipid rafts and the concept of the detergent-resistant-membranes (DRMs)

Some studies suggest that not all lipids diffuse freely in the plasma membrane (Brown *et al.*, 1998). Instead, discrete membrane domains appear to be enriched in certain lipids such as sterols and sphingolipids. The tight packing of sphingolipids long saturated acyl chains and intercalation of sterol allows for a more structured and rigid membrane organisation similar to the Lo phase of membranes. This tight lipid packing segregates from the surrounding unsaturated gylcerolipid environment. Such clusters were named "raft" because they were imagined to float as small Lo platforms within the larger part of the Ld plasma membrane. Raft microdomains move laterally within the plasma membrane and may associate with specific membrane proteins. Although their exact function remains to be understood, they may play important roles in processes such as cell signaling and uptake of extracellular molecules by endocytosis. They are now considered as specialized functional microdomains within plasma membrane. Since individual rafts could not be

visualized by light microscopy, the criterion for their existence was originally almost exclusively based on detergent insolubility (Brown *et al.*, 1992). Actually, the detergent molecules insert preferentially into the Ld phase and above a certain detergent concentration, the Ld phase solubilizes leaving the Lo phase intact. Thus, the material that can not be solubilized from membranes (for example with 1% Triton X-100 at 4°C) was assumed to be localized within rafts. Accordingly the corresponding fractions were called detergent-resistant membranes (DRMs) or detergent-insoluble membranes (DIMs).

While detergent insolubility in itself is artifactual and does not accurately reflect pre-existing raft formation in cell membranes, detergent insolubility remains a powerful first step method for assigning potential membrane raft association. Although being controversial, DRM isolation is probably the most widely used method to study membrane microdomains (Lichtenberg *et al.*, 2005). One of the most meaningful applications of DRM extraction is achieved if there is differential association of a protein with DRM before and after a stimulus, thereby linking it to a biological phenomenon (Lingwood *et al.*, 2007). This new promising research area justify why sterol- and sphingolipid-rich DRMs fractions have been extensively isolated from animal- and yeast plasma membranes, and recently also from plasma membranes of plant cells (Peskan *et al.*, 2000, Mongrand *et al.*, 2004, Borner *et al.*, 2005).

Objectives

The list of *Bacillus* strains reported as plant resistance inducers (and thereby as potential biopesticides) has grown rapidly over the last two decades (Fravel, 2005). However, little is known about the molecular determinants from *Bacillus* spp. that are responsible for ISR elicitation. As stated above in chapter 4.3.3., previous studies realized in our laboratory have demonstrated that cyclic lipopeptides retain such eliciting activity. However, the molecular mechanisms underlying ISR stimulation by these compounds are still poorly understood. To face this lack of knowledge, the main objective of this work is to further investigate the mechanisms governing the perception of such lipopeptidic biosurfactants at the plant cell surface. In the first part of our work, we have compared the ISR-eliciting potential of *Bacillus* lipopeptides on *Arabidopsis* and tobacco. This was performed by using whole plants and assessed by measuring disease reduction following challenge with the same pathogen *Botrytis cinerea*. These two plants were selected because stable cell suspension cultures, required for further investigation, were available in the laboratory. Moreover, both species are relevant model plants widely used for the study of molecular plant-microbe interactions, with rich data bases and molecular tools available. Based on these ISR results, *Nicotiana tabacum* was selected and cell cultures of this species were used throughout the work. The next objective was to characterize the molecular events that may occur within minutes after lipopeptide perception with the aim to select a relevant marker of the defense reaction in tobacco cells. Such markers were then used to compare the eliciting activity of the three main cLP families as well as of other secreted products from *Bacillus amyloliquefaciens*. We then placed special emphasis on surfactin which is by far the more active cLP for both early events and systemic resistance triggering. Our strategy was then to combine various biochemical and biophysic approaches to decipher the specific aspects of the interaction between the lipopeptide and plant cell plasma membranes. To that end we used a wide range of techniques/methodologies including structure-activity relationship, association kinetics, isothermal titration calorimetry, computational modeling and differential proteomics.

Results

Chapter 6. ISR triggered by the lipopeptide surfactin in tobacco and *Arabidopsis* plants

The first part of our work was dedicated to choose a handy plant model as deeply-investigated, easily available and genetically stable tobacco and *Arabidopsis* suspension cell cultures. We therefore firstly carried out a macroscopic investigation of disease reduction through ISR on these plant species before focusing *in vitro* on responses of their respective cell suspension culture to cLPs. We gave full attention to surfactin which may represent, as suggested by previous data, the most active cLP family on plants.

6.1. ISR on the pathosystem Nicotiana tabacum/Botrytis cinerea

Typical ISR experiments consisted of applying surfactin (10µM, as a mixture of C12 to C15 homologues) to the roots of tobacco plantlets before infecting leaves with the phytopathogen *Botrytis cinerea* in order to avoid any direct contact and thereby any possibility of antagonistic activity. Under our experimental conditions, infection rates in non-treated control plants were between 40% and 65%. In three independent experiments, treatment with the lipopeptide led to at least 25% reduction of disease incidence compared to untreated but infected controls (Figure 7). Results of disease reduction in the last two experiments are statistically significant with *p* values of 0.014 and 0.012 respectively according to t-test for independent samples using Statistica software, Stat Soft, Inc.

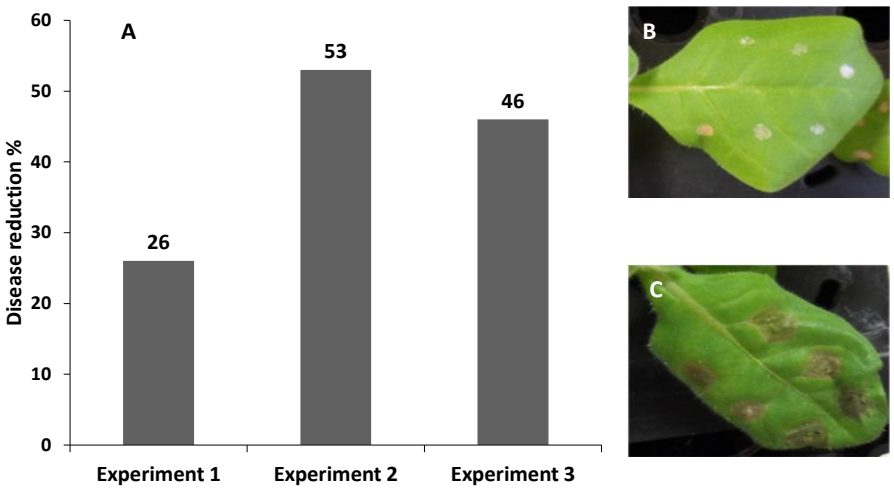


Figure 7 : A: Disease reduction rates on tobacco plantlets treated at the root level by surfactin mix at 10 µM. Leaves were 24 h later infected by the pathogen *Botrytis cinerea*. B: Illustration of ISR on tobacco leave treated by the pathogen *Botrytis cinerea* C: Representation of susceptible plant treated by the same pathogen.

6.2. ISR on the pathosystem Arabidopsis thaliana/Botrytis cinerea

ISR experiments were similarly performed on *Arabidopsis* plantlets. Disease reduction rates from 1% to 23% in four independent bioassays were obtained (Figure 8).

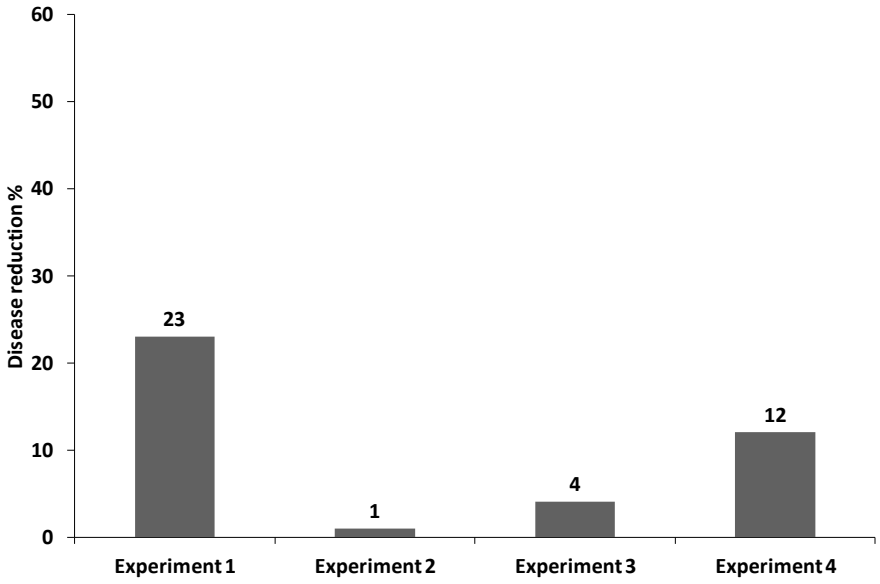


Figure 8: Disease reduction rates on *Arabidopsis* plantlets treated at the root level by surfactin mix at 10 µM. Leaves were 24 h later infected by the pathogen *Botrytis cinerea.*

6.3. Induction of localized resistance on the pathosystem *Arabidopsis thaliana/Botrytis cinerea*

The non reproducibility of ISR experiments on entire plants with surfactin lead us to test the local protection induced by surfactin at doses non toxic for the pathogen (Figure 9). In these assays, both the elicitor and the pathogen were applied on the same leaves. The possibility of direct inhibition of *Botrytis cinerea* by the lipopeptide was discarded by both Petri dishes confrontation experiments and microtiter plates assays that did not reveal any antifungal activity at concentrations up to 20 µM surfactin (data not shown).

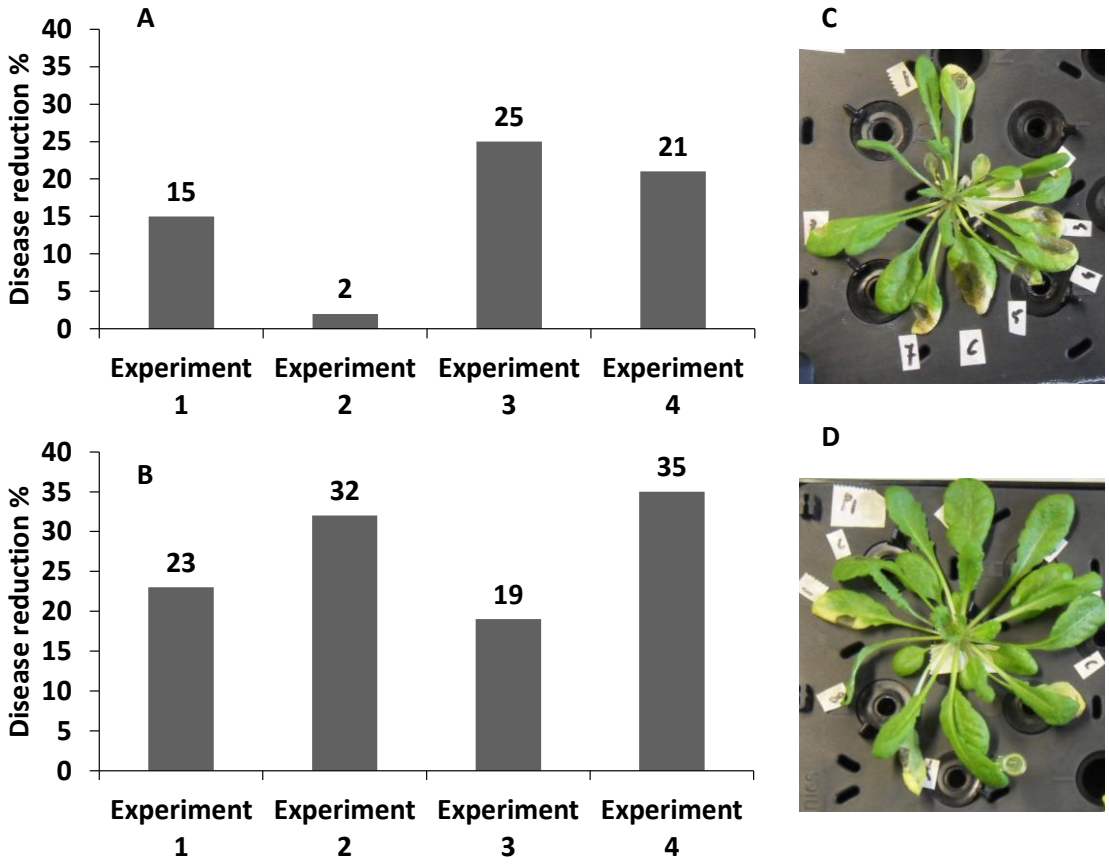


Figure 9: A. Disease reduction rates on *Arabidopsis* leaves treated on the bottom side by surfactin mix at 10 µM. Leaves were 24 h later infected by the pathogen *Botrytis cinerea.* B: Disease reduction rates on *Arabidopsis* leaves treated on the upper side by surfactin. C. Control non treated and infected plant. D. Surfactin treated and infected plant.

In view of these results, ISR is much more consistent on tobacco than on *Arabidopsis*. We therefore selected tobacco as plant model and used *Nicotiana tabacum* BY-2 as suspension cell line. Tobacco BY-2 cell suspension cultures are indeed easily available, with high genetic stability, homogeneity and growth rate. Moreover, as tobacco BY-2 cells behave very similarly to one another; the influence of neighboring cells behavior in the suspension is not as important as it would be in an

intact plant, leading to easier statistically interpretation of changes observed after a stimulus. Finally, BY-2 cells are relatively well understood and are encountered in numerous works relating to plant immunity research.

Chapter 7. Characterization of early defense events induced by surfactin in tobacco cultured cells

7.1. Extracellular medium alkalinisation

As mentioned in chapter 3.2., medium alkalinization takes part in the plant defense-associated early responses to various biotic elicitors (Boller, 1995, Felix *et al.*, 1999). We first used this consistent and easily measurable phenomenon on tobacco BY-2 cell suspension cultures to test the eliciting activity of surfactin, secreted by *B. amyloliquefaciens* S499.

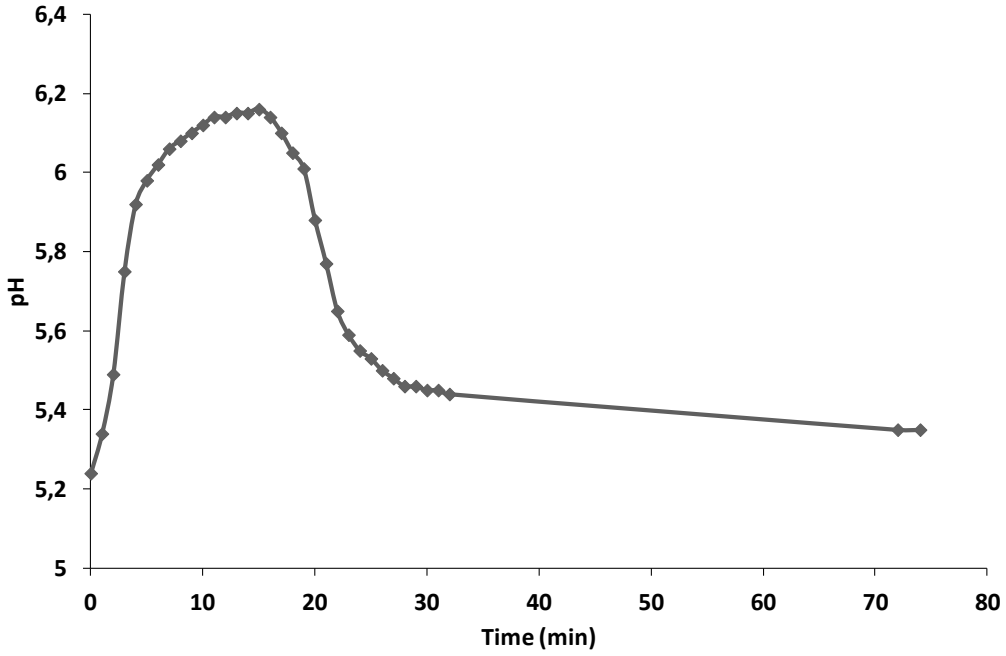


Figure 10: Extracellular pH change typically observed with *Nicotiana tabacum* BY-2 cell suspension cultures following treatment with 5 µM of the surfactin cLP family.

We observed that ΔpH_{max} varied with the age, the cell density and the initial pH of different batches of the cell culture. However, the typical response to a given dose of surfactin was highly reproducible within a given batch of cells (Figure 10). On another hand, tobacco cells were also treated with Triton X-100 used as positive control. Triton X-100 is a strong anionic detergent (such as surfactin) that could cause pore formation leading to medium alkalinization due to passive efflux/influx of ions through the plasma membrane. By contrast with the response induced by similar concentrations of surfactin, Triton X-100 only induced a constant, slow and limited response over the time of measurement (see below Figure 14A). ΔpH observed following surfactin treatment therefore originates from an active cell process.

7.2. Ion fluxes

The extracellular alkalinization response following elicitation usually occurs as a consequence of altered ion fluxes across the plasma membrane. Conductimetry after separation by ion chromatography and flame emission spectroscopy analyses from three independent experiments revealed a significant efflux of potassium, nitrate and chloride concomitant to pH increase. Indeed, the concentrations of K^+, NO^{3-} and Cl^- in the extracellular medium increased respectively by 680 ± 6 µM, 290 ± 21 µM and 24 ± 2 µM over the 15 min following surfactin addition (5 µM), while they remained fairly unchanged in the methanol-treated cell cultures used as controls.

7.3. Oxidative burst

The rapid generation of ROS (oxidative burst) has also been demonstrated to be typically involved in early events associated with the plant defense response following PAMP and MAMPs perception (Apel *et al.*, 2004). Generated ROS can act both directly as toxic substances against the pathogen and indirectly by reinforcing cell wall or by playing a signaling role to induce defense gene expression (Zipfel *et al.*, 2010, Torres, 2010). Hydrogen peroxide release by cLP-treated tobacco cells was investigated using the luminol-based chemiluminescence assay. A rapid and transient oxidative burst was clearly stimulated within minutes by pure surfactin. The surfactin-induced H_2O_2 accumulation peaked after 8-10 min when the lipopeptide was present at concentrations of 10 µM (Figure 11A). Oxidative burst also occurs in the cytoplasm of the cell suspension cultures with similar amplitude but more rapidly since fluorescence increase due to the oxidative stress-sensitive dye DCFH-DA was already visible after 1-2 minutes post-treatment (Figure 11B). Surfactin-induced ROS accumulation was similarly observed in root tissues of tobacco and tomato plantlets as determined with the same fluorimetric method (Figures 11C and 11D).

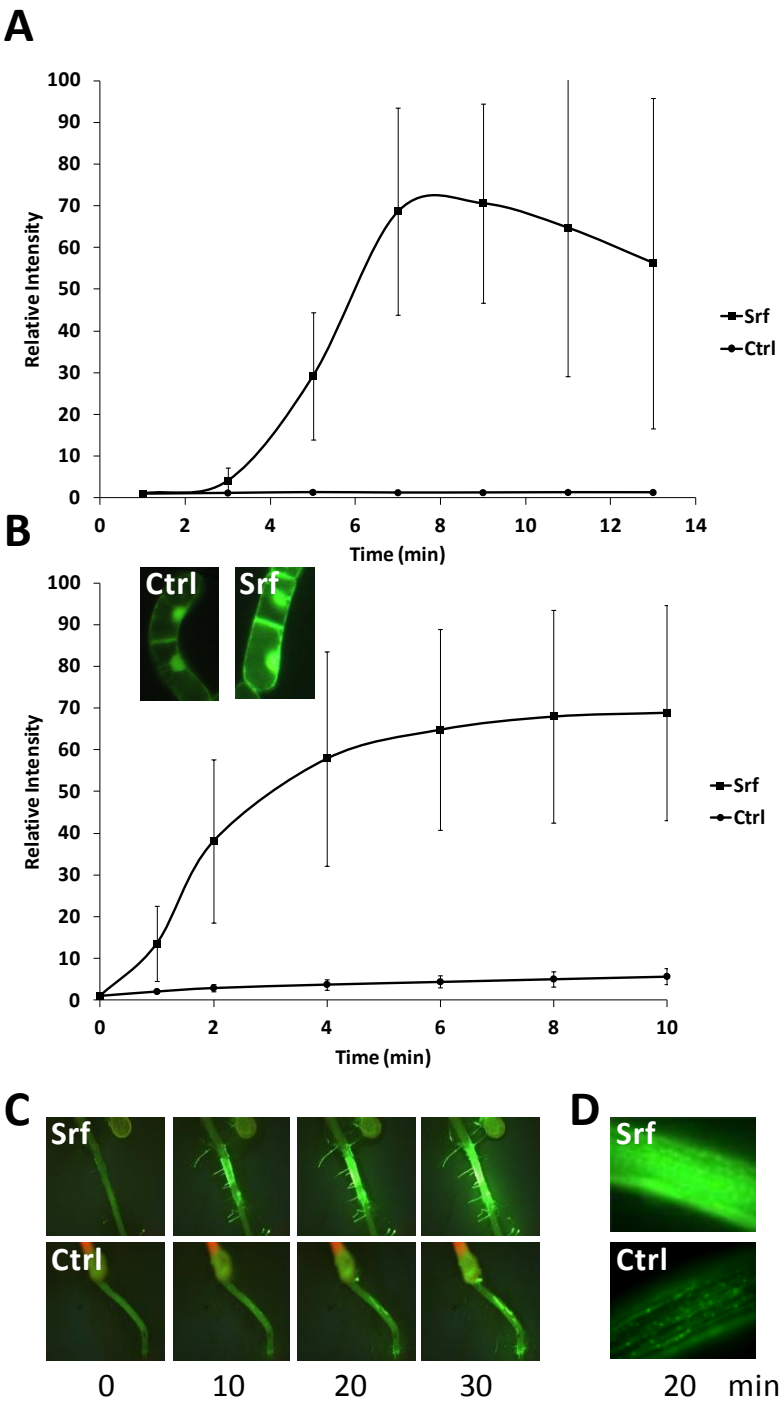


Figure 11 : A. Extracellular production of hydrogen peroxide by tobacco cells treated by 10 µM surfactin mix. B. Intracellular accumulation of ROS in tobacco cell suspension culture upon the same treatment. C. Kinetic of the intracellular accumulation of ROS in tobacco roots induced by 10 µM surfactin mix. D. Intracellular accumulation of ROS in tomato roots induced by surfactin. Extracellular H_2O_2 concentration was measured by chemiluminescence while intracellular ROS accumulation was detected by DCFH-DA staining. Control (Ctrl) consists in treatment with a similar volume of methanol. All images were acquired with the same exposition time and light sensitivity. A slight increase in fluorescence is observed in all cases due to light-induced auto-oxidation of the dye. Standard deviation bars result from three and seven independent experiments of intracellular and extracellular hydrogen peroxide production, respectively.

7.4. Regulation of early events

In plant-pathogen interactions, calcium is known to play an important role in the regulation of early events after elicitor perception (Lecourieux *et al.*, 2005). In order to determine if Ca^{2+} is also involved in surfactin-induced alkalinization and oxidative burst, the effects of both EGTA as calcium-chelator and $LaCl_3$ as calcium channel inhibitor were investigated in surfactin-treated tobacco cell suspensions. As shown in Figure 12, $LaCl_3$ addition in micromolar concentration (500

µM, inhibitory for channels in the plasma membrane but not those within the cells (Pineros *et al.*, 1997)) before treatment with surfactin totally inhibited both the oxidative burst and ΔpH change induced by the lipopeptide. A drastic effect on surfactin activity was also observed by pre-incubating tobacco cells in the presence of 1 mM EGTA, confirming the crucial role of Ca^{2+} influx in the occurrence of both phenomena. Incubation of plant cells in the presence of the serine/threonine protein kinase inhibitor K252a (2 µM) prior to surfactin addition also resulted in a marked decrease in the amplitude of the alkalinization (reduced by 54%) and the H_2O_2 accumulation which was almost completely inhibited (Figure 12A and B). In addition, at 2 µM ,diphenyleneiodonium (DPI), which is an inhibitor of NADPH oxidase involved in the oxidative burst (Pugin *et al.*, 1997), completely inhibited the H_2O_2 production stimulated by 10 µM surfactin but only partially affected (maximal inhibition 22%) the ΔpH.

Phospholipase A2 (PLA2) is thought to be involved in expression of the plant defense response to various stimuli (Chandra *et al.*, 1996, Lee *et al.*, 1997, Roos *et al.*, 1999). The effect of the PLA2 inhibitor arachidonic acid trifluoromethyl ketone ($AACOF_3$) was also tested to assess the involvement of the enzyme in signal transduction upon surfactin elicitation. $AACOF_3$ caused a 53% decrease in the ΔpH when added at 33 µM 30 min prior to the elicitation with 2 µM lipopeptide.

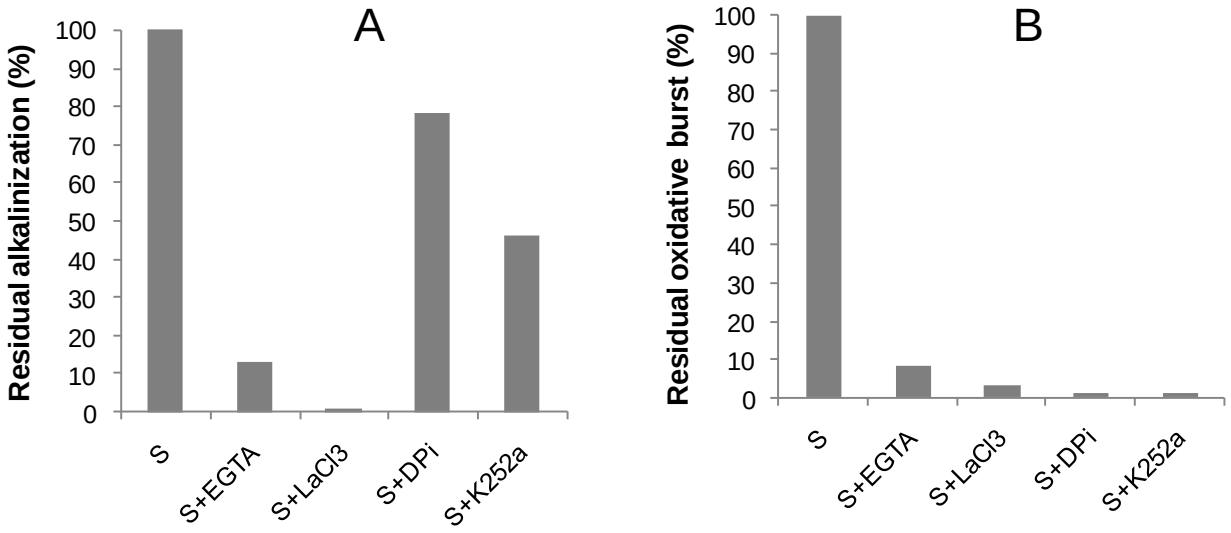


Figure 12 : Influence of chelating agent (EGTA) or enzyme inhibitors on surfactin-induced extracellular alkalinization (A) and oxidative burst (B). LaCl3: inhibitor of calcium channels. DPI: inhibitor of NADPH oxidase. K252a: Ser/Thr protein kinase inhibitors.

Chapter 8. Surfactin as main elicitor secreted by *Bacillus amyloliquefaciens*

B. amyloliquefaciens S499 is our laboratory strain having consistent ISR inducing activity and able to synthesize high amounts of several cLP families in liquid culture. Despite close structures and physico-chemical properties, these cLP families display significant differences in the broad array of biological activities they retain. Until now, their respective ability to trigger ISR on tobacco plant was not investigated. Consequently, we wanted to compare their respective potential to induce early stress responses by testing both supernatant from bacterial cultures and purified cLPs on tobacco BY-2 cell suspension cultures.

8.1. Activity of the secretome of *B. amyloliquefaciens* S499

Supernatant samples were firstly collected from a batch culture of *B. amyloliquefaciens* S499 at various time-points during *in vitro* growth and in a specific medium optimized for the production of cLPs. Crude supernatant samples were pre-purified on solid phase C18 cartridge to yield the corresponding MeOH40 extract (fractions eluted with 40% methanol) containing molecules with intermediate hydrophobicity, and the MeOH100 extract (fractions eluted with pure methanol), retaining more apolar compounds and cLPs of the surfactin, fengycin and iturin families. The effect on tobacco cells of C18 extracts prepared from samples collected every hour all over the culture were first estimated with indicator paper. MeOH40 extract did not induce any pH change (Figure 13A), suggesting that no metabolite with intermediate hydrophobicity produced by strain S499 upon such culture conditions are active in triggering an alkalinization response by tobacco cells. The same result was obtained with MeOH100 fractions from samples collected before surfactin production, indicating that apolar metabolic products that could accumulate early in the exponential growth phase are not active. By contrast, indicator paper color changes could be clearly visualized with MeOH100 extracts prepared from culture samples collected after the exponential growth phase (Figure 13A). ΔpH induced by these samples were more precisely measured with pH probe (Figure 13B). A weak but significant pH alkalinization was observed by treating tobacco cells with extracts from 16 h-old culture, corresponding to the apparition of surfactin in the supernatant. Stronger pH alkalinization was firstly significantly observed when MeOH100 extracts from samples containing higher surfactin concentrations were added to the cells (Figure 13B). These first results suggest that these lipopeptides are involved in the induction of alkalinization since it exactly correlates with their appearance and accumulation trend in the medium. However, a contribution of other metabolites with similar production kinetic or of fengycin, whose production is only 3-4 hours-

delayed compared to surfactin, could not be ruled out at this stage. By contrast, iturin, that accumulate later (not shown on graph), is seemingly not involved. Indeed, iturin-rich samples collected after 72 h of bacterial growth did not showed enhanced activity compared to those collected at 54 h with reduced iturin content (data not shown).

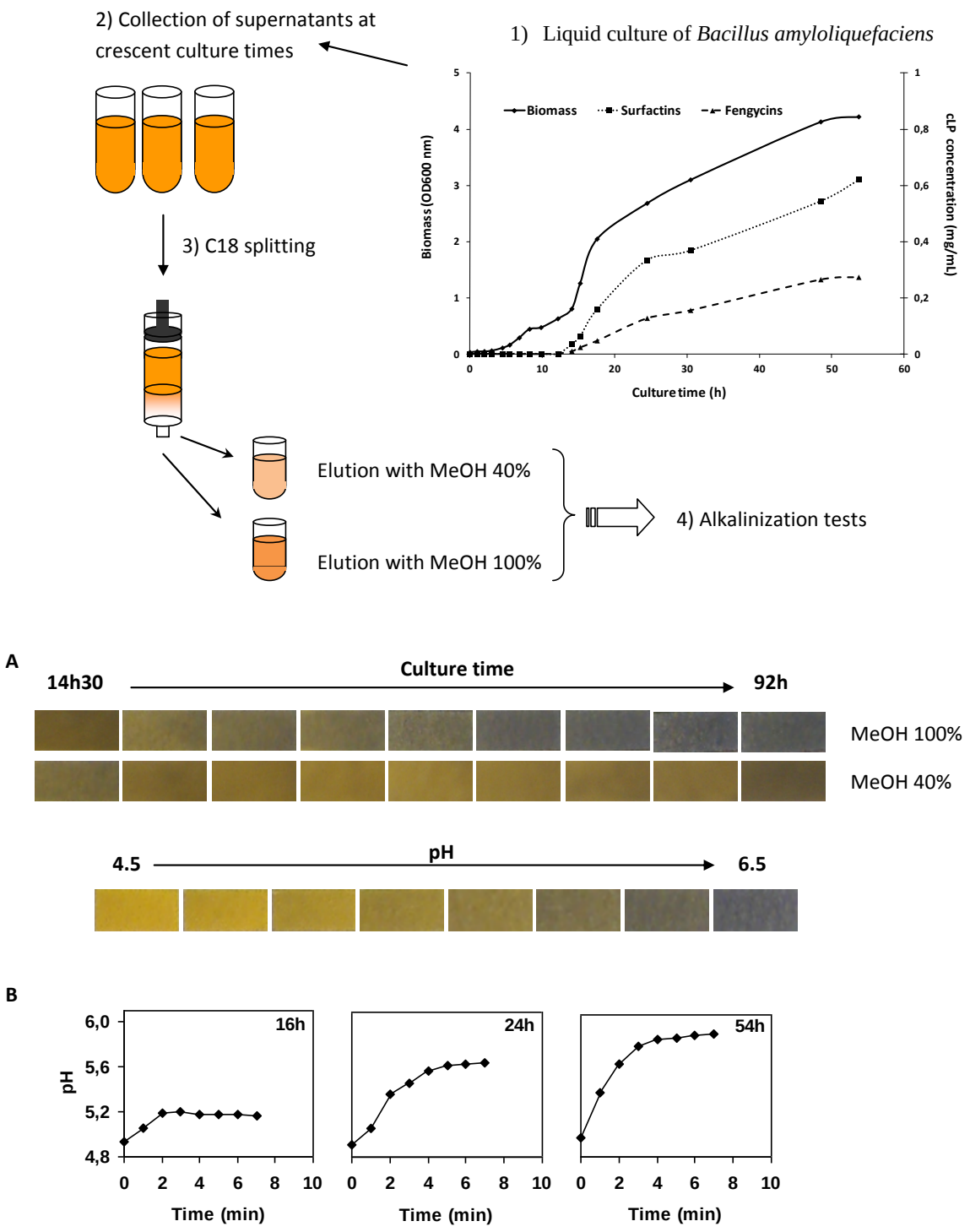


Figure 13 : Alkalinization response of tobacco cells induced by extracts from *Bacillus amyloliquefaciens* S499 cultures. Samples from liquid cultures were separated by solid-phase chromatography and eluted in two fractions to test the response induced by metabolites of crescent hydrophobicity. A. Color change of pH papers immersed in extracellular medium of tobacco cell cultures after addition of MeOH 40% and MeOH 100% fractions collected at different stages of bacterial growth. B. Tobacco cell suspension cultures treated with MeOH 100% fraction from supernatant samples collected at 16, 26, and 54 h of growth, corresponding to final surfactin concentrations of 0.7, 2.5, and 5.2 µM, respectively. Control consisted of cells treated with a similar volume of methanol.

8.2. Activity of purified cLPs

In a second approach to get more information about the relative alkalinization-inducing activities of the three lipopeptide families, we tested individually the alkalinization induced by surfactin, fengycin and iturin (as 99%-pure mixtures of homologues) purified from S499 cultures. As shown in Figure 14A, surfactin, but not iturin nor fengycin, induced a significant and transient pH increase upon addition at a concentration of 2 µM. Higher doses of both latter cLPs were also not effective with a weak ΔpH_{max} of 0.20-0.25 obtained by treating plant cells with compounds at 20 µM. However, as illustrated in Figure 14B, a first treatment with either fengycin or iturin did not preclude a full alkalinization response of the cells upon subsequent addition of surfactin.

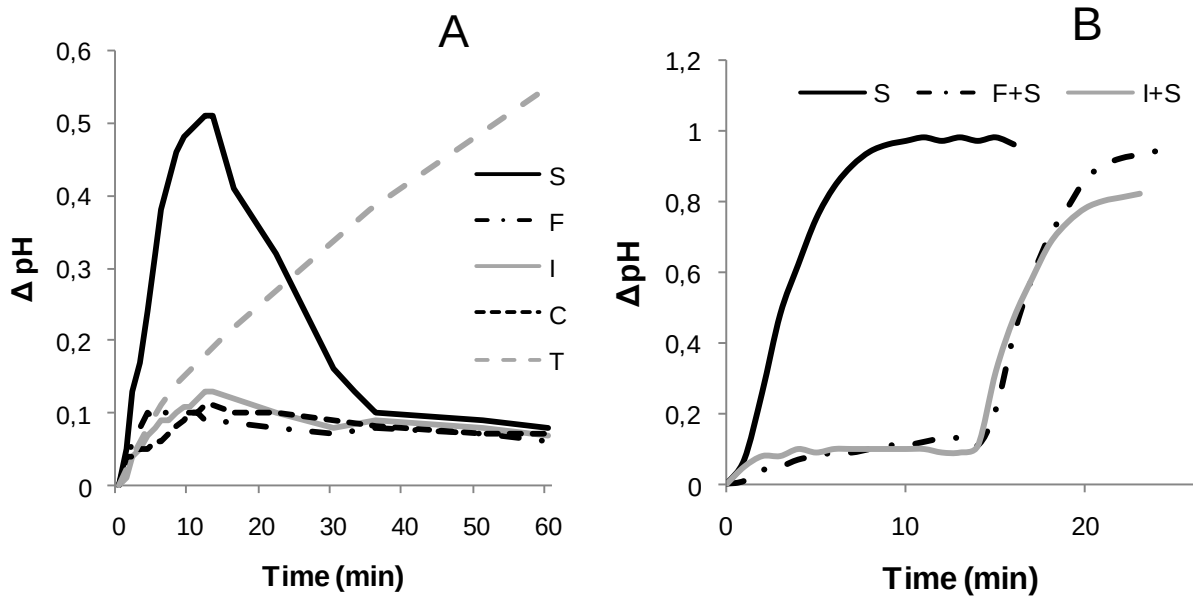


Figure 14 : A. Evolution of tobacco suspension culture medium pH in response to 2 µM of surfactin (S), fengycin (F), iturin (I), methanol control (C) and Triton X-100 as positive control (T). B. Influence of a prior treatment with either fengycin or iturin on response induced by surfactin.

By contrast, when fengycin and surfactin were used in combination, the total pH alkalinization was higher than the sum of their respective individual effects, suggesting some synergism between these two cLP families (Figure 15). This result was not observed with iturin.

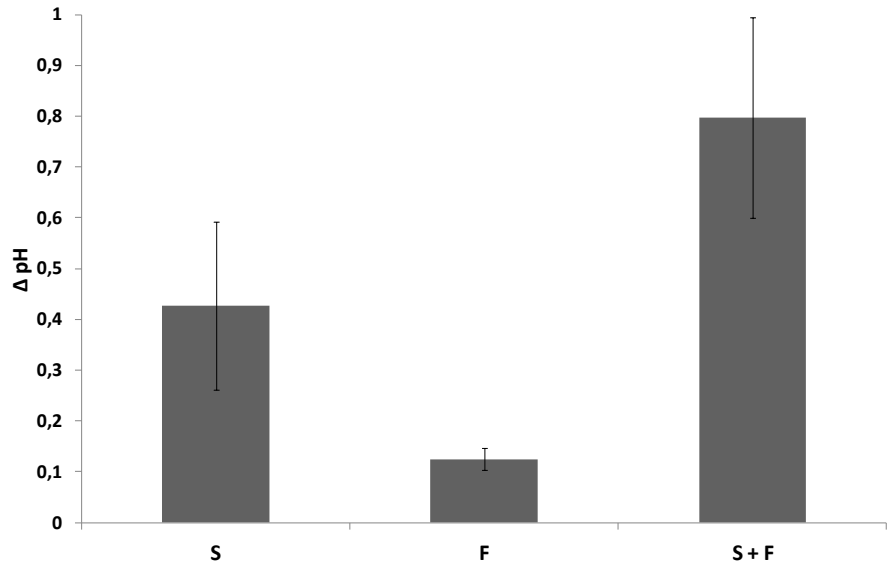


Figure 15 : pH alkalinization induced by surfactin and fengycin used separately or in combination.

A rapid and transient oxidative burst was also clearly stimulated within minutes by pure surfactin but neither by fengycin nor iturin added at the same concentration (Figure 16A). As in the case of alkalinization, no marked oxidative response could be observed following treatment with higher amounts of these last two cLPs.

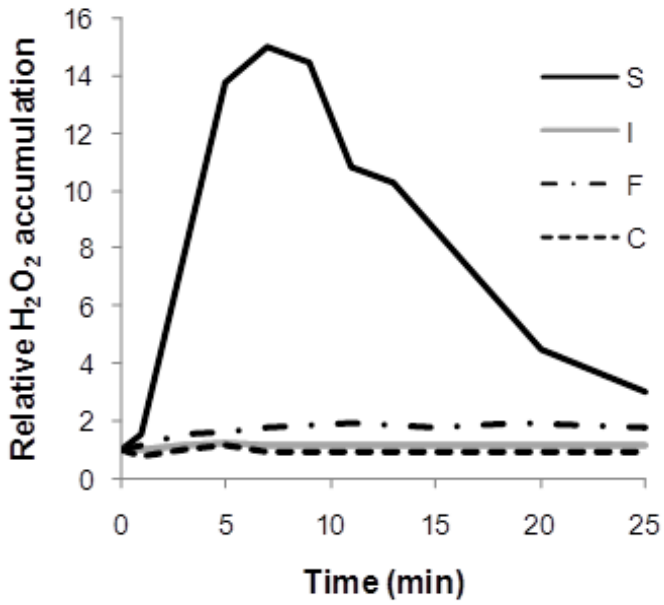


Figure 16 : Oxydative burst response (expressed in relative H_20_2 accumulation in tobacco culture medium) in response to surfactin (S), fengycin (F), iturin (I) and methanol as control (C).

8.3. Differential activities of bacterial mutants affected in cLP synthesis

In order to confirm the relative alkalinization-inducing activities of the three lipopeptide families, we tested MeOH100 extracts from various derivatives of the *Bacillus amyloliquefaciens* strain FZB42 affected in the biosynthesis of cyclic lipopeptides. The AK3 mutant efficiently produces surfactin but fengycin and iturin biosyntheses are suppressed. The CH1 and CH2 derivatives retain

fengycin and/or iturin synthesis but are impaired in the production of surfactin lipopeptide family. cLPs production by these strains was qualitatively assessed and quantified by HPLC. MeOH extracts were diluted in order to obtain a final cLP concentration into the tobacco cell suspension culture of 2 µM. A strong alkalinization response was observed following treatment with the AK3 extract but neither CH1 nor CH2 extracts induced a significant ΔpH compared with control cells treated with methanol (Figure 17).

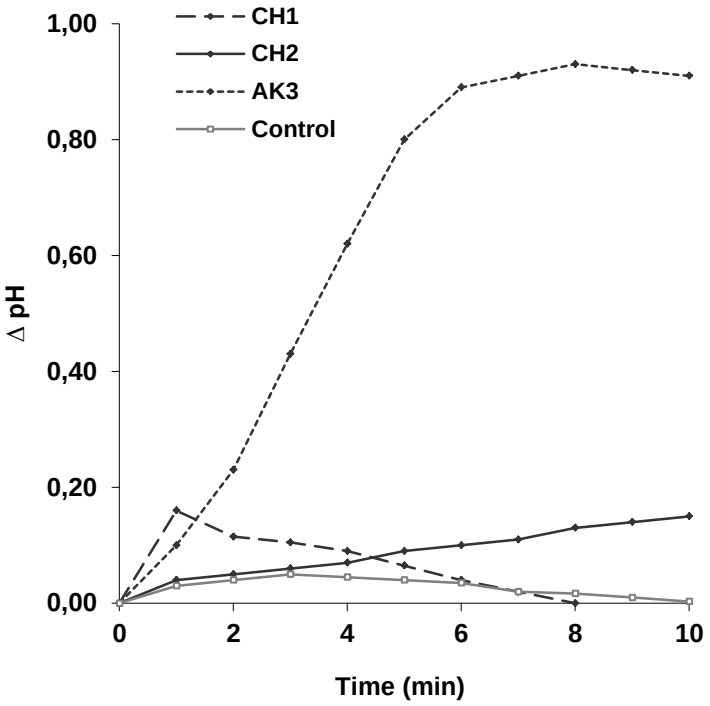


Figure 17 : pH variation of tobacco cell suspension culture medium in response to extracts from *Bacillus amyloliquefaciens* FZB42 mutants liquid cultures. The three mutants of strain FZB42 that are affected in surfactin (surf), fengycin (feng), or iturin (itu) production are CH2 (surf–/feng–/itu+), CH1 (surf –/feng+/itu+), and AK3 (surf +, feng–, itu–). Lipopeptides were extracted from culture broths by means of C18 solid phase extraction cartridges. Tobacco cell suspension cultures were treated with ~20 µl of culture extracts dissolved in methanol to obtain a final cLP concentration of 2 µM. Control consisted of cells treated with similar volume of methanol. Data presented result from one representative experiment.

These results confirm that among *Bacillus* cLP families, only surfactin behaves like an ISR elicitor on tobacco plants. This reinforces other results previously obtained in our laboratory showing that surfactin display the highest potential as ISR elicitor among all metabolites produced by *Bacillus*. It is worth to note that synergistic ISR activities between cLPs families may also occur as suggested by results obtained on tobacco cell suspension.

Chapter 9. Specific aspects of the interaction between surfactin and plant cells

We demonstrated in chapter 7 that early events induced by cLPs are in some aspects similar with those characterized with both PAMPs from pathogens and MAMPs from beneficial rhizobacteria. However, the mode of action of PAMPs is typically dependent of a receptor-driven perception while the one of MAMPs is still elusive. As amphiphilic compounds with specific structural features, cLPs may be perceived by an unusual mechanism. A deeper investigation of specific early responses of tobacco cells following surfactin perception could provide new information about the first step of surfactin-plant cell communication. The impact of structural modifications of surfactin on their activity is also particularly interesting to unravel structural treats required for their perception at the membrane level.

By contrast with most of the other elicitors but similarly to other biosurfactants, surfactin is known to spontaneously interact with lipid bilayers by means of hydrophobic interactions (Maget-Dana *et al.*, 1995). The first step of membrane disturbance mechanism underlying most of their biological activities is the insertion into the lipid bilayer. It is followed by their aggregation, leading to the formation of pores breaking down the underlying structure (Liu *et al.*, 2005). The last part of this chapter would therefore be devoted to consider their insertion within membranes of tobacco cells.

9.1. The eliciting activity of surfactin relies on specific structural traits

The synthesis/purification of a broad array of surfactin variants, differing in the peptide or/and in the lipid part of the molecule, was firstly achieved. These variants were then tested for their activity on tobacco cell suspension cultures to get more information about structural requirements for optimal early responses induction.

9.1.1. Synthesis and isolation of surfactin structural derivatives

For some bacteria, the NRPS machinery allows precursor-directed modulation of the amino acid content of the peptide produced by a given strain. It results from the reduced specificity of adenylation domains regarding the nature of the amino acid residue it can activate (Kowall *et al.*, 1998, Grangemard *et al.*, 1997). We wanted to take advantage of such flexibility to generate surfactin variants after growing the strain in a medium supplemented with L-Leu, L-Val or L-Ileu as sole amino acid source. Feeding with these residues led to additional peaks in the surfactin signature (Figure 18), determined by LC-ESI-MS analysis of supernatant extracts (see Annexe I). Amino acid

analysis of hydrolysates and in-source fragmentation in the mass spectrometer confirmed the nature of the substitutions at positions 4 and 7. All surfactin variants purified from these cultures and used for the structure-activity relationship are detailed in Table 5.

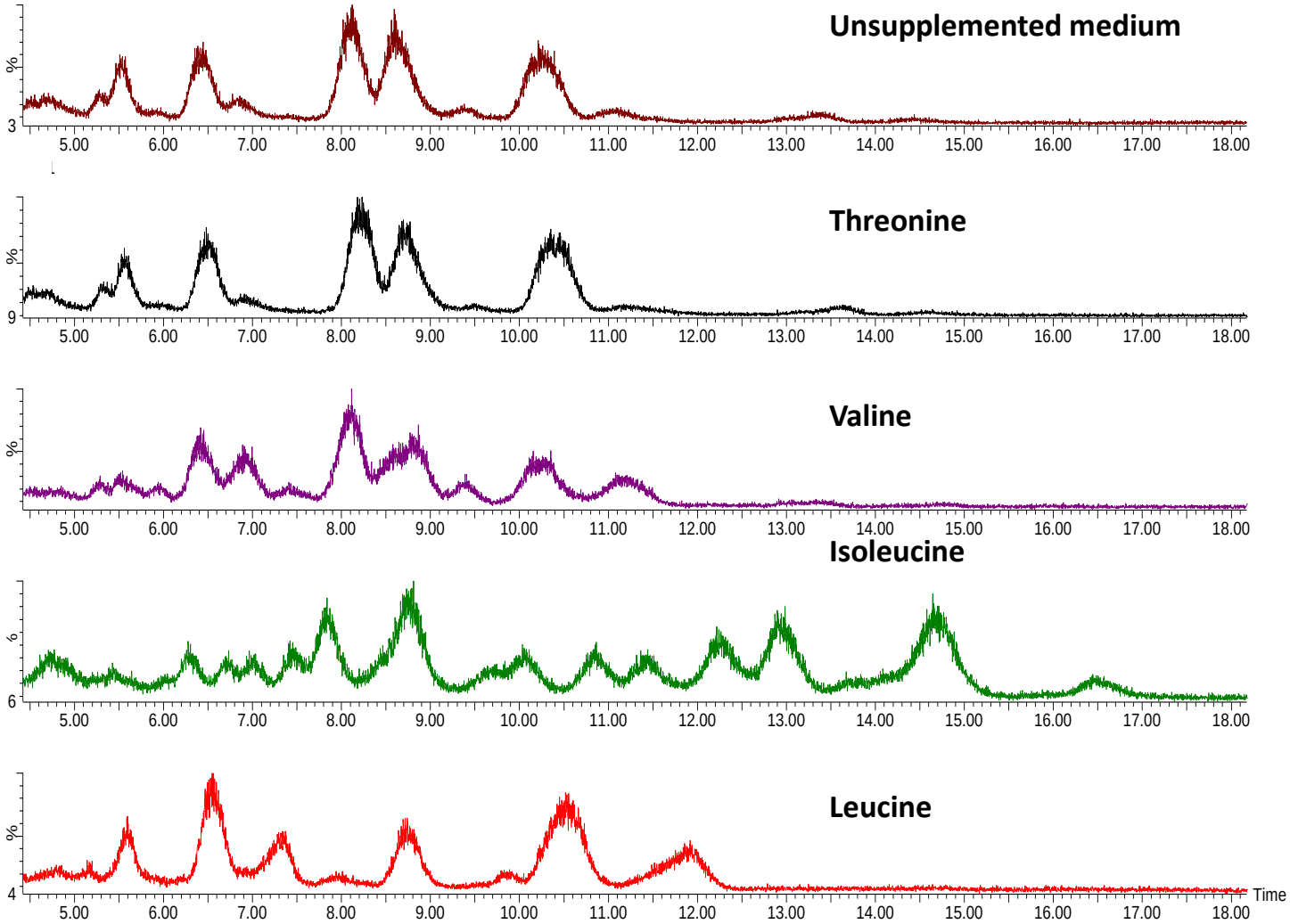


Figure 18 : LC-MS chromatograms of surfactin isoforms from *Bacillus amyloliquefaciens* S499 grown in unsupplemented medium or in medium supplemented with 0.5 g/L of L-Threonine, L-Valine, L-Isoleucine, or L-Leucine. Only these three last amino acids lead to new surfactin profiles.

Table 5 : Surfactin variants used in this study. For C14 Ileu and C15 Ileu, the real position of the Ileu residue within peptide cycle was not determined.

	Origin	Structure
C12	Natural prod.	$CH_3-(CH_2)_8-CH-CH_2-CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Leu-O$
C13	Natural	$CH_3-(CH_2)_9-CH-CH_2-CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Leu-O$
C14	Natural	$CH_3-(CH_2)_{10}-CH-CH_2-CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Leu-O$
C15	Natural	$CH_3-(CH_2)_{11}-CH-CH_2-CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Leu-O$
C14 m	Chemical	$CH_3-(CH_2)_8-CH-CH_2-CO\rightarrow GluCH_3\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow AspCH_3\rightarrow Leu\rightarrow Leu-O$
C14 l	Chemical	$CH_3-(CH_2)_8-CH-CH_2-CO\rightarrow GluCH_3\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow AspCH_3\rightarrow Leu\rightarrow Leu-O$
C14 lm	Chemical	$CH_3-(CH_2)_{10}-CH-CH_2-CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Leu-O$
C14 Leu4	Precursor-directed	$CH_3-(CH_2)_{10}-CH-CH_2-CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Leu\rightarrow Asp\rightarrow Leu\rightarrow Leu-O$
C14 Ile	Precursor-directed	$CH_3-(CH_2)_{10}-CH-CH_2-CO\rightarrow Glu\rightarrow Leu/Ile\rightarrow Leu/Ile\rightarrow Val\rightarrow Asp\rightarrow Leu/Ile\rightarrow Leu/Ile-O$
C15 Val7	Precursor-directed	$CH_3-(CH_2)_{11}-CH-CH_2-CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Val-O$
C15 Leu4	Precursor-directed	$CH_3-(CH_2)_{11}-CH-CH_2-CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Leu\rightarrow Asp\rightarrow Leu\rightarrow Leu-O$
C15 Ile	Precursor-directed	$CH_3-(CH_2)_{11}-CH-CH_2-CO\rightarrow Glu\rightarrow Leu/Ile\rightarrow Leu/Ile\rightarrow Val\rightarrow Asp\rightarrow Leu/Ile\rightarrow Leu/Ile-O$

9.1.2. Effect of structural changes on the alkalinization response

As revealed after separation by HPLC and identification by MALDI-TOF mass spectrometry, the surfactin (all of the A form) mixture from strain S499 is mainly composed of six homologues: C12 to C15 linear saturated acyl chain homologues as well as iso-C14 and iso-C15 with ramified and saturated acyl chains. These homologues were purified by semi-preparative HPLC and tested individually on tobacco cells except for the C14 and C15 homologues for which linear and ramified forms could not be completely resolved, and were tested as single samples. Means calculated from three independent experiments revealed that homologues with the shortest lipid chains (C12 and C13) failed to induce any significant pH shift (Figure 19A). By contrast, C14 and C15 surfactin triggered a significant alkalinization response that probably account for most of the activity of the mixture sample. Interestingly, chain ramification may also be important for elicitor activity since a limited but significant difference was observed between cells treated with linear (C14n) or ramified (C14i) surfactin homologues (Figure 19B). The active C14 homologue was further chemically modified via alkylation (C14 M) and/or saponification (C14 L and C14 L+M) in order to appreciate the importance of cyclization and presence of ionic charges (two acidic residues) in the peptide. As shown in Figure 20B, both modifications considered individually led to a significant decrease of the eliciting activity on tobacco cells. Moreover, the linearized and methylated form (L+M) only retained about 25% of the activity.

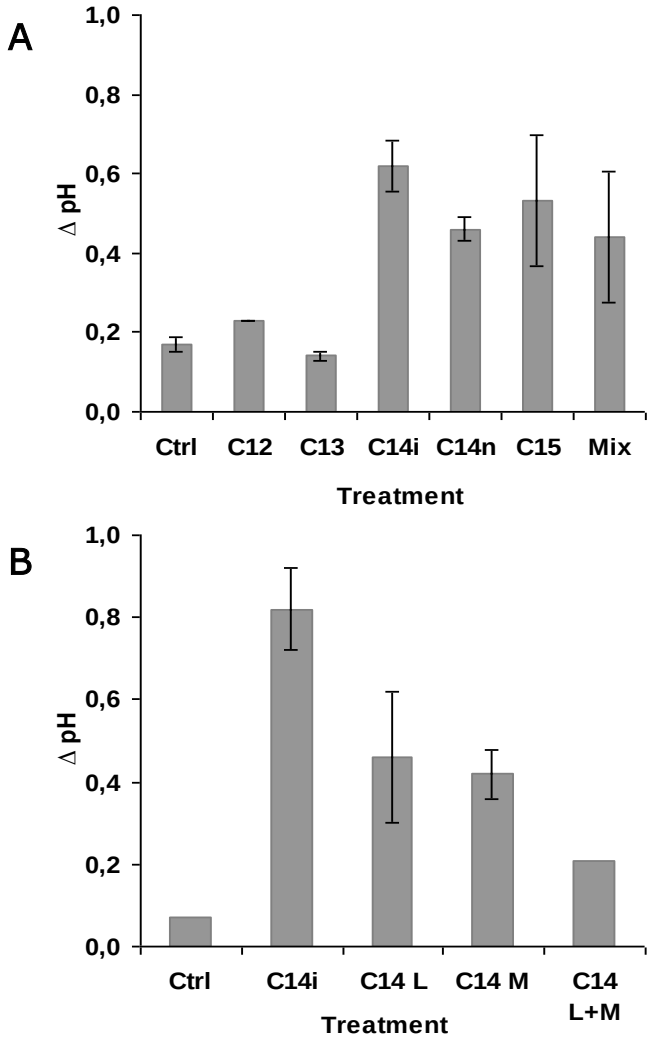


Figure 19 : Structure-activity relationship of surfactin in eliciting pH alkalinization of tobacco cell suspension culture medium. A. Medium alkalinization response to treatment of tobacco cells with 2 µM surfactin homologues with lipid chain length from 12 carbons (C12) to 15 carbons (C15). For surfactin with 14 carbons in the lipid chain, linear chain (C14n) and ramified chain (C14i) homologue were tested. The mixture of the different surfactin homologues was used as a positive control (Mix). Data and standard variations calculated result from three independent experiments. B. Impact of lipid acid chain conformation and peptide core charge of C14i surfactin on cell response. This iso form of the C14 homologue was either methylated on the acidic amino acids (C14 M) or synthesised in a linearized form (C14 L) or both (C14 L+M). The natural C14i homologue and modified surfactin were added to obtain a final concentration of 4 µM in the cell suspension culture medium. Control consisted of cells treated with similar volume of methanol (Ctrl). Data are means and standard variations calculated result from three independent experiments.

9.1.3. Effect of structural changes on the oxidative response

As ROS accumulation represents a sensitive and appropriate marker for defense activation by surfactin in host plant cells, we further used this phenomenon to study the structure/activity relationship of surfactin. Similarly as the alkalinization response, our data revealed that homologues with the shortest lipid chains (C12 and C13) failed to induce any hydrogen peroxide release (Figure 20A). By contrast, C14 and C15 surfactin triggered a significant response that probably accounts for most of the activity of the surfactin mix sample. Unlike the alkalinization response, all the linear, methylated and linear/methylated derivatives of the active C14 homologue completely lost their eliciting activity on tobacco cells.

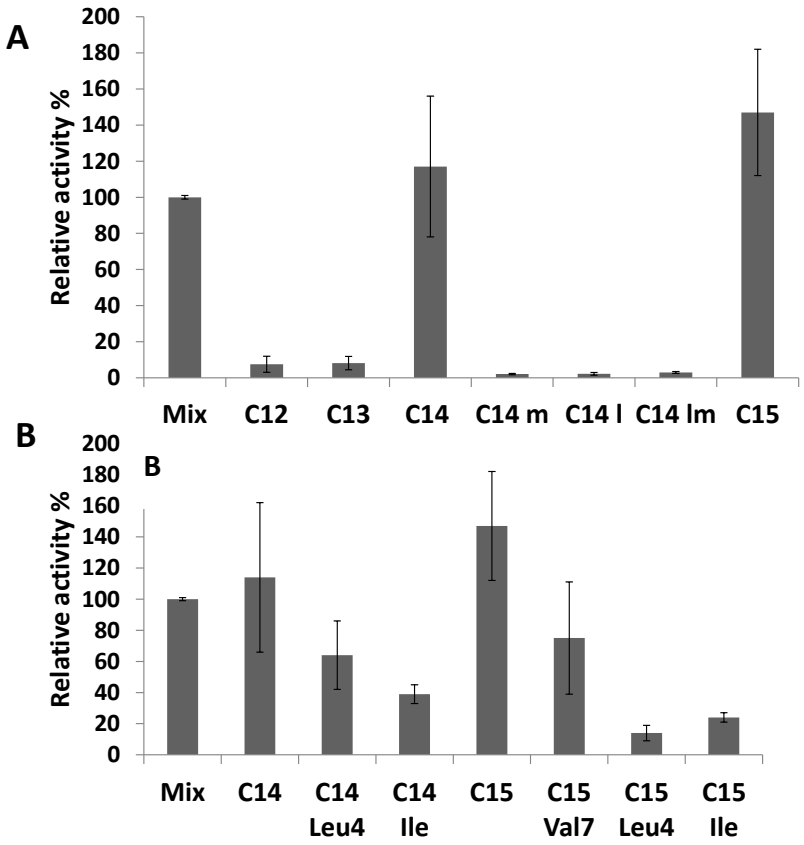


Figure 20 : Hydrogen peroxide release by tobacco cells upon treatment with 15 µM surfactin variants. A. Compounds with variable lipid chain from 12 (C12) to 15 carbons (C15) and methylated and linear derivative of the C14 homologue. B. Compounds varying in the peptide sequence. The 15 µM concentration lead to the highest responses with active surfactin homologues and was therefore selected in order to highlight the difference of activity between active and less or non active isoforms. Data are expressed as relative activity compared to the one of surfactin mix containing naturally produced homologues. Data and standard deviations calculated result from three independent experiments.

In all cases, activities of surfactin peptidic variants were reduced compared to the one of unmodified surfactin homologues with similar fatty acid chain length (Figure 20B). A substitution of L-Val by L-Leu in position 4 affected the activity of the C15 homologue more profoundly than the one of the C14. This suggests that efficacy of the compound relies on a combined effect of acyl chain length and the residue position in the peptide. In contrast, a substitution of Leu residue by a less hydrophobic amino acid was less detrimental for the activity than a substitution by a more hydrophobic residue. Indeed, both C15 Leu4 and C15 Ile showed a three-time lower activity than C15 Val7 which itself lost 50% of the natural C15 homologue activity.

9.2. No competitive effect between homologues

We also evaluated the competition between active and inactive surfactin variants for potential recognition sites on cultured cells. Figure 21 shows that subsequent addition of C15 homologue after a first treatment with inactive C13 results in an integral production of ROS. This was also observed following a first addition of C14 that has been completely inactivated by methylation or, to a slightly lower extent, upon cell pre-treatment with C15 homologue with Leu/Ile substitution. Results obtained in experiments performed with other inactivated forms (C12, cyclized C14 surfactin) were similar (data not shown).

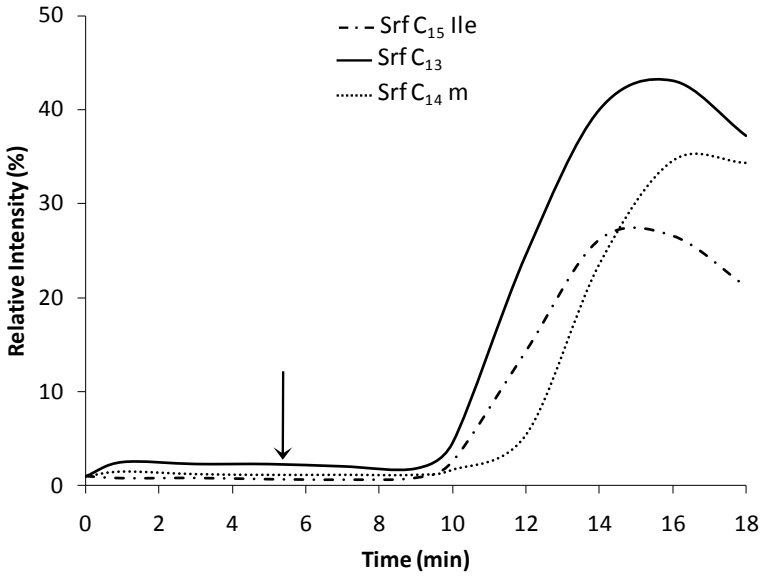


Figure 21 : Extracellular release of hydrogen peroxide expressed in relative intensity of maximum activity. 10 µM of surfactin C15 homologue was applied 10 min after a first stimulation at the time indicated by the arrow with non or less active C13/C14m/C15Ile isoforms. Presented data result from one representative experiment.

9.3. Micromolar surfactin concentrations are required for eliciting activity

As shown in Figure 22, surfactin induced a significant and transient pH increase upon addition at a concentration as low as 2 µM. A dose-dependent response was clearly observed and an EC_{50} concentration corresponding to the half-maximal alkalinisation inducing activity of 2.5 µM was calculated. A concentration of 20 µM was not more effective than 10 µM, suggesting a saturation of the phenomenon as already observed in other studies (Pugin *et al.*, 1998, Felix *et al.*, 1999).

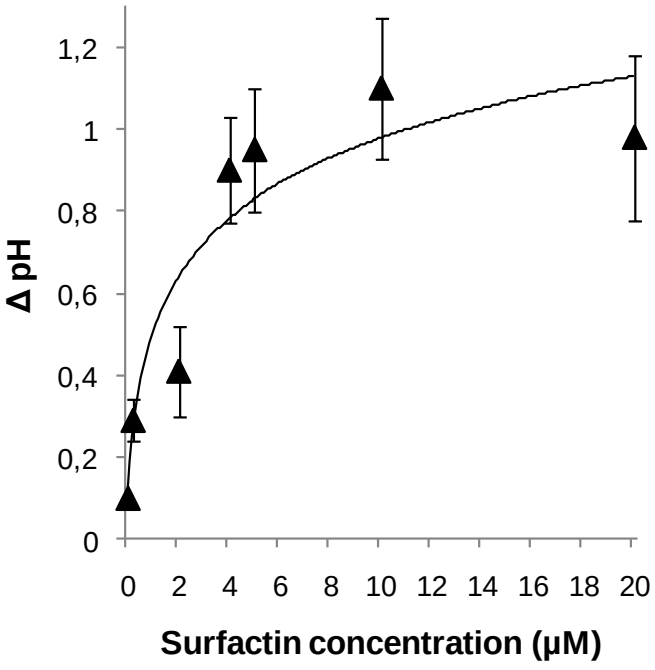


Figure 22 : Extracellular alkalinization of tobacco cell suspension cultures as function of surfactin concentration.

Treatment of tobacco cells with surfactin at 10 µM or 20 µM triggered oxidative burst corresponding to the formation of H_2O_2 concentrations in the range of 3.5-4.0 µM. At lower doses (2-5 µM), reduced but significant H_2O_2 accumulation was also measured allowing to calculate an EC_{50} value of 2.3 µM (Figure 23).

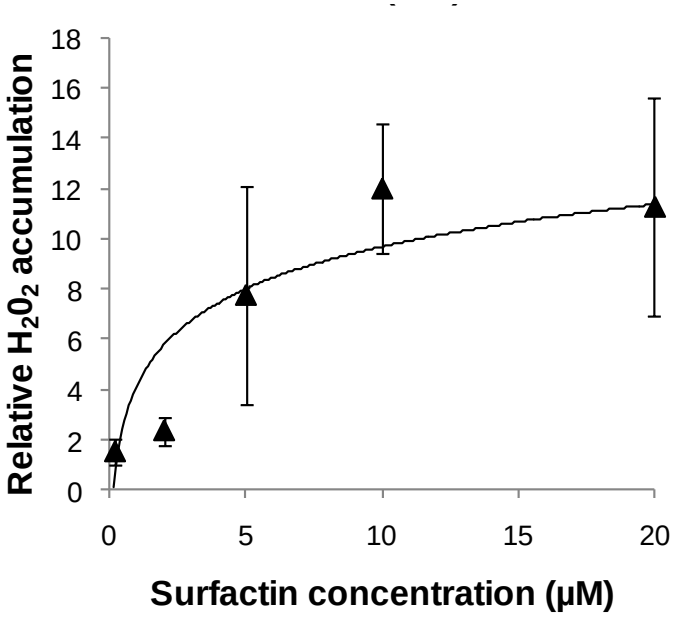


Figure 23 : Hydrogen peroxide accumulation in tobacco cell suspension culture as function of surfactin concentration. Results are expressed in relative units of chemiluminescence.

9.4. Surfactin does not trigger secondary oxidative burst associated with HR and cell death

Viability of tobacco cells was determined in the presence of increasing concentrations of surfactin by using Evans blue coloration (Baker *et al.*, 1994). Both microscopic observation and spectrophotometric quantification did not reveal any significant cell mortality in the first hours following surfactin addition at the concentrations used compared with the methanol-treated control (data not shown). Data obtained for mortality assessment 24 h after cLP addition showed a limited adverse effect of surfactin on tobacco cell viability (Figure 24A) compared with benzylamine, reported as a powerful cell-death-inducer agent (Kawano *et al.*, 2000) (data not shown), or Triton X-100, used for calibration. No significant cell death was observed following treatment with 2 µM surfactin, and mortality rates of approximately 25% were induced by surfactin added at a concentration of 10 µM to 20 µM (Figure 24B). In addition, the integrity of cells was evaluated by measuring intracellular protein release and only minor leaks, if any (up to 4 to 7% increase in extracellular proteins), were induced during the first 5 h following treatment with 2 µM to 20 µM surfactin (data not shown).

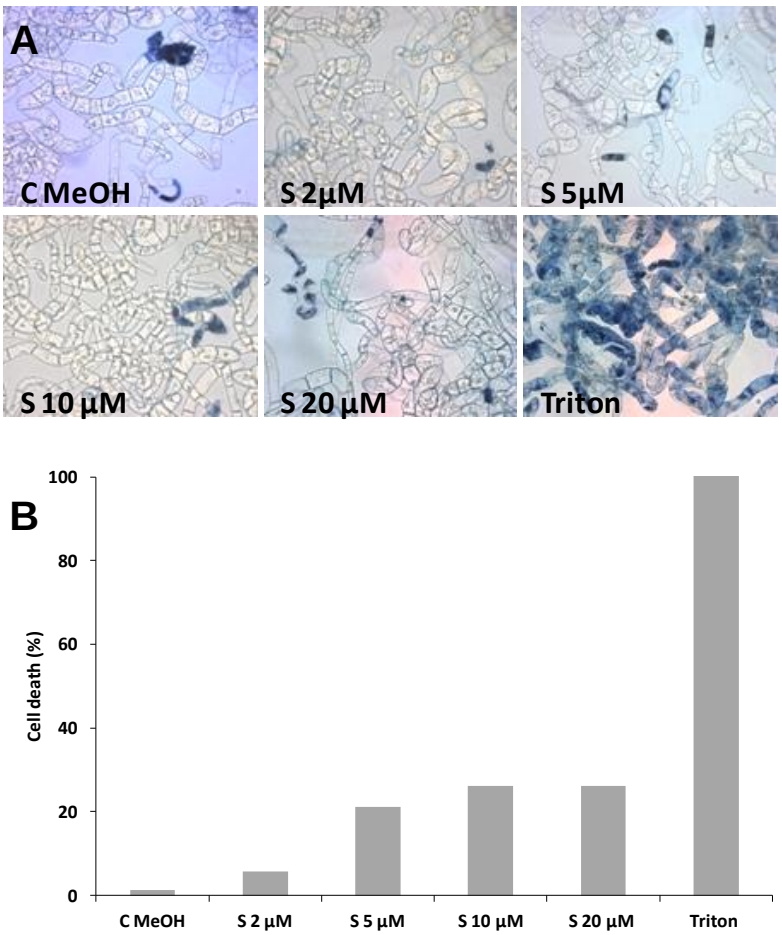


Figure 24 : A. Visual evaluation of tobacco cell mortality upon treatment with surfactin. Cells were treated with 100% methanol as control (C MeOH), with concentrations of surfactin from 2 µM to 20 µM and with Triton X-100 as positive control. Pictures were taken 24 h after the treatment of cells. Dead cells are dyed with Evans blue. B. Tobacco cells death induced 24 h following treatment with rising surfactin concentrations.

9.5. No refractory state associated with surfactin perception

The presence of high-affinity protein receptors for PAMPs in the plasma membrane is typically associated with the occurrence of some refractory state. Host cells are no longer able to react to a second stimulation by the same compound because of irreversible saturation of the binding sites (Felix *et al.*, 1993, Freudenberg *et al.*, 1998). As illustrated in Figure 25 for two different concentrations and timings, such a refractory state is not observed in the interaction with surfactin, as a second treatment resulted invariably in a consistent oxidative burst response.

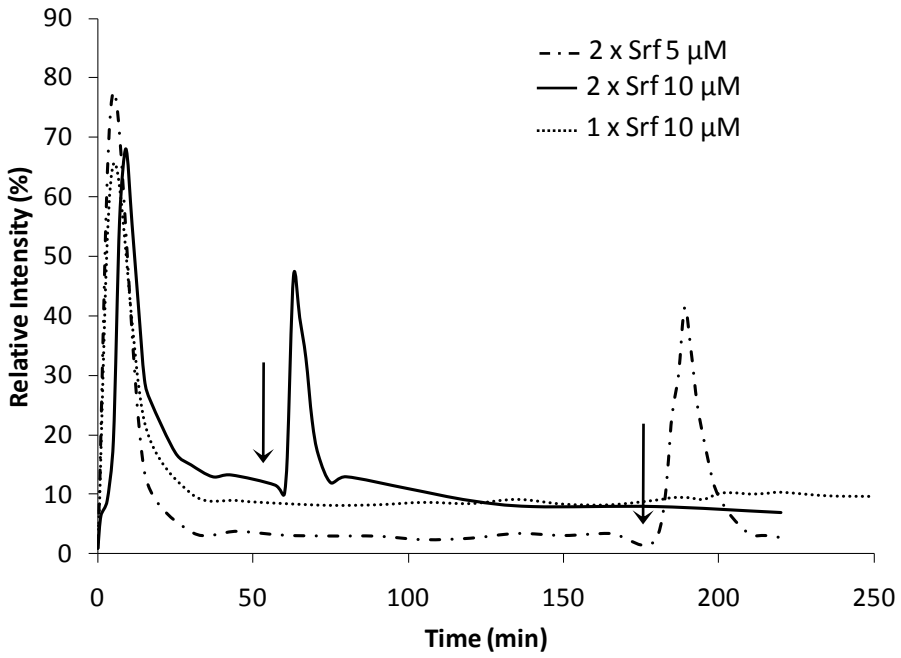


Figure 25 : Extracellular release of hydrogen peroxide induced by two successive treatments of tobacco cells with surfactin mix. For two different concentrations and timings, re-addition of surfactin leads to a consistent albeit slightly lower response, while no secondary burst has been observed upon single addition of surfactin.

9.6. High affinity of surfactin for plasma membrane

At various times following addition of surfactin, the supernatant of tobacco cultures was subjected to HPLC-MS for quantification of residual concentration of the lipopeptide. At the end of the experiment, remaining concentration in the supernatant was measured. The intracellular amount of surfactin was evaluated at the same time after grinding and washing in a polar solvent the cell pellet. The amount of surfactin into the membrane fraction was finally evaluated after washing the resultant pellet in an apolar solvent. Upon addition to a tobacco cell suspension at a concentration similar to the one inducing extracellular alkalinization and oxidative burst, the long-chain homologues C14 and C15 almost completely disappear from the medium within 10 min. Most of the initial concentrations are recovered from membranes fraction of the treated cells (Figures 26A and B). Surfactin, and to a greater extent long chain isoforms, thus likely integrally insert into lipid bilayers or associate with lipid constituents of tobacco cells.

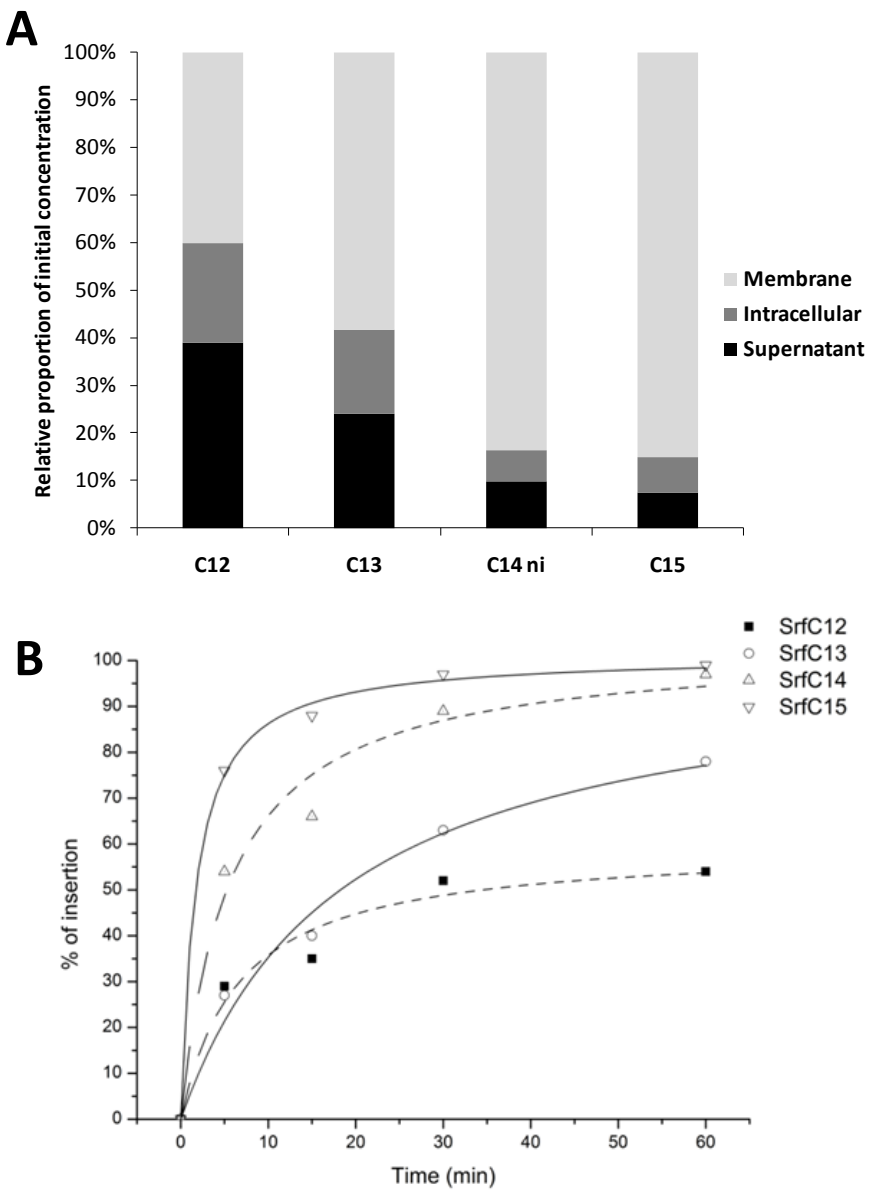


Figure 26 : A. Relative abundance (% of the initial concentration) of the various surfactin homologues in supernatant, intracellular and cell membrane fractions recovered 3h after their addition (5 µM in supernatant) to a tobacco cell suspension. B. Kinetics of insertion of various surfactin homologues within tobacco cells based on decrease of the concentration (initial 5 µM) measured in the supernatant. In both cases, data presented are means calculated from two independent experiments yielding similar results.

By contrast, the binding kinetic of inactive isoforms or derivative (linearized and methylated) is significantly slower and they remain mostly in the supernatant fraction (data not shown) suggesting a much lower affinity for the plant cell membrane. So the differential ROS-inducing activity among the various homologues and derivatives of surfactin is also reflected by differences in their level of interaction with tobacco cells. In all cases, lipopeptide amounts recovered from the intracellular pool are very low indicating that these compounds do not readily penetrate through the tobacco cell wall. Similar kinetic and partitioning trends were obtained by using intact roots of tobacco plantlets instead of cell suspension cultures. The surfactin-induced oxidative burst is transient while binding of the lipopeptide to the cell membrane is long-lasting, as extraction performed five days later (on both cells and roots) allowed to recover most of the initial amounts. In all instances, the initial amount of surfactin added to cells or roots was fully recovered at the end of the experiment by

summing supernatant, intracellular and membrane pools. Moreover, surfactin was extracted from membranes in its intact form as revealed by unmodified molecular masses observed in HPLC-MS. It suggests that transiency of the oxidative burst response does not result from lipopeptide degradation at the membrane level.

Finally, there is no significant change in insertion rate of the purified C14 homologue after pre-treatment of cells with proteases (trypsin and pronase), added to inactivate putative plasma-membrane associated proteic binding site or after heating at 80°C. Similar results were obtained on tobacco protoplasts generated by treating tobacco cells with cell-wall degrading enzymes cocktails (data not shown). This suggests that the lipid part of the plasma membrane is, if not the binding site that directly triggers the observed early events, the site in which active cLPs preferentially insert.

Chapter 10. Interaction of surfactin with model vesicles

In order to get further insights into the nature of the interaction between surfactin and plant cell membranes, ITC experiments were performed using large unilamellar vesicles (LUV). Binding isotherms of lipopeptide-lipid interactions were determined by calorimetric measurements upon titration of lipid dispersions into peptide solutions. Briefly, the technique consists in measuring heat variation in the LUV containing measure cell after each injection of the lipopeptide. The heat deviation is characteristic of the kind of interaction and its intensity and integration of isotherms could give binding parameters.

10.1. Thermodynamic parameters of the binding

We first compared binding of surfactin to palmitoyl oleoyl phosphatidyl choline (POPC) and palmitoyl linoleoyl phosphatidyl choline (PLPC), the predominant phospholipids of mammalian and plant membranes, respectively. Parameters obtained from the fitting of cumulative reaction heats as a function of lipid concentration demonstrate that surfactin interacts spontaneously($\Delta G_D^{w\to b} < 0$) with both types of vesicles in an endothermic ($\Delta H_D^{w\to b}$>0) and entropy-driven ($\Delta S_D^{w\to b} > 0$) process (Figure 27A and Tables 6-8). Based on the affinity constant (K) (Figure 27A) the binding of surfactin to POPC is only slightly lower than to PLPC (Table 6).

In the aim to approach the *in vivo* lipid environment encountered by surfactin at the plasma membrane level of tobacco cells, ITC-based thermodynamic studies were conducted on lipid vesicles mimicking the plasma membrane of root cells (phosphatidyl choline/phosphatidyl ethanolamine/stigmasterol/sitosterol/glucosylceramide; 40/30/8/5/13 in %). Our results also confirmed a clearly different behaviour between long bioactive and shorter inactive surfactin homologues (Figure 27B). Membrane partitioning for both C13 and C14 forms tested individually is basically endothermic and entropy-driven, as observed by using the surfactin mix sample. However, based on K values, the affinity of the C14 homologue for root cell membrane model is 4 times higher than the one calculated for C13 (Table 7).

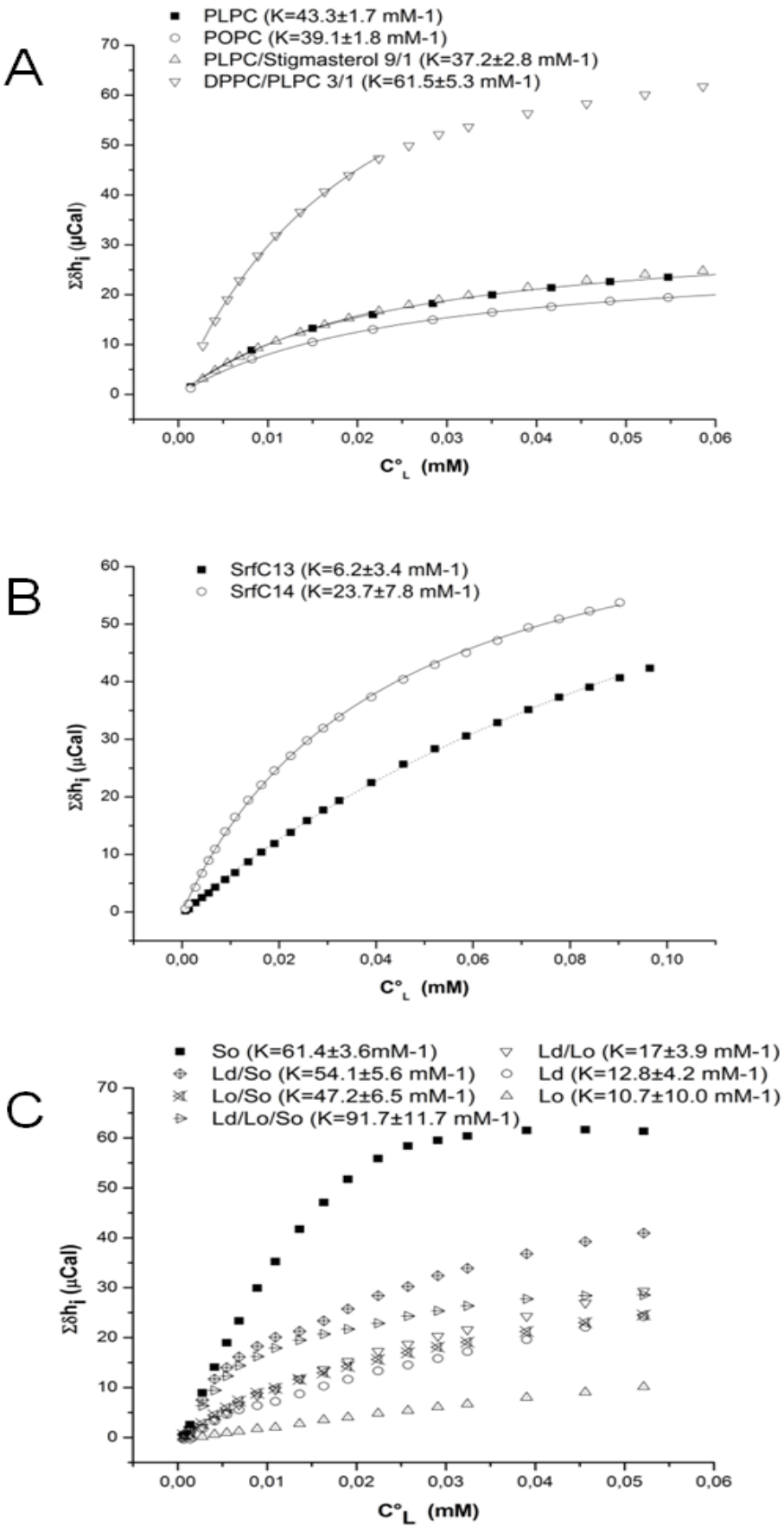


Figure 27 : (A) Titration of 10 µM surfactin mix with 1 mM vesicles of different composition, derived from ITC measurements at 25°C. (B) Titration of 10 µM surfactin C13 and C14 homologues with 1 mM vesicles mimicking plant root plasma membrane composition from ITC measurements at 23°C. (C) Titration of 10 µM surfactin mix with 1 mM vesicles with various lipid phases composition from ITC measurements at 23°C. So: solid ordered phase; Lo: liquid ordered phase; Ld: liquid disordered phase. The solid line corresponds to mean theoretical fits from three independent experiments and thermodynamic parameters of membrane binding are calculated according to the cumulative heat model (Heerklotz *et al.*, 2001, Felix *et al.*, 1999).

Table 6 : Thermodynamic parameters for membrane binding of surfactin mix at 25°C on 1 mM large unilamellar vesicles.

Lipid composition	K (mM^{-1})	ΔH (Kcal/mol)	ΔG (Kcal/mol)	TΔS (Kcal/mol)
POPC 1mM	39.1±1.8	1.9±0.1	-8.6±0.1	10.5±4.2
PLPC 1mM	43.1±1.7	2.3±0.1	-8.7±0.0	11.0±0.1
PLPC/Stigmasterol 9/1 1mM	37.2±2.8	2.5±0.1	-8.6±0.1	11.1±3.8
POPC/DPPC 1/3 1mM	87.8±5.1	5.0±0.1	-9.1±0.0	14.5±0.0

Table 7 : Thermodynamic parameters for membrane binding of two surfactin homologues at 23°C on 1 mM large unilamellar vesicles mimicking the plant plasma membrane composition.

Homologues	K (mM^{-1})	ΔH (Kcal/mol)	ΔG (Kcal/mol)	TΔS (Kcal/mol)
Srf C13	6.2±3.4	7.8±3.8	-7.5±0.3	15.3±3.7
Srf C14	23.7±7.8	5.4±1.3	-8.3±0.2	13.7±8.0

Table 8 : Thermodynamic parameters for membrane binding of surfactin mix at 23°C on 1 mM large unilamellar vesicles with various lipid phases composition.

Lipid composition	Phase	K (mM^{-1})	ΔH (Kcal/mol)	ΔG (Kcal/mol)	TΔS (Kcal/mol)
POPC/PSM 0.1/0.9	So	61.4±0.1	6.1±0.1	-8.8±0.1	-13.6±0.2
POPC	Ld	12.8±4.2	4.1±0.9	-7.9±0.1	-11.4±1.0
POPC/Chol 0.45/0.55	Lo	10.7±10.0	1.8±1.2	-7.8±0.3	-8.6±1.5
POPC/PSM/Chol 0.5/0.25/0.25	Ld+Lo	17.0±3.9	4.2±0.6	-8.1±0.2	-11.5±0.8
POPC/PSM/Chol 0.2/0.5/0.3	Lo+So	47.2±6.5	3.7±0.2	-8.8±0.1	-10.9±0.3
POPC/PSM 0.6/0.4	Ld+So	54.1±5.6	2.6±0.3	-8.7±0.1	-9.6±0.4
POPC/PSM/Chol 0.4/0.5/0.1	Ld+Lo +So	91.7±11.7	2.4±0.1	-9.1±0.2	-9.3±0.3

10.2. Preferential interaction of surfactin with specific lipid organization

In the context of lipid domains formation, the most important phase transition in lipid bilayers under equilibrium conditions is the so-called main transition. It corresponds to the change of the membrane from a solid phase with conformationally ordered lipid acyl chains (solid ordered, So) to a liquid phase with conformationally disordered lipid acyl chains (liquid disordered, Ld) (Simons *et al.*, 2004). In the presence of sterols, a third liquid-ordered phase (Lo) has been shown. All these phases may also coexist in some ternary lipid mixtures (De Almeida *et al.*, 2003).

Interestingly, a much higher binding affinity (based on K values) of surfactin on LUVs was observed by using a mixture of PLPC with a high melting temperature lipid, dipalmitoyl phosphatidyl choline (DPPC) (Figure 27A, Tables 6-8), which are thought to form coexisting Ld and So phases. Some lipopeptides other than surfactin strongly interact with sterols, forming destabilizing complexes within the cytoplasmic membrane of target cells (Maget-dana *et al.*, 1994). The influence of sterols was thus tested by adding stigmasterol (the main representative of plant membrane sterols) in appropriate proportions within LUV lipids, but no significant effect on surfactin binding was observed (Figure 27A).

In order to investigate the mode of interaction of surfactin with different patterns of lipid domains, we used a ternary system POPC/sphingomyelin/cholesterol to generate different phases: liquid ordered (Lo), liquid disordered (Ld), solid ordered (So) or the coexistence of phases (De Almeida *et al.*, 2003). Our results show that association of surfactin with the bilayers is favoured for LUVs containing So domains (Figure 27C). Affinity for other coexisting domains (Lo+So< Ld+So< So< Ld+Lo+So) decreases even if binding is still entropy-driven and endothermic. Binding to “raft like” domains (Lo+Ld) is low in comparison to domains containing So phase.

These observations are reinforced by results obtained by computer simulation of surfactin insertion within lipid mixtures. The theoretical approach used allows predicting the behavior of a molecule in the presence of a lipid monolayer composed of one or more lipid components. Briefly, the energies of all molecular paired interactions are calculated for a huge number of positions, an interaction matrix is created and then used to assemble 200x200 molecules. The system is minimized by a Monte Carlo procedure. Calculations show that surfactin has a preferential location at the boundary between both lipid phases POPC and DPPC (Figure 28A) or sphingomyelin (Figure 28F) and to interact with itself, rather to disperse in one or other lipid domain (Figure 28C). This is consistent with results obtained for DOPC/DPPC/surfactin systems in AFM (Brasseur *et al.*, 2008). The concomitant presence of cholesterol and surfactin reduces the size of each domain, the lipid phase

separation being then less obvious (compare Figures 28A and D), which could be correlated to the lowest affinity of surfactin for POPC/cholesterol vesicles as previously observed. Furthermore, we predict from the calculations that surfactin has a cholesterol-like behavior, since cholesterol and surfactin locate in a very similar way at the interface between lipid domains in POPC/sphingomyelin (compare Figures 28E and F) or POPC/DPPC systems (compare Figures 28A and B).

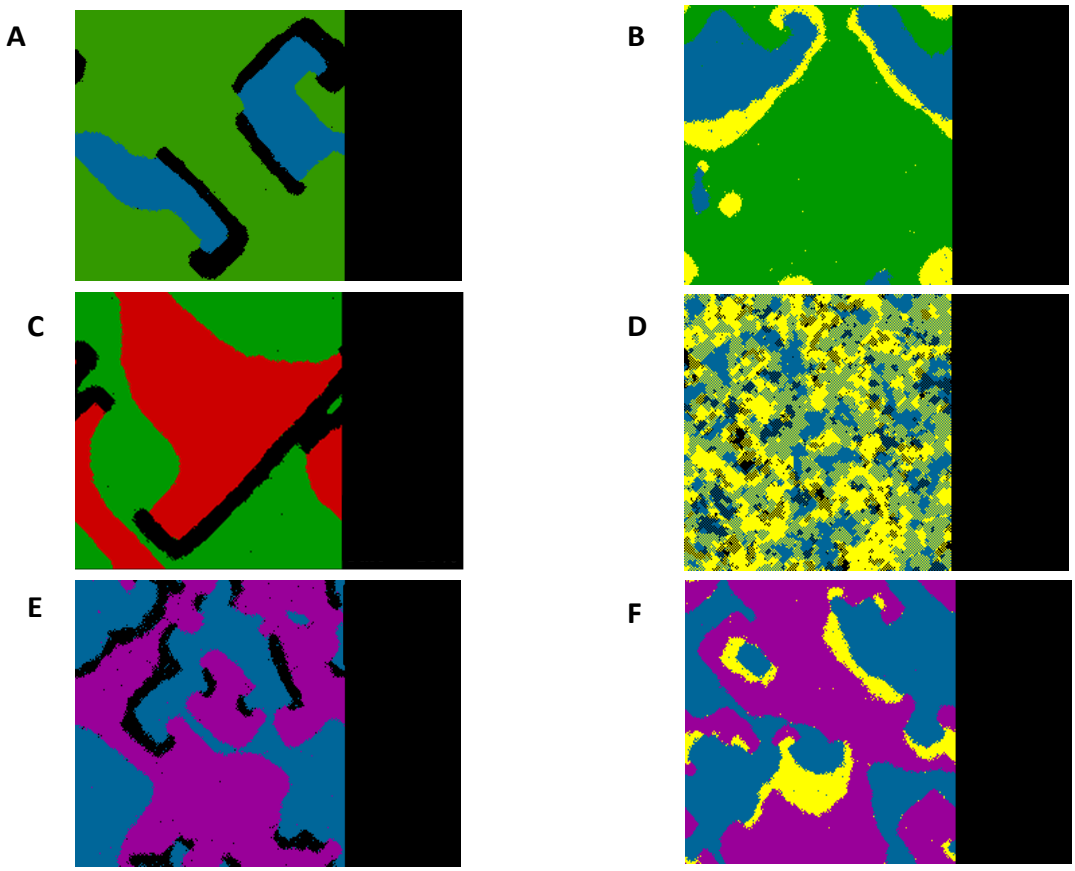


Figure 28 : monolayer grid of 200X200 lipids calculated by the BM theoretical procedure (see material and methods). Each pixel represents a molecule. Blue: POPC; yellow: cholesterol (chol); green: DPPC; red: DOPC; mauve: SPM; black: surfactin (srf)

A. **Monolayer of DPPC/POPC/srf at 4.5/4.5/1.0 molar ratio**
B. **Monolayer of DPPC/POPC/chol at 4.5/4.5/1.0 molar ratio**
C. **Monolayer of DPPC/DOPC/srf at 4.5/4.5/1.0 molar ratio**
D. **Monolayer of POPC/Chol/srf at 4.5/4.5/1.0 molar ratio**
E. **Monolayer of POPC/SPM/srf at 4.5/4.5/1.0 molar ratio**
F. **Monolayer of POPC/SPM/chol at 4.5/4.5/1.0 molar ratio**

Chapter 11. Modification of DRM-associated proteome following interaction with surfactin

As mentioned previously in chapter 5, the various types of lipids forming the PM are not uniformly distributed inside the bilayer but rather spatially organized. PM may be thus considered as spatially highly organized and dynamic platforms which could be disturbed by stresses and compounds having affinity for their lipid structure. Proteins are believed to dynamically associate with membrane microdomains allowing the formation of protein complexes and synchronization efficiency and specificity of cellular responses (Simons *et al.*, 2000).

As previously shown, early responses induced within seconds to minutes after surfactin perception depend on protein phosphorylation events. No specific perception system has been characterized for surfactin but we demonstrated a specific affinity with lipid bilayers and some lipid organizations. This could lead to a reorganization of PM proteins leading to response cascades. As the direct interaction of surfactin with PM proteins is not measurable with classical binding experiments due to steric effects, we looked for an alternative way to investigate their interaction with the non-lipid part of PM. We wanted therefore to study possible early protein dynamics in the plant PM in response to surfactin. Surfactin perception involves lipid interaction and induces a broad array of early responses. Therefore the proteins implicated in these responses may be activated by a dynamic association within specific subdomains of the whole plasma membrane after the interaction of surfactin with some lipid environments.

11.1. Tobacco plasma membrane microdomains extraction

The structural characterization of these specialized PM microdomains renders them theoretically resistant to solubilization by non-ionic detergents, leading to the isolation of detergent resistant membranes (DRMs). While DRM isolation in itself is artifactual and does not accurately reflect pre-existing raft microdomains formation in cell membranes, it remains a powerful first step method for assigning potential membrane raft association and its most meaningful application is achieved if there is differential DRM association of a protein before and after a stimulus, thereby linking it to a biological phenomenon (Lingwood *et al.*, 2007).

We realized a first subcellular fractionation of tobacco BY-2 cells by preparing a highly purified PM fraction. This fraction was submitted to a further step of fractionation by treatment with the non-ionic detergent Triton X-100 to isolate DRMs. The procedure of extraction of PM and DRM from cultured tobacco BY-2 cells is schematized in Figure 36 in the material and methods section. PM-enriched fractions were obtained by differential centrifugation and aqueous polymer two-phase

partitioning as described in the work of Santoni (Santoni, 2007) to obtain highly enriched PM fraction. We confirmed the purity of the isolated PM by measuring the activity of ATPases from PM and two other cell compartments. The activity of the PM ATPase at pH 6·7 was strongly inhibited by the PM-ATPase inhibitor vanadate (62% inhibition at 0.5mM) while an inhibition of 3% and 7.5% was induced by the mitochondria-ATPases inhibitor (sodium azide at 0.5mM) and the tonoplast-ATPases inhibitor (sodium nitrate at 50mM), respectively. These results indicate a low contamination by membranes of these other cell compartments. The absence of the mitochondrial voltage-dependent chloride channel protein (CLC-Nt1, uniprot accession number Q40485) and cytochrome *c* oxidase in our proteome analysis (see below), also supports the lack of contamination by endomembranes (Lurin *et al.*, 2000).

The differential quantitative analysis of PM and DRM proteins was then performed by 2D UPLC-MS/MS using the statistical software developed by Waters. Statistical analysis includes identification of the peptides and proteins and their relative quantification on the base of co-analysis of a second internal standard composed of several proteins digests, present in both samples to be compared at different abundance ratio. The classical 2D electrophoresis step was replaced by two C18 UPLC performed at different pH. As no labeling step was required, possible bias due to efficiency of labeling chemical reaction and loss of sample are eliminated. All the proteins present at concentration within the detection range are moreover available for analysis and not solely those which are labeled. Proteins were retrieved from the National Center for Biotechnology Information (NCBI) database (version September 2011) using *Nicotiana tabacum* keyword. This led to the identification, in BY-2 cell DRM, of 335 proteins whose functional and physicochemical characteristics are further discussed. 102 proteins listed in Table 9 were found more abundantly in the DRM fraction (Figure 29). Some of these proteins were previously characterized as specific DRM proteins of *Nicotiana tabacum* (Morel *et al.*, 2006), confirming that our fractionation method was suitable to further analysis of DRM proteome after surfactin stimulation.

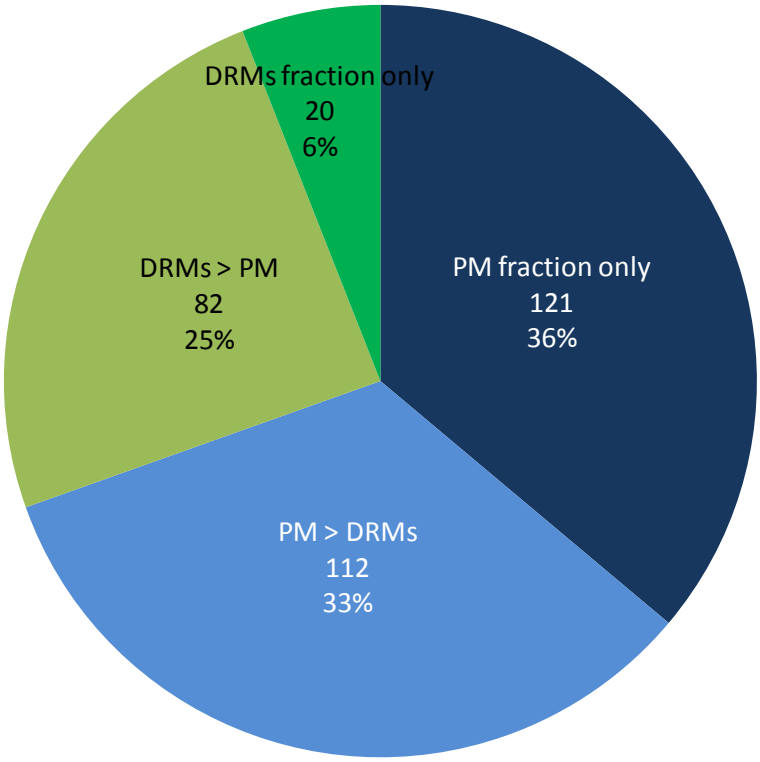


Figure 29: Number and proportion of proteins identified by 2D LC-MS/MS in the whole plasma membrane of tobacco cells and in lipid microdomains resistant to detergent (DRM) isolated from the plasma membrane.

Table 9 : Inventory of proteins associated with tobacco PM DRM. Proteins are ranked from slightest enriched in DRM fraction until those that were only present in DRM fraction. Acc. No., accession number in Uniprot database. DRM/PM: ratio of normalized abundance in DRM and PM, respectively;

Acc. No.	Protein name	DRM /PM
P93371	Actin 93 Fragment OS Nicotiana tabacum PE 3 SV 1	1,010
P93375	Actin 104 Fragment OS Nicotiana tabacum PE 3 SV 1	1,041
C7ENV6	Actin Fragment OS Nicotiana tabacum PE 2 SV 1	1,073
Q05214	Actin OS Nicotiana tabacum PE 3 SV 1	1,073
B5M4V6	Actin OS Nicotiana tabacum PE 3 SV 1	1,073
C7F8N2	Actin Fragment OS Nicotiana tabacum PE 3 SV 1	1,083
P93373	Actin 54 Fragment OS Nicotiana tabacum PE 3 SV 1	1,083
Q8H1T6	Phospholipase D beta 1 isoform Fragment OS Nicotiana tabacum PE 2 SV 1	1,094
P93372	Actin 66 Fragment OS Nicotiana tabacum PE 3 SV 1	1,105
Q6F4H4	Actin OS Nicotiana tabacum PE 2 SV 1	1,105
Q43801	Inorganic pyrophosphatase OS Nicotiana tabacum PE 2 SV 1	1,116
Q40495	N ethylmaleimide sensitive fusion protein OS Nicotiana tabacum GN NtNSF 1 PE 2 SV 1	1,127
Q8GTJ3	Phospholipase D beta 1 isoform Fragment OS Nicotiana tabacum PE 4 SV 1	1,150
Q07761	60S ribosomal protein L23a OS Nicotiana tabacum GN RPL23A PE 2 SV 1	1,174
D9IP68	Pto like protein Fragment OS Nicotiana tabacum PE 2 SV 1	1,185
Q6TKQ9	Ribosomal protein L3B OS Nicotiana tabacum GN RPL3B PE 2 SV 1	1,197
B5TWD2	Calcium dependent protein kinase CDPK5 OS Nicotiana tabacum GN CDPK5 PE 2 SV 1	1,197

Q43797	Inorganic pyrophosphatase OS Nicotiana tabacum GN ppa PE 2 SV 1	1,246
P41918	GTP binding nuclear protein Ran A1 OS Nicotiana tabacum GN RAN A1 PE 2 SV 1	1,246
Q40487	Cationic peroxidase isozyme 40K OS Nicotiana tabacum PE 2 SV 1	1,271
A1XEL2	CYP73A47v3 OS Nicotiana tabacum PE 2 SV 1	1,284
A1XEL1	CYP73A47v1 OS Nicotiana tabacum PE 2 SV 1	1,323
Q07760	60S ribosomal protein L23 OS Nicotiana tabacum GN RPL23 PE 2 SV 1	1,336
Q40462	NTGB1 Fragment OS Nicotiana tabacum PE 2 SV 1	1,336
P41919	GTP binding nuclear protein Ran B1 OS Nicotiana tabacum GN RAN B1 PE 2 SV 1	1,350
Q8VXD0	Alpha tubulin OS Nicotiana tabacum GN tubA2 PE 2 SV 1	1,350
A1XEL3	CYP73A47v2 OS Nicotiana tabacum PE 2 SV 1	1,419
Q9FSF2	Putative DNAJ protein OS Nicotiana tabacum GN dnaJ PE 2 SV 1	1,433
Q8VXC9	Alpha tubulin OS Nicotiana tabacum GN tubA3 PE 2 SV 1	1,448
Q40522	Ras related protein Rab11D OS Nicotiana tabacum GN RAB11D PE 2 SV 1	1,477
Q285L8	40S ribosomal protein S3a OS Nicotiana tabacum GN cyc07 PE 2 SV 1	1,492
Q40520	Ras related protein Rab11C OS Nicotiana tabacum GN RAB11C PE 2 SV 1	1,492
Q6TKR0	Ribosomal protein L3A OS Nicotiana tabacum GN RPL3A PE 2 SV 1	1,507
Q9ZRE0	NTFP2 Fragment OS Nicotiana tabacum PE 2 SV 1	1,537
Q76BK8	PERK1 like protein kinase Fragment OS Nicotiana tabacum GN NtPERK1 PE 2 SV 1	1,537
P43643	Elongation factor 1 alpha OS Nicotiana tabacum PE 2 SV 1	1,553
P93377	40S ribosomal protein S14 Fragment OS Nicotiana tabacum GN RPS14 PE 3 SV 1	1,568

E2ESP4	Cytosolic class I small heat shock protein 3B OS Nicotiana tabacum GN sHSP3B PE 3 SV 1	1,584
A1IGC6	PERK1 like protein kinase OS Nicotiana tabacum GN NtPERK1 PE 2 SV 1	1,682
Q76CU2	Pleiotropic drug resistance protein 1 OS Nicotiana tabacum GN PDR1 PE 2 SV 1	1,716
Q9FRU1	Alpha tubulin OS Nicotiana tabacum GN BYtuba PE 2 SV 1	1,840
O81390	Calcium dependent protein kinase OS Nicotiana tabacum GN CDPK1 PE 2 SV 1	1,878
Q76CU1	PDR type ABC transporter 2 Fragment OS Nicotiana tabacum GN NtPDR2 PE 2 SV 1	1,896
A0A9R6	Histone H4 OS Nicotiana tabacum GN NtH4 PE 3 SV 1	1,916
A8J6V0	Histone H2B OS Nicotiana tabacum GN NtH2B2 PE 2 SV 1	1,935
P49627	60S ribosomal protein L13 OS Nicotiana tabacum GN RPL13 PE 2 SV 1	1,994
Q9M7A8	LRR receptor like protein kinase OS Nicotiana tabacum PE 2 SV 1	2,014
Q67BD2	Heat shock protein 70 1 OS Nicotiana tabacum GN HSP70 1 PE 3 SV 1	2,096
Q9ZSD5	Syntaxin related protein Nt syr1 OS Nicotiana tabacum GN Nt Syr1 PE 2 SV 1	2,096
Q9M663	Harpin inducing protein OS Nicotiana tabacum GN hin1 PE 2 SV 1	2,117
Q9M5Z8	Vacuolar H ATPase B subunit OS Nicotiana tabacum PE 2 SV 1	2,160
Q40468	Eukaryotic initiation factor 4A 15 OS Nicotiana tabacum PE 2 SV 1	2,181
B7X6S6	Secretory carrier associated membrane protein 2 OS Nicotiana tabacum GN NtSCAMP2 PE 2 SV 1	2,316
Q40595	Aquaporin OS Nicotiana tabacum GN NT2 PE 2 SV 1	2,340
O82702	V type proton ATPase subunit G 1 OS Nicotiana tabacum GN VATG1 PE 3 SV 1	2,363
C0STW5	NtRab11D OS Nicotiana tabacum GN NtRab11D PE 2 SV 1	2,509
P93374	Actin 53 Fragment OS Nicotiana tabacum PE 3 SV 1	2,535

Q6E2W3	ATP synthase subunit alpha Fragment OS Nicotiana tabacum GN atp1 PE 3 SV 1	2,638
Q8W561	Calcium calmodulin dependent protein kinase CaMK1 OS Nicotiana tabacum PE 2 SV 1	2,718
Q5M9V4	ATP synthase subunit alpha OS Nicotiana tabacum GN atp1 PE 3 SV 2	2,915
Q6TXM4	Proton P ATPase OS Nicotiana tabacum GN nha1 PE 2 SV 2	2,974
Q8GT44	Putative rac protein OS Nicotiana tabacum GN rac4 PE 2 SV 1	3,004
Q9SQI4	Centrin OS Nicotiana tabacum GN CEN2 PE 2 SV 1	3,127
Q8W507	Aquaporin OS Nicotiana tabacum GN NtPIP1 1 PE 2 SV 1	3,158
O24662	Aquaporin 1 OS Nicotiana tabacum PE 2 SV 1	3,158
Q06BK4	Water channel protein OS Nicotiana tabacum PE 2 SV 1	3,158
Q9SDQ5	Small GTP binding protein Sar1BNt OS Nicotiana tabacum PE 2 SV 1	3,222
Q8RVJ9	NADPH oxidase NtrbohD OS Nicotiana tabacum GN rboh PE 2 SV 1	3,320
P93769	Elongation factor 1 alpha OS Nicotiana tabacum GN tel1 PE 2 SV 1	3,320
Q8LRN5	NADPH oxidase OS Nicotiana tabacum GN rbohD PE 2 SV 1	3,525
P93353	Hin1 protein OS Nicotiana tabacum GN hin1 PE 2 SV 1	3,561
Q9S820	NTGP2 OS Nicotiana tabacum GN rac PE 2 SV 1	3,669
Q762F4	14 3 3 protein OS Nicotiana tabacum GN Nt14 3 3epsilon PE 3 SV 1	3,706
Q9SMB6	Phragmoplastin OS Nicotiana tabacum GN phragmoplastin PE 2 SV 1	3,743
Q93X84	Rac like GTPase 1 OS Nicotiana tabacum PE 2 SV 1	3,743
O24138	NtSar1 protein OS Nicotiana tabacum GN NtSAR1 PE 2 SV 1	3,743
Q9ZRD2	NTGP3 OS Nicotiana tabacum GN rac2 PE 2 SV 1	3,896

P52885	GTP binding protein SAR1 OS Nicotiana tabacum GN SAR1 PE 2 SV 1	3,975
O24142	Rop subfamily GTPase OS Nicotiana tabacum GN rop1 PE 2 SV 2	4,015
Q9LLS5	Inorganic phosphate transporter OS Nicotiana tabacum GN PT1 PE 2 SV 1	4,437
Q41246	14 3 3 like protein OS Nicotiana tabacum PE 2 SV 1	5,155
P25998	60S ribosomal protein L8 OS Nicotiana tabacum GN RPL8 PE 2 SV 1	5,312
Q9XG71	Putative coat protein OS Nicotiana tabacum PE 4 SV 1	DRM only
Q40590	TFHP 1 protein OS Nicotiana tabacum PE 2 SV 1	DRM only
Q93YE4	Putative coat protein OS Nicotiana tabacum PE 4 SV 1	DRM only
B7X6S8	Syntaxin OS Nicotiana tabacum GN NtSYP22 PE 2 SV 1	DRM only
Q25C91	Tobacco fibrillarin homolog OS Nicotiana tabacum GN NtFib1 PE 2 SV 1	DRM only
Q6I692	Isochorismate synthase Fragment OS Nicotiana tabacum GN ics PE 2 SV 1	DRM only
O50038	Short chain alcohol dehydrogenase OS Nicotiana tabacum GN SCANT PE 2 SV 1	DRM only
Q56AY3	Calcium dependent protein kinase OS Nicotiana tabacum GN CPK5 PE 2 SV 1	DRM only
A0T3D8	PT3 OS Nicotiana tabacum GN PT3 PE 2 SV 2	DRM only
Q9ST22	Phosphate transporter OS Nicotiana tabacum PE 2 SV 1	DRM only
Q8W506	Aquaporin OS Nicotiana tabacum GN NtPIP2 1 PE 2 SV 1	DRM only
Q8W559	Calcium calmodulin dependent protein kinase CaMK3 OS Nicotiana tabacum PE 2 SV 1	DRM only
Q40543	Protein serine threonine kinase OS Nicotiana tabacum PE 2 SV 1	DRM only
Q40464	NTGB3 Fragment OS Nicotiana tabacum PE 2 SV 1	DRM only
Q9ZRE1	NTFP1 Fragment OS Nicotiana tabacum PE 2 SV 1	DRM only

P29687	Ras related protein Rab5 OS Nicotiana tabacum GN RAB5 PE 2 SV 1	DRM only
Q9AYT3	Phosphate transporter OS Nicotiana tabacum GN NtPT2 PE 2 SV 1	DRM only
Q9XLX8	F1 ATP synthase subunit alpha Fragment OS Nicotiana tabacum GN atp1 PE 4 SV 1	DRM only
Q40570	Ras related GTP binding protein OS Nicotiana tabacum PE 2 SV 1	DRM only
Q5MJW5	Avr9 Cf 9 rapidly elicited protein 76 Fragment OS Nicotiana tabacum GN ACRE76 PE 2 SV 1	DRM only

The most representative integral protein of the plant PM, the P-type H^+-ATPase, was identified in our tobacco DRM fraction. This result is in agreement with previous analysis indicating an enrichment of this protein in tobacco DRM (Mongrand *et al.*, 2004, Borner *et al.*, 2005).
As expected, several members of the aquaporin family (PM intrinsic proteins PIPs) are also present in DRMs (Morel *et al.*, 2006).
The proportion of proteins involved in signaling is significantly higher in the DRM fraction than in the whole PM, in agreement with results obtained on the same plant model (Morel *et al.*, 2006). A significant enrichment of signaling proteins such as calcium dependent protein kinase, calcium calmodulin-dependent protein kinase and small GTP-binding proteins was observed. The leucine rich repeat (LRR)-containing receptor kinase, a member of the receptor-like kinase (RLK) family, is also more abundant in DRM fraction than in the whole PM. LLR RLKs play roles in diverse processes such as growth and development, setting up of symbiosis, or plant pathogen recognition (Dievart *et al.*, 2004). In addition to signaling proteins, the NADPH oxidase NtrbohD, known to be involved in plant responses to the fungal elicitor cryptogein and previously detected in tobacco DRM (Stanislas *et al.*, 2009), the phospholipase D isoform β, a lipid-utilizing enzyme whose function is clearly established in plant signaling (Wang, 2002), as well as various proteins implicated in intracellular trafficking, metabolism or related to cell wall, are abundant in our DRM fraction.

11.2. Differential DRM association of proteins before and after surfactin stimulation

Plant DRMs could be involved in the regulation of physiological functions due to the enrichment in these domains of proteins implicated in signaling and responses to biotic/abiotic stresses (Simon-Plas *et al.*, 2011). The incorporation of other proteins in these specialized microdomains may thus lead to the formation of multiproteins complexes and modulate the activity of these enzymes. As no high affinity binding site to surfactin has been characterized so far, and since their high affinity for some lipid organizations, it was therefore particularly biologically relevant to investigate if their incorporation inside the lipid bilayer could promote the trafficking of proteins. The incorporation of proteins into specific membrane microdomains and the formation of multiproteins complexes may lead to the triggering of early events previously observed upon surfactin stimulation.
To reveal surfactin-induced protein dynamics at the PM of tobacco cells, we performed a quantitative proteomic analysis of DRM in a time-course experiment. Since early defense responses occur within 2 to 20 min after surfactin elicitation, surfactin-induced membrane protein compartmentalization is expected to occur within or even prior to this time frame, while changes in

protein *de novo* biosynthesis are excluded at this early time points. We stimulated tobacco cell cultures with 10 µM surfactin or control treatment and collected samples after 15 minutes. PM fraction of treated (DRMS) and control (DRMC) tobacco cells were extracted by two-phase partitioning and subsequently isolated DRMs by Triton X-100 treatment. The DRM proteome was then analyzed similarly than previously, and ratios of the protein abundance in control (DRMC) on protein abundance in treated (DRMS) cells were calculated. Differential quantitative relative analysis was performed using the 3 biological replicates per sample type (3 DRMC and 3 DRMS), to reach statistical significance. The differential analysis was done by selecting significantly differentially distributed proteins within DRMC and DRMS with p values (associated to the difference of LogE ratio) ≤ 0.05 (indication lower abundance) or $\geq$ to 0.95 (indicating higher abundance).

We first controlled the ratio obtained with the 4 protein digests spiked: MPDS mix. We used data normalization on the ADH (P00330) of MPDS mix (expected ratio = 1/1) and calculated the pair wise comparisons. The max percentage of deviation observed is of 11% on the GPB protein (P00489), which is acceptable.

MPDS mix	**ratio expected E1/E2**	**ratio obtained DRMT/DRMS**	**Deviation (%)**
ADH (P00330)	1	1	0
GPB (P00489)	1.66	1.48	11
ENO (P00924)	0.79	0.88	8.8
BSA (P027769)	0.183	0.18	1.6

Proteins identified, quantified and found significantly differentially distributed in DRMC and in DRMS samples, for 3 replicate over 3, are listed in Table 10. On 73 proteins differentially distributed, 22 are enriched in DRMS samples, 13 are similarly abundant and 38 are depleted. On all proteins identified, only two were not found in the previous PM proteome investigation. Enrichment or depletion factors are ranging from 0.4 to 1.8, except for the 14-3-3 i 2 protein that was only found in DRMC. This is in agreement with factors obtained in other studies in animal (MacLellan *et al.*, 2005, Dhungana *et al.*, 2009) and plant cells (Stanislas *et al.*, 2009). Proteins identified as differentially associated to tobacco DRMs after surfactin challenge are mainly involved in signaling, protein targeting and vesicular trafficking (Figure 30).

A 14-3-3 protein is the most enriched in DRMS. Ubiquitin fragments and luminal binding proteins are other prominently enriched proteins following surfactin stimulation. 14-3-3 proteins could specifically interact with NtrbohD, the tobacco oxidase responsible for ROS production (Simon-

Plas *et al.*, 2002) that was previously demonstrated to be associated with tobacco DRM fraction. Ubiquitination is one regulatory mechanism that serves to target proteins to different pathways (Bonifacino *et al.,* 1998). Monoubiquitination of some cell surface proteins has been shown to act as a sorting determinant for internalization from the plasma membrane (Hicke, 2001). Ubiquitination also signals internalization of membrane proteins into multivesicular bodies (Katzmann *et al.,* 2002). Finally, ubiquitination mediates sorting of select proteins from the secretory pathway to the endosomal/vacuolar pathway in response to nutritional signals (Magasanik *et al.,* 2002, Umebayashi *et al.*, 2003). Luminal binding proteins (BiP) are involved in the folding of proteins and also acts in the endoplasmic reticulum (ER) quality control mechanism that recognizes unfolded or abnormally folded proteins and exports them out of the organelle for degradation (Ma *et al.,* 2004). Overexpression of BiP in tobacco protoplasts (Leborgne-Castel *et al.,* 1999) increases cell tolerance to ER stress, whereby ER stress is defined as an imbalance between the cellular demand for ER function (proper protein folding) and the capacity of the ER to carry out this function. Overexpression of BiP has for example been found to confer tolerance to drought stress in tobacco (Alvim *et al.,* 2001).

Calmodulins and calcium/calmodulin-dependent kinases are among the main proteins with signaling functions that are depleted from DRM following treatment with surfactin. These proteins are implicated in calcium ion binding and transduction of calcium signals by modifying its interaction with target proteins. The kinetics of the transient increase in calcium binding proteins at the plasma membrane coincides with that of transitory calcium-dependent extracellular alkalinization and ROS production, occurring within the first 15 min after elicitation.

Four proteins involved in cell trafficking belonging to the dynamin-related protein family have been shown to undergo a decrease of their relative importance in DRMs 5 minutes following crytpogein elicitation (Stanislas *et al.,* 2009). We observed that phragmoplastin, the tobacco dynamin-related protein, was more present in DRM fraction than in the whole PM. However, its presence is not significantly affected 15 min after surfactin treatment in our experiments.

Table 10: Proteins identified, quantified and found significantly differentially distributed in DRMC and in DRMS samples, for 3 replicates over 3. Proteins are classified from the most enriched to the most depleted in DRM of treated cells. Enriched proteins have a ratio DRMC/DRMS ≤0.8 while protein depleted in DRM after surfactin treatment have a ratio ≥1.2. DRMC: protein abundance in DRM of control cells. DRMS: protein abundance in DRM of surfactin treated cells.

DRMC/DRMS ratio	Acc. No	Protein name	Associated function
Enriched	Q75XU9	14 3 3 f 2 protein Fragment	Signaling (Activate the auto-inhibited plasma membrane P-type H^+-ATPases and implicated in ROS generation)
Enriched	Q76ME6	Calmodulin NtCaM10	Signaling (Calcium ion binding)
Enriched	Q03683	Luminal binding protein 3 Fragment	Other function
Enriched	Q40596	Pentameric polyubiquitin	Other function (Regulate the interaction of proteins)
Enriched	Q40543	Protein serine threonine kinase	Signaling
Enriched	Q76MF4	Calmodulin NtCaM1	Signaling (Calcium ion binding)
Enriched	Q8RVF7	Polyubiquitin like protein Fragment	Other function (Regulate the interaction of proteins)
Enriched	P93379	Ubiquitin	Other function (Regulate the interaction of proteins)
Enriched	O49907	Polyubiquitin	Other function (Regulate the interaction of proteins)
Enriched	Q76MF3	Calmodulin NtCaM11	Signaling (Calcium ion binding)

Enriched	C7ENV4	Polyubiquitin Fragment	Other function (Regulate the interaction of proteins)
Enriched	O49905	Polyubiquitin	Other function (Regulate the interaction of proteins)
Enriched	Q03681	Luminal binding protein 1 Fragment	Other function
Enriched	Q03682	Luminal binding protein 2 Fragment	Other function
Enriched	O49906	Ubiquitin extension protein	Other function
Enriched	Q45FL8	Ubiquitin extension protein	Other function
Enriched	Q03686	Luminal binding protein 8 Fragment	Other function
Enriched	O49998	14 3 3 like protein F	Signaling (Activate the auto-inhibited plasma membrane P-type H^+-ATPases and implicated in ROS generation)
Enriched	B6A8D0	Ubiquitin Fragment	Other function (Regulate the interaction of proteins)
Enriched	Q03685	Actin 53 Fragment	Intracellular trafficking
Enriched	Q03684	Luminal binding protein 5	Other function
Enriched	P93377	Luminal binding protein 4	Other function
Neutral	C7ENV6	Actin Fragment	Intracellular trafficking
Neutral	Q40511	Heat shock protein 70 Fragment	Other function
Neutral	A7LP32	RbohF	Biotic/abiotic stress
Neutral	Q84QJ3	Heat shock protein 70	Other function
Neutral	Q9SQI4	Centrin	Intracellular trafficking

Neutral	Q67BD0	Heat shock protein 70 3	Other function
Neutral	P93376	Actin 103 Fragment	Intracellular trafficking
Neutral	P93373	Actin 54 Fragment	Intracellular trafficking
Neutral	Q6F4H4	Actin	Intracellular trafficking
Neutral	Q6TXM4	Proton P ATPase	Transport
Neutral	Q8RVJ9	NADPH oxidase NtrbohD	Biotic/abiotic stress
Neutral	P93769	Elongation factor 1 alpha	Metabolism-apoptosis
Neutral	D9IP68	Pto like protein Fragment	Biotic/abiotic stress
Depleted	Q6IVK8	ADH like UDP glucose dehydrogenase	Metabolism-apoptosis
Depleted	Q709M1	Protein kinase 1	Signaling
Depleted	Q9XG67	Glyceraldehyde 3 phosphate dehydrogenase	Metabolism-apoptosis
Depleted	P09094	Glyceraldehyde 3 phosphate dehydrogenase cytosolic Fragment	Metabolism-apoptosis
Depleted	Q8W561	Calcium calmodulin dependent protein kinase CaMK1	Signaling (Calcium ion binding)
Depleted	O49911	Plasma membrane polypeptide	Other function
Depleted	Q76CU2	Pleiotropic drug resistance protein 1	Metabolism-apoptosis
Depleted	P43643	Elongation factor 1 alpha	Biotic/abiotic stress
Depleted	Q9M7A8	LRR receptor like protein kinase	Biotic/abiotic stress
Depleted	Q1G0Z1	Putative spindle disassembly related protein CDC48	Transport
Depleted	Q40495	N ethylmaleimide sensitive fusion protein	Transport
Depleted	P41918	GTP binding nuclear protein Ran A1	Transport
Depleted	Q9M663	Harpin inducing protein	Biotic/abiotic stress
Depleted	D9IP70	Pto kinase interactor 1 protein Fragment	Biotic/abiotic stress

Depleted	O24142	Rop subfamily GTPase	Signaling
Depleted	Q9M3U5	DREPP2 protein	Signaling
Depleted	Q9S820	NTGP2	Signaling
Depleted	Q93X84	Rac like GTPase 1	Signaling
Depleted	A1IGC6	PERK1 like protein kinase	Biotic/abiotic stress
Depleted	Q76BK8	PERK1 like protein kinase Fragment	Biotic/abiotic stress
Depleted	Q8VXC9	Alpha tubulin	Intracellular trafficking
Depleted	Q9ZRD2	NTGP3	Other function
Depleted	P41919	GTP binding nuclear protein Ran B1	Other function
Depleted	C7ENV5	Glyceraldehyde 3 phosphate dehydrogenase Fragment	Metabolism-apoptosis
Depleted	Q6L7J8	Harpin inducing protein 1 like 9	Biotic/abiotic stress
Depleted	Q8GT44	Putative rac protein	Signaling
Depleted	Q8GT45	Putative rac protein Fragment	Signaling
Depleted	Q8VXD1	Alpha tubulin	Intracellular trafficking
Depleted	Q9FV95	Cellulose synthase Fragment	Cell-wall related protein
Depleted	Q40569	Ras related GTP binding protein	Signaling
Depleted	Q8VXD0	Alpha tubulin	Intracellular trafficking
Depleted	Q75ZE0	14 3 3 e 1 protein	Signaling (Activate the auto-inhibited plasma membrane P-type H^+-ATPases and implicated in ROS generation)
Depleted	P93353	Hin1 protein	Biotic/abiotic stress
Depleted	Q6L7J7	Harpin inducing protein 1 like 18	Biotic/abiotic stress

Depleted	Q9FRU1	Alpha tubulin	Intracellular trafficking
Depleted	Q40595	Aquaporin	Transport
Depleted	Q84RP9	Putative calcium dependent protein kinase Fragment S	Signaling
Depleted	Q75ZD3	14 3 3 i 2 protein	Signaling (Activate the auto-inhibited plasma membrane P-type H^+-ATPases and implicated in ROS generation)

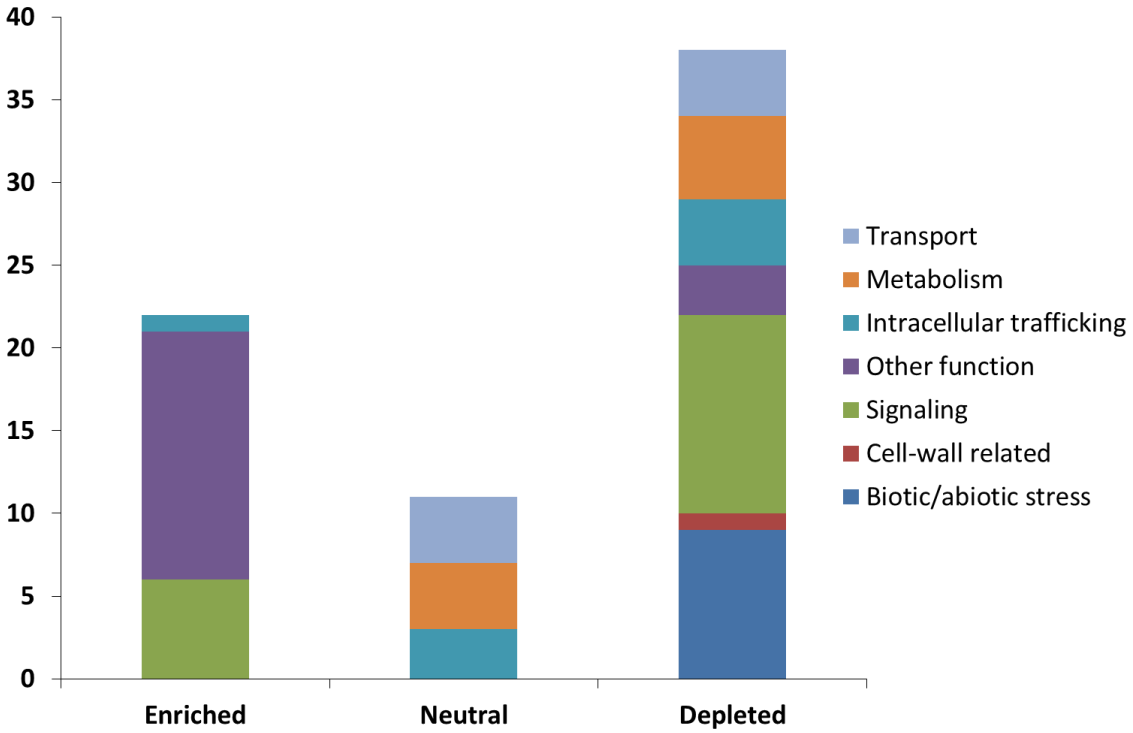


Figure 30 : Amount of proteins that were enriched, depleted or not affected within DRMs 15 minutes following surfactin perception by tobacco cells, classified by their associated function.

Chapter 12. Activity of other ISR elicitors on tobacco cells and on plantlets

Amphiphilic secondary metabolites synthesized by rhizobacteria could be considered as a specific class of ISR elicitors. In the last part of this work, we wanted to compare the activity of surfactin with NABD and rhamnolipids that are other amphiphilic compounds also recently reported as elicitors. NABD (N, N-diméthyl-N-tetradécyl-N-benzylammonium) is produced by *Pseudomonas putida* and was previously shown in our laboratory as an ISR elicitor against *Botrytis cinerea* on both bean and tomato and against *Pythium aphanidermatum* on cucumber (Ongena *et al.*, 2005c, Ongena *et al.*, 2002, Ongena *et al.*, 1999). Rhamnolipids were isolated from *Pseudomonas aeruginosa* and reported to induce resistance against *Botrytis cinerea* on grape (Varnier *et al.*, 2009). The rhamnolipids mixture used contains two types of rhamnolipids: 60% have two rhamnose residues linked to two 10 carbons lipid chains while the remaining 40% only have one rhamnose residue.

12.1. Oxidative burst on tobacco suspension cells

We firstly used tobacco BY-2 cell suspension cultures to compare the activity of these three elicitors for the induction of the oxidative burst. The extracellular hydrogen peroxide accumulation was measured as previously for increasing concentrations of each elicitor dissolved in DMSO (dimethylsulfoxyde). Figure 31 shows that they all induced high responses compared to those obtained by a well-known oxidative burst-inducer, the PAMP laminarin. Surfactin at 20 µM retains an activity nearly two times higher than the one obtained with rhamnolipids and twenty times higher than the one of laminarin. By contrast, NABD only displays low oxidative burst activity. This may suggest than fatty acid chains present in both surfactin and rhamnolipid structures but not in NADB are structural treats having an important potential to stimulate a transient production of ROS by plant cells.

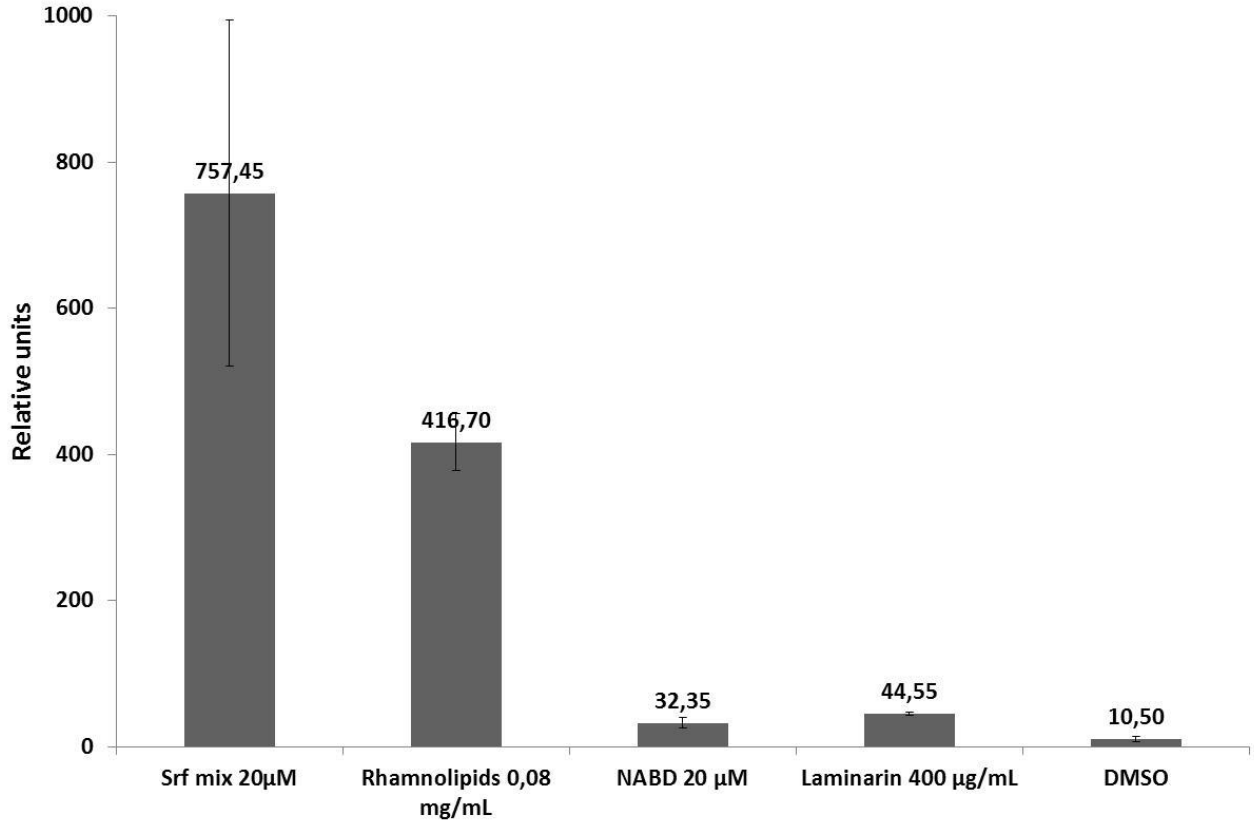


Figure 31 : Oxidative burst (expressed in relative units of chemiluminescence) induced after treatment of tobacco cell suspension cultures with different ISR elicitors at their most active concentration. Laminarin, an oxidative burst-inducer PAMP, was used as reference control while the solvent DMSO was the negative control. Data are means and standard variations are calculated from three independent experiments.

12.2. ISR experiments on tobacco plantlets

Given the results previously obtained with rhamnolipids and NABD, ISR experiments were realized with the same elicitors on tobacco plantlets against the phytopathogen *Botrytis cinerea*. First results are summarized in Figure 32. As all experiments were not done by using the same method, these results should not be compared together but should be considered as a global picture of the disease reduction these amphiphilic elicitors could confer. In all cases, elicitor treatments lead to a significant protection against the pathogen but, as observed for cultured cells, surfactin and rhamnolipids seemingly retain a higher eliciting activity than NABD. Further experiments must be realized to confirm these results.

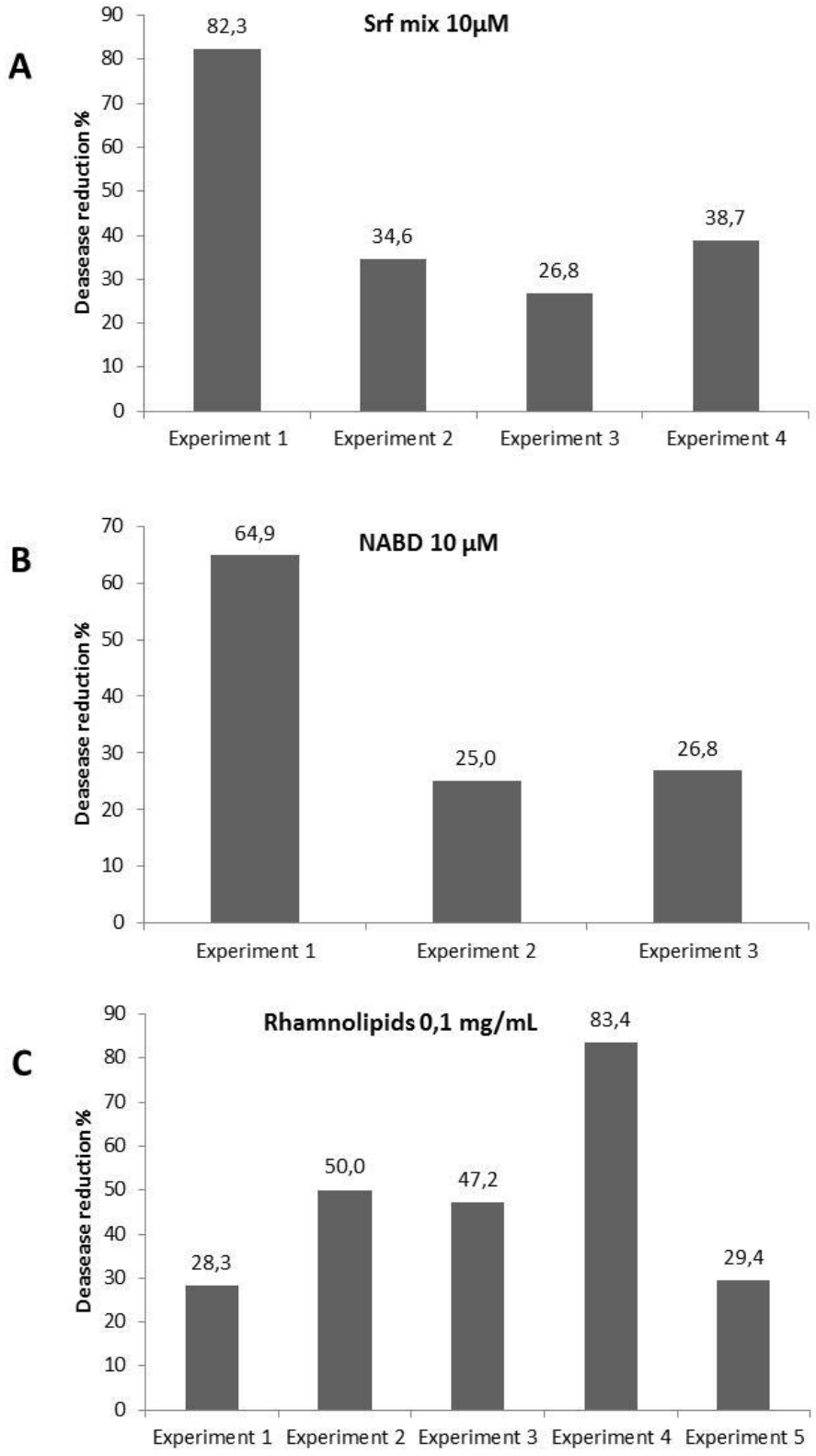


Figure 32: Disease reduction obtained on tobacco plants treated at the root level with ISR elicitors and infected on leaves with the phytopathogen *Botrytis cinerea*. A. Results from 4 repetitions with tobacco plants pretreated with 10 µM surfactin. B. Results from 3 repetitions tobacco plants pretreated with 10 µM NABD. C. Results from 5 repetitions with tobacco plants pretreated with 0.1 mg/mL of rhamnolipids.

Discussion

i. Surfactin as elicitor of ISR: a new biological function for *Bacillus* cLPs in the mutualistic plant-microbe interaction

Due to their strong interaction with biological membranes, cyclic lipopeptides in general and particularly those from *Bacillus* are typically considered as compounds inducing pores and lysis of target cells. Surfactin displays some antiviral (Kracht *et al.*, 1999), antimycoplasma and antibacterial (Huang *et al.*, 2008) activities but evidences for other biological functions of these cLPs have accumulated in the past decade. For instance, a role of surfactin as molecular signal involved in *Bacillus* cell communities' behaviour (quorum sensing, cannibalism, biofilm formation) has been demonstrated (Ongena and Jacques, 2008a). In the context of plant-*Bacillus* interaction, cLPs are clearly implicated in the root colonization process as well as in direct antagonism toward phytopathogens (see chapter 4.3). Results from our laboratory have highlighted in 2007 that they also retain the ability to induce plant immunization. This was demonstrated in bean and tomato by using pure compound and by showing that overexpression of both surfactin and fengycin biosynthetic genes in the naturally poor producer *B. subtilis* strain 168 was associated with a significant increase in the potential of the derivatives to induce resistance (Ongena *et al.*, 2007). Besides, treatment of potato tuber cells with purified fengycin but not surfactin and iturin, resulted, as observed in tobacco, in the accumulation of plant phenolics derived from the phenylpropanoid metabolism (Ongena *et al.*, 2005a, Ongena *et al.*, 2005b). Given these results, it is clear that *Bacillus* cLP families retain differential abilities to stimulate plant defense reactions, suggesting that specific features of the peptide part of these molecules are important for their perception.

Surfactin is the most efficient cLP family from *Bacillus* to stimulate plant immunity. Our results presented in chapter 6 and 7 demonstrate that among the three *Bacillus* cLP, only surfactin family retains consistent ISR-stimulating activity on two additional plant models used to study plant biology: tobacco and *Arabidopsis*. For the latter, the ISR-eliciting potential of surfactin was reinforced by data from recent assays performed in the laboratory (unpublished results). Also coming from recent trials, elicitation of systemic resistance by surfactin has been evidenced in wheat against *B. graminis* (unpublished results) and in sugar beet to control infection by *Polymyxa betae,* the vector of rhizomania disease caused by the *Beet necrotic yellow vein* virus (Dessoignies *et al*., submitted). Results presented in chapter 8 corroborate the elicitor potential of surfactin by showing that it is the main metabolite produced by the strain *B. amyloliquefaciens* S499 responsible

for plant defense response induction in tobacco. As a matter of fact, such a crucial role of surfactin for the ISR triggering potential of *Bacillus* can be extended to other strains as strongly suggested by a comparative study performed with 20 different isolates in the laboratory (Cawoy et al. submitted). Data related to ISR activities on various plants of the different cLPs families produced by *Bacillus* are summarized in Table 11.

Table 11: ISR activity of the three cLP families produced by *Bacillus* strains on different plant species. Besides these data, a strong eliciting activity on tobacco suspension cultures has only been observed with the surfactin producing strain and with the surfactin family. * Statistically different from disease control; n.t.: not tested.

ISR activity				
	***Bacillus amyloliquefaciens* S499**	**Surfactin**	**Fengycin**	**Iturin**
Tomato	**++***	**++***	**+**	**-**
Bean	**++***	**++***	**+**	**-**
Cucumber	**++***	**-**	**-**	**-**
Tobacco	**++***	**++***	**-**	**-**
Arabodopsis	**+**	**+***	**n.t.**	**n.t.**
Wheat	**++***	**++***	**n.t.**	**n.t.**
Sugar beet	**++***	**++***	**n.t.**	**n.t.**

Given their dissimilar properties and biological activities and despite close strucutures and physico-chemical features, each *Bacillus* cLP family likely possesses a privileged role in the mutualisitc interaction between plants and *Bacillus* species (Figure 33).

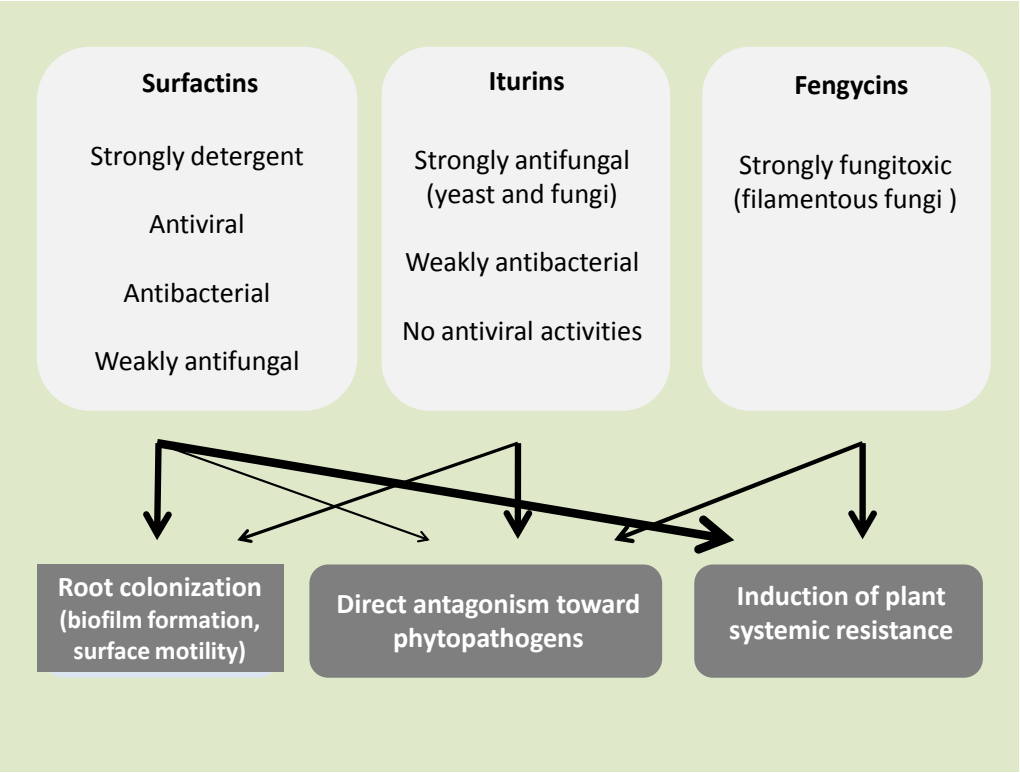


Figure 33 : Different biocontrol-related activities of *Bacillus* cLP families. Each family displays specialized activities implicated in the mutualistic interaction between the rhizobacteria and the host plant. In parallel, synergistic effects have been demonstrated between the different cLP. They are thought to rely on how these compounds interact both together and mutually with the membranes of target organisms.

Interestingly, we demonstrated that fengycin and surfactin in combination induce a higher total pH alkalinization than the sum of their individual effect, suggesting for the first time a synergistic activity of these two cLP families. A similar strong synergistic outcome has been previously observed between surfactin and iturin (Maget-Dana *et al.*, 1992) as well as between iturin and fengycin (Koumoutsi *et al.*, 2004, Romero *et al.*, 2007b) and may rely on how these compounds interact together and/or how they mutually interact specifically with target membranes. This result added to the different ISR activity spectrum of the three cLP families highlight thus that both peptide and lipid part of cLPs are important features for perception and ISR activity. It also suggests a central role of the membrane lipids of the target cells.

ii. Molecular mechanisms associated with surfactin-triggered ISR: from early events to defense compounds

a. Early events

In light of works reported in the past decade, a global picture of the ISR induced by beneficial rhizobacteria is being dresseds depicting the cascade of signaling events from the activation of transcription factors to the phenotypic expression of the phenomenon (For a review, see Van Wees *et al.*, 2008). However, molecular mechanisms underlying early steps in the perception of bacterial elicitors by the host plant are still poorly understood (van Loon, 2007). We have therefore further investigated in this work some aspects of the responses of plant cells induced by surfactin cLP family.

We demonstrate in chapter 7 that surfactin induces strong and early pH alkalinization of the extracellular medium of tobacco cell cultures. The extent and the kinetic of the alkalinization are consistent with those induced on the same plant model by the cell walls of the three rhizobacterial strains and the siderophore of *Pseudomonas putida* WCS374 (van Loon *et al.*, 2008). The medium alkalinization response may be considered as one of the first step in the cascade of events leading to the establishment of defense response. Upon treatment with PAMPs/DAMPs, the alkalinization is usually related to a general modification of ion fluxes and to plasma membrane depolarization, mainly resulting from calcium influx and chloride efflux (Boller, 1995). Early ion fluxes then typically lead to the activation of enzymes localized to plasma membrane fractions: the NADPH oxidase (or respiratory burst oxidase homologs Rboh) responsible for ROS production (Simon-Plas *et al.*, 2002) and the mitogen activated protein kinase homologs (Nurnberger *et al.*, 2001, Kadota *et al.*, 2004). ROS are indeed generated in plant cells by the NADPH oxidase, which forms superoxide anion radicals that are further converted into H_2O_2 via superoxide dismutase (Sagi *et al.*, 2006). The activation of this enzyme and the related redox system by elicitors may therefore be responsible for (1) an acidification of the cytosol due to the release of protons from the reducing agent; (2) an alkalinization of the extracellular medium due to the consumption of protons by different reactions; and (3) a decrease of the plasma membrane potential due to the electron transfer at the extracellular surface (Grabov *et al.*, 1993). These secondary effects could in turn participate in signal transduction and amplification leading to the establishment of ISR.

In surfactin-elicited cells, we demonstrate that both calcium influx and chloride efflux phenomena occur and are coupled with K^+ efflux while the alkalinization was only slightly affected by pre-

treatment with the NADPH oxidase inhibitor DPI. Therefore the increase of extracellular pH is not due to proton consumption for H_2O_2 production via the NADPH oxidase/superoxide dismutase system as observed for other elicitors (Pugin *et al.*, 1997). Medium alkalinization following lipopeptide addition likely results from other mechanisms and a Ca^{2+} activation of the plasma membrane H^+-ATPase could be involved since blocking calcium entry resulted in complete inhibition of pH increase upon surfactin treatment. In agreement with this hypothesis, the decrease in proton concentration corresponding to a ΔpH of 0.95 units is similar to the concomitant increase in the amounts of potassium ions we measured in the external medium over the same 15 min laps of time. This suggests that alkalinization provides a driving force for cellular K^+ efflux by secondary mechanisms, such as K^+ channels or H^+/K^+ symporters.

We also observed significant production of ROS within minutes upon surfactin treatment. ROS play multiple roles in plant defenses. In addition to a direct protective effect due to their toxicity for the invader, they may favor cell wall cross-linking, induce cell death or activate an array of defense genes and thereby later accumulation of antimicrobial proteins and phytoalexins (Apel *et al.*, 2004). ROS take also part in systemic signaling leading to the establishment of SAR (Fobert *et al.*, 2005). Data obtained from the complete inhibition of surfactin-induced oxidative burst by DPI show that the phenomenon mainly originates from the NADPH oxidase system. Unlike the PAMP cryptogein, which triggered a long-lasting production of ROS by tobacco cells, surfactin induced a rapid, but transient, oxidative burst lasting less than 30 min. It is similar to the one induced by cell wall preparations of the rhizobacterial strains *Pseudomonas putida* WCS358, WCS374, and WCS417, the pseudobactin siderophore of WCS374; flagella of WCS358, the β-1,3-glucan laminarin and the active flagellar peptide flg22 (van Loon *et al.*, 2008a, Menard *et al.*, 2004, Aziz *et al.*, 2004). The first burst upon surfactin stimulation, considered as non-specific, is not followed by a second one as typically observed in incompatible interactions. Treatment with protein elicitors such as elicitin, harpin or cryptogein led to both biphasic burst and cell death (Huitema *et al.*, 2005, Peng *et al.*, 2004) even if some studies indicate that H_2O_2 is necessary but not sufficient to induce cell death in tobacco (Koehl *et al.*, 2003, Dorey *et al.*, 1999, Glazener *et al.*, 1996). By contrast, in the presence of surfactin, cells or plants remain fully viable suggesting that the hypersensitive response is not required for expression of the systemic resistance induced by the lipopeptide.

As revealed by the use of chelator and channel blocker, both pH change and oxidative burst induced by surfactin are regulated by Ca^{2+} influx into surfactin-elicited tobacco cells. Therefore, in the case of surfactin as for other MAMPs, elicitor-induced Ca^{2+} spiking is one of the earliest events that may act as a master messenger for almost all downstream reactions. On another hand, oxidative burst and extracellular alkalinization are respectively totally suppressed and significantly reduced by the

protein kinase inhibitor K252a. The occurrence of these surfactin-elicited early events is thus closely related to dynamic changes in the protein phosphorylation status. In addition, specific inhibitor of phospholipase A2 significantly reduces extracellular alkalinization. This enzyme should thus also play a regulatory role in early events as it was shown for the expression of plant defense mechanisms associated with pathogenesis, wound-induced signal transduction or elicitation by systemin, oligosaccharides and ergosterol (Roos *et al.*, 1999, Chandra *et al.*, 1996, Lee *et al.*, 1997, Narváez-Vásquez *et al.*, 1999). On basis of all these observations, a hypothetical model of the cascade of surfactin early-induced events is presented in Figure 34.

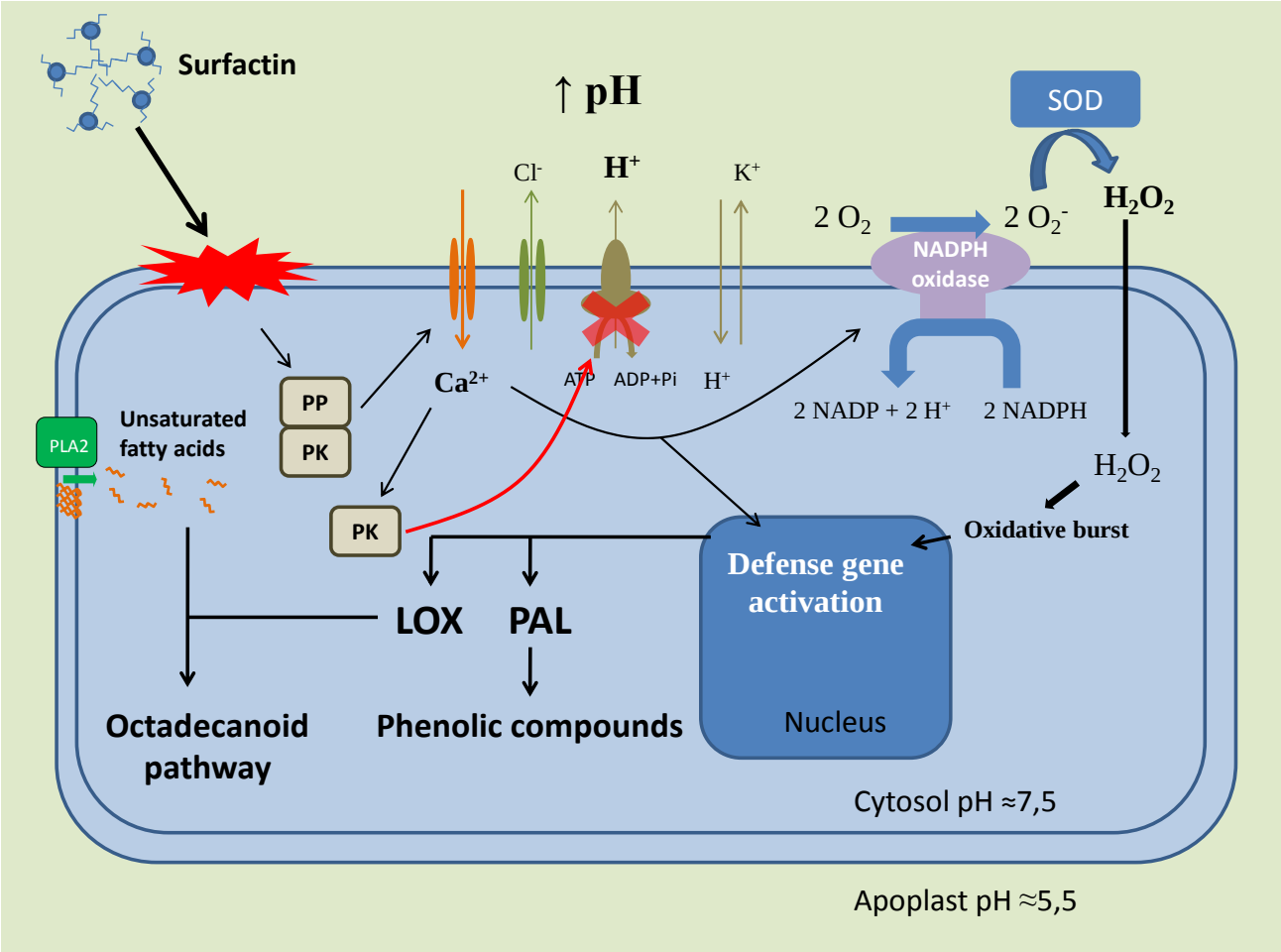


Figure 34 : Hypothetical model of events occurring following surfactin perception at the tobacco cell surface. Early, strong and reversible ions fluxes, extracellular alkalinization and hydrogen peroxide have been characterized following surfactin perception. Plasma membrane located H^+-ATPase, NADPH oxidase, PLA_2 enzymes as well as calcium ions and phyosphorylation events were shown implicated in the occurring of these typical stress responses. Other results obtained in our laboratory on the same plant model previously demonstrated the latter activation of enzymes leading to the production of a broad array of antimicrobial compounds linked to defense response. Surfactin perception at the plasma membrane level therefore transiently activates surrounding enzymes which will in turn launch the cascade of events likely leading to the systemic signal of the ISR.

b. Signaling

Previous results from the laboratory have shown that inoculation of cucumber and tomato plants with strain S499 producing surfactin as elicitor resulted in a more efficient defense reaction to subsequent pathogen infection (Ongena *et al.*, 2005a). cDNA-AFLP analysis of control and bacterized plants provided conclusive evidence that such priming effect is associated with a modified expression of some (unidentified) genes in the host plant (Ongena *et al.*, 2005a). Such resistance is not accompanied by the expression of *PR* genes or by SA accumulation that are both trademarks of the SAR-type signaling pathway. By contrast, a clear stimulation of *PR8* gene (coding for chitinase) following treatment with lipopeptide has been observed in *Arabidopsis* and in

sugar beet. In the last case, systemic disease reduction also correlated with enhanced expression of the transcription activator NPR-1. More work is thus required to further elucidate the signaling route that is responsible for the systemicity of the surfactin-induced ISR.

c. Defense mechanisms

Phospholipase A2 catalyses the hydrolysis of glycerophospholipids resulting in the liberation of free fatty acids, among which the LOX substrates linoleate and linolenate. Phospholipase involvement may thus also be viewed as start point for the expression of the defense-related LOX pathway. Activation of LOX enzyme is a common feature of the plant defense response to pathogen, wounding and stress (Baysal *et al.*, 2007, Feussner *et al.*, 2002, Shah, 2009). Stimulation of the entire metabolic route leading to oxylipins is also tightly related to the occurrence of ISR in bean (Ongena *et al.*, 2004) and tomato (Ongena *et al.*, 2008b) treated with the rhizobacterium *Pseudomonas putida* BTP1 (Mariutto *et al.*, 2011). LOX stimulation also occurs upon surfactin treatment in tobacco cells (Jourdan *et al.*, 2009) and key enzymes of the LOX pathway are also activated in resistant tomato plants following induction by surfactin (Cawoy et al. submitted; Ongena *et al.*, 2007). Time-course monitoring showed that LOX and hydroperoxide-degrading enzyme activities were significantly enhanced and it correlated with disease symptom reduction. It indicates a possible induction of this oxylipin pathway leading to a wide range of bioactive metabolites among which signalling jasmonates or various compounds with fungitoxic activity (Matsui *et al.*, 2009, Prost *et al.*, 2005).

The plant model *Arabidopsis*, having demonstrated response potential to cLPs stimulation and for which numerous mutant lines already exist, could be used to further investigate the signaling pathways of the ISR phenomenon triggered by lipopeptides.

iii. Perception of surfactin by plant cells: a new mechanism?

Little information exists about how MAMPs from PGPR are recognized by plant cells and concerning the link between this first contact and the triggering of signaling pathways leading to the establishment of ISR. Different observations from our work highly suggest that the perception of surfactin may rely on an unusual mechanism, compared to those characterized for other MAMPs from PGPR.

a. Structural traits drive eliciting activity

Surfactin typically displays a "horse saddle" topology harboring a minor polar and a major hydrophobic domain in the peptide chain. For an isolated molecule in polar solvent, the aliphatic tail of the fatty acid is probably in a folded configuration along the hydrophobic domain of the peptide, stabilized by interactions with leucine and valine side chains (Bonmatin *et al.*, 2003, Shen *et al.*, 2009, Peypoux *et al.*, 1999). It is thus obvious that both the nature and place of the various amino acid residues in the cyclized peptide moiety strongly contribute to the stability of this conformation. Metabolically-engineered surfactin derivatives with Val/Leu, Leu/Val, Leu/Ile or Val/Ile substitutions in the peptide displayed a partial but significant decrease of their defense-inducing activity. A substitution of Val in position 4 by Leu or Ile is known to significantly improve surface properties (Bonmatin *et al.*, 2003, Grangemard *et al.*, 1997). It reinforces surfactant behavior and probably causes changes in the organization of surfactin molecules at the air-water interface, and thus possibly at the membrane level (Bonmatin *et al.*, 1995). The significant loss of functionality of the peptide variants of surfactin might therefore be explained by a more subtle destabilizing effect regarding the tri-dimensional structure of the molecule. Such an impact of individual substitutions on the spatial positioning of the acyl chain relative to the peptide backbone is obvious in the case of surfactin and other closely related lipopeptides (Bonmatin *et al.*, 2003, Volpon *et al.*, 2007).

It is worth noting here that, even if multiple studies have characterized structures and surface properties of new surfactin variants, very few have focused on biological activities of such variants. By purifying for the first time a sufficient amount of metabolically engineered surfactin variants, we demonstrate that a theoretical amplification of physical properties (surface tension decrease and critical micelle concentration) of variants with Leu in position 4 does not necessarily correlate with an increased activity on living organisms, at least on plant cells.

By contrast, alkylation of acidic amino acids and breakdown of the peptide cycle ring completely suppressed the burst-inducing activity of surfactin on tobacco cells. This reveals the importance of a specific hydrophilic/lipophilic balance and spatial conformation for perception of surfactin at target sites on plant cell membranes. Both membrane activity and lytic potential on erythrocytes were also shown to increase with the number of ionic charges (Morikawa *et al.*, 2000, Francius *et al.*, 2008) and the cyclic structure (Dufour *et al.*, 2005). Cyclization thus benefits not only stability and rigidity of the compound, but also its biological activity. It is clear now that negative charges and cyclic structure also benefit to perception by plant cells.

A complete loss of activity is observed for surfactin with fatty acid chains shorter than 14 carbons and a higher activity is observed for the predominant C15 homologue. Such an important role for the aliphatic tail has already been documented for other biological activities of lipopeptides, and is explained by the fact that it readily inserts into phospholipid bilayers (Bonmatin *et al.*, 2003, Carrillo *et al.*, 2003, Eeman *et al.*, 2006, Heerklotz *et al.*, 2007). All studies showed that the longer the fatty acid chain of surfactin is, the higher is its biological activity (Kracht *et al.*, 1999, Tendulkar *et al.*, 2007, Snook *et al.*, 2009). A longer lipophilic acid chain increases the intermolecular interactions and thus decreases the critical micelle concentration (Razafindralambo *et al.*, 1998). The active concentration of surfactin is in the same order of magnitude than the CMC, suggesting that surfactin with long fatty acids exist, at least partially, as micelles at the concentrations at which they are perceived by plant cells. Despite the ball-like 3D conformation of surfactin in solution, some specific dynamic changes may thus occur in the fatty acid position relative to the peptide moiety to favour its insertion in lipid layers (Deleu *et al.*, 2003, Tsan *et al.*, 2007).

Globally, our results show that both a typical hydrophilic/hydrophobic balance and the tridimensional conformation of the lipopeptide are crucial features for efficient recognition by tobacco cells. Moreover, as the most active isoforms are also those retaining the highest surface activity, lipid bilayer is likely the privilegied target of the early interaction of surfactin.

b. Surfactin is likely not recognized by high affinity receptors

PAMPs are typically perceived at the plant cell surface by high-affinity membrane-anchored pattern recognition receptors, while no protein receptor have been characterized to date for MAMPs from rhizobacteria. The three *Bacillus* cLP families retain differential abilities to stimulate defense reactions (fengycin induces later response, iturin is not active), confirming that specific functions in the peptide part of these molecules are important for their perception. As there are also stricking

similarities in early events induced by surfactin with those stimulated by receptor-perceived PAMPs, the peptide moiety may serve as epitope for the binding to a receptor.

However different clues are against the hypothesis of a receptor-mediated perception (Table 12). In addition to the structure-activity investigation, we show that there is no competitive effect between different surfactin homologues with identical peptide cycle. It again suggests that their recognition does not rely on very specific structural traits in that part of the molecule. Finally, neither secondary oxidative burst associated with HR nor refractory states have been observed upon treatment with surfactin. This contrasts with results of Van Loon and collaborators, who observed that elicitation with either cell wall, siderophore or flagella of other PGPR, led to a state of complete or at least substantial refractoriness (van Loon *et al.*, 2008). Interestingly, addition of cryptogein after those bacterial compounds led to a full cryptogein-specific production of ROS, demonstrating that the cells had retained their full capacity to develop an oxidative burst.

The micromolar concentrations required (EC_{50} ~ 2.5 µM) for surfactin activity are also much higher than the nanomolar amounts necessary for PAMPs perception by high-affinity associated receptors. All these observations are consistent with results obtained in other experiments. For instance, treatment of *Arabidopsis* plantlets with micromolar concentrations of surfactin does not induce growth retardation (Dorey *et al*., unpublished data), which is typically observed with flagellin when a FLS2 receptor is implicated (Albrecht *et al.*, 2012). A possible induction by surfactin of ethylene in *Arabidopsis* leaves, which is also a trademark event associated with receptor-mediated perception of PAMPs, was also tested (Felix *et al*., unpublished data). However, no increase in ethylene release by treated leaves was observed upon treatment with surfactin, while a significant rise was observed upon addition of the harpin elicitor used as positive control. Interestingly, there was also no significant change in insertion rate of C14 surfactin in heat-treated or protease-treated cells/protoplasts (not shown). A first contact of tobacco cells with surfactin does not preclude subsequent reactivity to a second application, meaning that possible saturation of high-affinity fixation sites is not involved.

At this point, the existence of specific receptor proteins for bacterial cLPs in plant cells is thus questionable. The lipid part of the plasma membrane should therefore be considered as both the privilegied target site of cLPs and the starting point of all the early events observed on tobacco cells.

Table 12: Some characteristics of the interaction between surfactin cLP family and plant cells may suggest a protein receptor-driven perception. However, numerous clues from our results imply that plant lipid bilayers are the privilegied target binding site of these amphiphilic compounds. Then early responses may be launched through a precise disturbing effect of the bilayer organization. Some organized lipid platforms inside the whole plasma membrane may therefore be viewed as specific binding sites able to modulate the activity of proteins displaying affinities for lipid environment.

Characteristics of the interaction surfactin-plant cells

Suggesting a specific protein receptor perception	**Suggesting a specific interaction with lipid bilayer**
Similarity surfactin-induced defense responses / PAMPs-induced defense responses	Numerous other biological activities are related to a lytic activity of plasma membranes
Differential ISR activity of the three *Bacillus* cLP families	High [] required for eliciting activity (µM)
Effect of the peptide cycle composition on the activity	No refractory state / No competition between surfactin isoforms
	Importance of a specific hydrophilic/lipophilic balance and of the spatial conformation
	Most active isoforms are also those having the highest surface activities
	Tight binding to plant plasma membrane fraction
	Close relationship between the physical structure of the lipid bilayer and the affinity of surfactin for the bilayer

iv. A specific interaction with membrane lipids is required for surfactin perception

The hypothesis of a preferential interaction of surfactin with the lipid phase of the target membrane is supported by correlation obtained between eliciting activity and affinity of the multiple variants of the cLP for plasma membrane. Indeed, surfactin fully insert into plasma membrane fraction but with higher insertion rate for long- chain surfactin, and a significant decrease for methylated and/or linear surfactin.

Isothermal calorimetry experiments yielded further information about the interaction of surfactin with lipid bilayers. Surfactin interaction appeared to be an entropy-driven process. This may be explained by a gain of water disorder after dehydration induced by surfactin incorporation into the bilayer hydrophobic core and by deformation and structural changes of the lipid bilayers. Morover, binding is also exothermic, suggesting close van der Waals interactions of the lipopeptides side chain with the bilayer hydrophobic cores. These results are in accordance with those from Maget-Dana showing that surfactin has a high affinity for membrane lipids driven by hydrophobic interactions (Maget-Dana *et al.*, 1995).

In agreement with results obtained on early events induction and kinetic insertion, the affinity of the C14 homologue for model membranes is much higher than the one calculated for C13. This may be explained by an optimal interaction with acyl chains of phospholipids that have a similar length as the surfactin hydrocarbon tail (Grau *et al.*, 1999). However, this interaction is not favored in the presence of lipids typical from plant cells, and addition of stigmasterol in binary mixtures with PLPC did not affect the level of binding. On the contrary, a higher sterol proportion in lipid ternary mixtures, leading to the formation of Lo domains, significantly decreased binding of surfactin. Sterols are intercalated between fatty acyl chains of phospholipid molecules and extend their side chain deeply into the interior of the bilayers. This could explain that they restrict the access of lipopeptides to the inner side of membranes. It is also clear from computer stimulation that surfactin does not behave similarly in the presence of sterols with a less obvious lipid phase separation. This apparent lowest destabilizing effect of surfactin for sterols, containing environments was previously observed (Carrillo *et al.*, 2003) and was evidenced for a number of other membrane-destabilizing compounds (Maula *et al.*, 2009, Pott *et al.*, 1996), but it contrasts with the other *Bacillus* lipopeptides such as iturin (Maget-dana *et al.*, 1994) and fengycin (Eeman *et al.*, 2009). This may partly explain the differential bioactivities of these structurally close-related lipopeptide families on various cell types.

More generally, our results highlight the close relationship between the physical structure of the lipid bilayer and the affinity of surfactin for the bilayer. The lipopeptide clearly exhibits enhanced binding to So containing vesicles. It is known that even if the phase state is uniform, the lipid packing inside these domains is heterogeneous (Bernchou *et al.*, 2009). It gives a highly structured and condensed membrane with regular corrugations in its topography called ripple phase. Even if at physiological temperatures, cellular membranes coexist mainly as fluid and liquid-ordered domains, it is likely that rippled solid ordered phases transiently form, arising from periodic arrangements of linear ordered and disordered lipid domains.

The higher apparent affinity of surfactin for bilayers containing So and Ld phases may result from a higher segregation at the boundary between both lipid phases as observed by computer stimulation of surfactin insertion. Interaction with highly packed lipids of So phases, highly mobile and unsaturated lipid chains of the Ld phases or sterol congested Lo phases is likely less entropy favored than self interaction of surfactin in aggregates. Surfactin aggregation is believed to generate a strong entropy-favored dehydration of the surrounding phospholipid carbonyl groups and a gain of water disorder resulting from a decrease in the hydrogen bonding of water to these groups (Carrillo *et al.*, 2003). The decrease in hydrogen bonding with water reduces the water penetration into the polar head groups of the membrane and may cause membrane fusion processes. Such a behavior is supported by the observed higher K values obtained in ITC with saturated phospholipids and So phases, as well as by results from computer simulation of surfactin insertion.

It would be biologically relevant to investigate the impact of surfactin insertion on lipid modifying enzymes activities and the indirect consequence of this insertion on defense pathways activation. As the hypothesis of a protein-mediated perception of cLPs is still not fully discarded, it would be valuable to compare the binding profiles of surfactin on LUV vesicles from tobacco microsomal membranes before and after proteolytic treatments. In this way, similar binding profile would suggest that a complete proteic structure is not implicated in the first step of surfactin binding at the membrane level and would reinforce the hypothesis of the role of plasma membrane bilayer as a receptor.

a. Surfactin-driven lateral compartmentalization involved in plant cell signaling?

The limited miscibility of surfactin with fluid membrane lipids gives thus rise to segregation into surfactant-depleted bilayer and surfactant-rich, defect regions, at the boundary between the two lipid phases. This is clearly illustrated by our computer stimulation data. Surfactin segregation into preferential area may then indirectly induce some lipid phase separation, further affecting the behavior of proteins having affinity for lipid environment. In turn, this may lead to the recruitment of specific defense-related membrane proteins into newly created lipid platforms. Surfactin-induced disturbing of lipid bilayer organization could favor the activation of lipid-binding enzymes and the formation of functional complexes, e.g. where the interaction with some kinases and phosphatases is favoured. From these functional platforms can subsequently be launched downstream signaling events.

One proposed explanation for the dynamic association of proteins to specific lipid platforms is that surfactin insertion induces local membrane curvature. Indeed, in a planar membrane mostly built from lipids with equal diameters of head group and fatty acid moiety, and thus considered as cylindrical shaped, the insertion of lipopeptides with conical or inverse-conical geometries might induce curvature strain. Membrane curvature is nowadays seen as an active way to create membrane domains and to organize centers for membrane trafficking (McMahon *et al.*, 2005). Such domains then become preferential adsorption sites for amphiphilic molecules and for proteins containing specific recognition motifs. These proteins have a greater affinity for positively curved membranes through the recognition of curve-induced defects in lipid packing (Figure 35A). This hypothesis is consistent with earlier studies postulating that *Bacillus* cLP families are all known to prefer aggregates with positively curved interface (Nazari *et al.*, 2012) and that the segregation of certain amount of surfactin into concave regions of membrane undulations stabilizes ripple phases, due to periodic variations of gel and fluid surfactin-rich regions (Brasseur *et al.*, 2007). It is also in agreement with results obtained by Heeklotz and coworkers. They demonstrated that surfactin exhibits a different behavior from conventional detergents. The latest acts by increasing the lateral pressure in the headgroup region and by disordering the acyl chains. By contrast, the strong membrane activity of surfactin is not reflected in an extreme disordering of the fatty acyl chains but rather results from this inverted cone-like structure that causes the tilting of the acyl chains of the surrounding lipids, leading to a reorientation of the lipid headgroups (Heerklotz *et al.*, 2004). Thinner membranes with tilted chains are likely to break at packing defects between more ordered clusters, which may also accumulate surfactant. Nazari and coworkers recently confirmed that

surfactin mixes poorly with some lipids and segregates at specific membrane areas into detergent-rich clusters. These clusters upset the membrane locally whereas most of the remaing membrane area is little affected, resulting in the so-called “heterogeneous perturbation” (Nazari *et al.*, 2012). Interestingly, the interaction of iturin A with multilamellar vesicles was shown to induce the formation of two laterally separated phases, one formed by pure phospholipid and the other by lipopeptide–phospholipid complexes (Grau *et al.*, 2000). It was concluded from this study that the membrane barrier properties are likely to be damaged in the area where the lipid complexes are accumulated, due to structural fluctuations. This may be one of the bases of iturin A biological activity. Moreover, this cLP can also modify the curvature of the membrane, what could also partly explain its biological activity. This set of observation is globally in accordance with our hypothesis represented in Figure 35A.

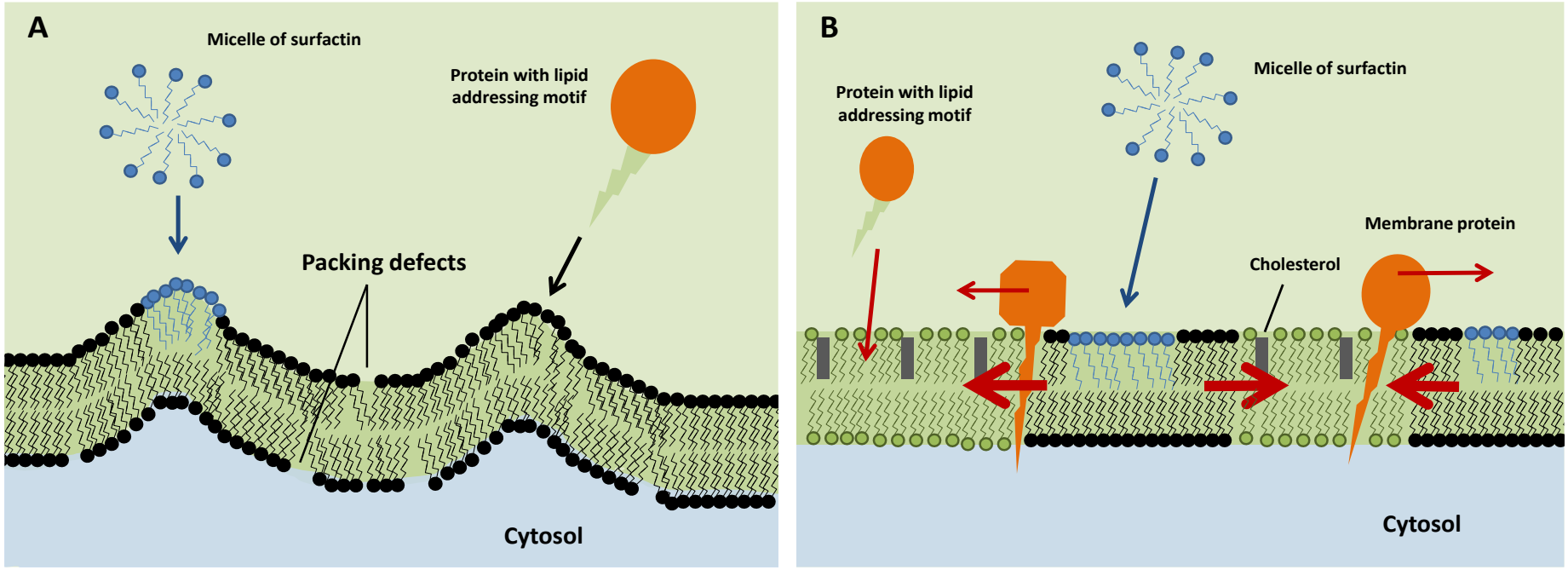


Figure 35: From the set of observations obtained after our work, we depict possible explanations of the surfactin eliciting activity. The strong membrane activity of surfactin is not reflected in an extreme disordering of the fatty acyl chains, but would rather result from segregation at specific membrane areas, leading to the tilt of the acyl chains of surrounding lipids. The entropy-driven nature of the process may be explained by a gain of water disorder after dehydration and a lower freedom of movement of lipid chains, but also by deformation and structural changes of the lipid bilayer. Surfactin insertion could then induce the reorganization of the plasma membrane lipids and proteins. A. The segregation of the inverted cone-like structure of surfactin generates membranes with tilted chains and leads to packing defects between more ordered clusters. The membrane perturbation is localized to these membrane defects whereas the order in the bulk of the membrane is little affected. This "heterogeneous perturbation" favors structures with different local curvatures into plasma membranes which would become adsorption sites for proteins with lipid addressing motifs. Membrane curvature may thus regulate the localization of proteins that are thought to have greater affinity for positively curved membranes through recognition of curve-induced defects in lipid packing. B. Surfactin segregation into specific membrane area indirectly induces lipid phase separation leading to a displacement/insertion of plasma membrane proteins depending on their affinity for newly formed lipid platforms. The presence of lipid domains can affect the specificity of the hydrolytic activity of lipid-modyfing enzymes such as phosholipases, resulting in marked differences in the physical properties of the membrane end-product. In turn, the hydrolysis of phospholipids leads to second messengers involved in cell signaling.

On the other hand, surfactin ability to trigger ISR could rely on the ability to temporally and spatially organize protein complexes. The dynamic association of some proteins to membrane domains could be part of the sensitization pathway, leading to the organization of certain cellular processes. This hypothesis is based on the concept of dynamic membrane compartmentalization. Although the molecular basis of this compartmentalization remains largely undeciphered, two non-exclusive mechanisms might be involved: (1) clustering of nanoscale assemblies into more stable, selective and functional platforms; or (2) association-dissociation of specific proteins to particular domains (Simon-Plas *et al.*, 2011). To date, data obtained with plants are in favor of the latter mechanism, with the modification of protein content in specific membrane microdomains upon different biological stimuli. Very few things are known about the mechanisms underlying the dynamic association of proteins into functional entities. Concerning DRM microdomains ressembling Lo phase, it is hypothesized that proteins having high affinity for ordered lipid environment might spontaneously partition into it. In this perspective, the formation of large microdomains in a Lo-like phase due to segregation of surfactin at lipid phases interfaces could enhance the association of proteins.

Beside this direct effect, protein association into lipid microdomains and their activation may also indirectly result from the segregation of surfactin into membranes. The best candidates which could be affected by surfactin-induced lipid bilayer perturbations are enzymes with lipid substrates such as phospholipases. Phospholipase A2 is sensitive to membrane structure and curvature, and the presence of particular lipid domains can induce some specificity in their hydrolytic activity, resulting in marked differences in the physical properties of the membrane end-product (Leidy *et al.*, 2011). In turn, hydrolysis of phospholipids by phospholipases may result in the release of lipid secondary messengers involved in cell signaling (Chapman, 1998, Munnik *et al.*, 1998). That may explain how surfactin perception induces early events on tobacco cells and major changes in protein association to lipid microdomains. Activation of phospholipase C has been observed during the oxidative burst induced by different elicitors (Legendre *et al.*, 1993, Kasparovsky *et al.*, 2004), and the resulting increase in inositol-3-phosphate could explain the changes in cytosolic calcium measured in treated plants (Mithöfer *et al.*, 1999). Phosphatidic acid (PA), the product of phospholipases C and D, is also currently seen as a major second messenger in plant responses to a wide variety of stress treatments including ethylene, wounding, pathogen elicitors, osmotic and oxidative stress, and abscisic acid (Munnik, 2001). Several downstream targets of phosphatidic acid have been identified, including protein kinases, ion channels and the tobacco NADPH oxidase RbohD (Munnik *et al.*, 2009, Zhang *et al.*, 2009). Interestingly, phosphatidic acid (PA) has been

shown to accumulate upon elicitation by PAMPs, and the direct application of PA to plants induces pathogen-related gene expression and cell death (Testerink *et al.*, 2011). In tomato cells treated with xylanase, PA and its phosphorylated derivative diacylglycerolpyrophosphate, are quickly synthesized after elicitation. Similarly, the free unsaturated fatty acids generated by phospholipase A2 are thought to act as precursors for the synthesis of jasmonic acid, an active inducer of secondary metabolite synthesis in response to pathogen attack (Munnik *et al.*, 1998). In this context, it is worth noting that surfactin could inhibit or enhance phospholipase A2 activity (Kim *et al.*, 1998), while plipastatin cLP family inhibits phospholipases A2, C and D (Umezawa *et al.*, 1986). This substantiates the hypothesis that cLPs may directly affect phospholipase activity by modifying lipid bilayer properties. This could lead to further perturbation of plasma membrane organization and second messenger's production. Consequently, proteins involved in signaling cascades may either be recruited within target lipid microdomains, where they constitute protein complexes, or be affected by second messenger's activities (Figure 35B).

b. Significant changes in plasma membrane proteome upon surfactin perception

Specific segregation of surfactin may thus both directly affect the lipidic organization of the plasma membrane (Figure 35A) and indirectly induce displacements of some membrane-associated proteins involved in early defense events pathways (Figure 35B). Many proteins may indeed be activated or inactivated by their localization in or out of membrane microdomains displaying different physical phases. Because of the heterogeneity of the cell membrane, proteins partition between different lipid domains and form functional complexes only when recruited to specific locations that allow the proper tridimensional configuration for activity (Hanzal-Bayer *et al.*, 2007, Sprong *et al.*, 2001, Van Meer *et al.*, 2008). This lateral organization is very important for various cellular processes, such as membrane fusion (Chamberlain *et al.*, 2001, Lang *et al.*, 2001), protein trafficking (Bretscher *et al.*, 1993), and signal transduction (Kawabuchi *et al.*, 2000, Moffett *et al.*, 2000, Simons *et al.*, 2000).

The lipid raft hypothesis proposes that preferential interactions between lipids generate domains of specific lipid compositions to drive the sorting of membrane proteins showing a clear preference for a particular lipid phase (Van Meer *et al.*, 2008). For coexisting liquid and solid phases in model bilayers, a peptide or protein that is anchored to the membrane by a α-helix typically prefers the liquid phase (Stopar *et al.*, 2009). Partitioning into the solid phase in preference to the liquid phase

only occurs for precise hydrophobic matching with the solid. For coexisting Lo and Ld domains (typically the lipid rafts), most membrane-bound peptides partition out of Lo and into Ld.
Based on this concept and on interpretation of our data from ITC and modelisation, a surfactin-induced lipid reorganization triggering the recruitment and activation of key defense-related enzymes (such as the NADPH oxidase) in particular membrane microdomains has been investigated in the last part of our work by the detergent insolubility method. We firstly identified 102 proteins that were more abundant in the DRM fraction purified from tobacco plasma membranes among the 335 proteins that we identified in the whole plasma membrane. Among these 102 proteins, numerous are similar to those characterized in the DRM fraction of the 145 DRM proteins identified by Morel and coworkers on the same plant model (Morel *et al.*, 2006). Most of DRM proteins are involved in transport, signaling and response to biotic and abiotic stress, such as calcium dependent protein kinases, H^+-ATPase and the tobacco NADPH oxidase NtrbohD. The incorporation of other proteins in DRMs in response to a stimulus may thus lead to the formation of multiprotein complexes and trigger defense pathways.

The subsequent characterization of the proteic content of the DRM fraction isolated from tobacco cells previously treated with surfactin (DRMS) or not (DRMC) give us more information on protein trafficking than ever previously obtained. On 73 proteins differentially distributed between the two samples, 22 are shown enriched in DRMS, 13 are similarly abundant and 38 are depleted. This is nearly ten times higher than in the work of Stanislas in which 4 proteins were shown enriched for 1 depleted in the DRM fraction upon cryptogein (a well-known PAMP elicitor) stimulation (Stanislas *et al.*, 2009). To our knowledge, this is the first time such a change of the protein association to DRM, and to a greater extent to lipid microdomains of the whole plasma membrane, is observed in response to an active substance. Even if limited to DRM, our proteomic investigation highlights that surfactin perception by tobacco cells induces association-dissociation processes of specific proteins to particular membrane microdomains. These results also reinforce the current hypothesis that DRM are involved in the regulation of physiological functions. As already observed in studies performed in animals cells upon biological stimuli, proteins identified as differentially associated to tobacco DRM after surfactin challenge are mainly involved in signaling, protein targeting and vesicular trafficking. The way by which the dynamic association of proteins to DRM could participate in the regulation of the signaling process may therefore be a conserved process between plant and animals.

Moreover, our set of data is in total agreement with recent quantitative proteomic approaches obtained on three different plant/PAMP models: *Arabidopsis*/bacterial flagellin 22 (flg22), tobacco/oomycetal cryptogein and rice/ fungal chitin (Keinath *et al.*, 2010, Stanislas *et al.*, 2009, Fujiwara *et al.*, 2009). Shortly after elicitation, the FLS2 receptor to the PAMP flagellin was shown to shift from non DRM to DRM and others enzymes, among which H^+- and Ca^{2+}-transporting ATPases, vacuolar H^+-ATPase, Ca^{2+}-dependant protein kinases, also underwent relocalization (Keinath *et al.*, 2010). We found a differential abundance of calmodulin proteins and calcium/calmodulin-dependent protein kinase in DRM. These proteins are implicated in calcium ion binding and the transduction of calcium signal by modifying its interaction with target proteins. The kinetic of the transient increase in calcium binding proteins at the plasma membrane is biologically coherent since it coincides with that of transitory calcium-dependent extracellular alkalinization and ROS production that we observed within the first 15 min after elicitation.

Among proteins enriched in DRM upon surfactin treatment, 14-3-3 protein is the most abundant. Studies over the past 20 years have proven 14-3-3 to be ubiquitous and play a central role in the regulation of many cellular processes such as control of the cell cycle, differentiation, apoptosis, targeting of proteins to different cellular locations, and coordination of multiple signal transduction pathways (Roberts, 2000). These proteins could achieve such functions by regulating the activity of proteins in a signal transduction cascade, promoting the formation of multiprotein complexes, or modulating the activity or localization of transcription factors. In plants, 14-3-3 proteins could specifically interact with NtrbohD responsible of ROS production (Simon-Plas *et al.*, 2002). We demonstrated that NtrbohD was transiently activated upon surfactin stimulation and we observed that NtrbohD mainly localizes in tobacco DRMs. The enrichment or depletion in this DRM fraction of a 14-3-3 protein able to act as a regulator of this oxidase at a timing corresponding to ROS production is therefore particularly biologically relevant. Once again, this result is consistent with the rapid phosphorylation of RbohD observed after flg22 treatment (de la Fuente van Bentem *et al.*, 2007) and the fact that regulators of RbohD, such as 14-3-3 and small G proteins, are also recruited in DRM following PAMP perception (Stanislas *et al.*, 2009, Fujiwara *et al.*, 2009).

Ubiquitin fragments and luminal binding proteins are other prominently enriched proteins in DRM following surfactin stimulation. Ubiquitination is a mechanism for protein modification leading to their degradation and studies in the past several years have revealed that this system is important for a broad range of plant developmental processes and responses to abiotic and biotic stresses (Zeng *et al.*, 2006, Lee *et al.*, 2011). Luminal binding proteins are implicated in regulatory mechanisms that

serve to target proteins to different pathways. This suggests that protein degradation, sorting or internalization through vesicular trafficking could be a key component of defense reactions following elicitor perception, which is in accordance with our hypothesis that surfactin-induced membrane curvature creates membrane domains and organizes centers for membrane trafficking. It is worth noting here that the triggering of protein sorting pathway and ER stress may meanwhile be the result of important changes occurring within the first 15 minutes following the interaction of surfactin with the plasma membranes.

The further study of the dynamic association of proteins to the entire plasma membrane and to microdomains at earlier and later times following surfactin challenge would allow a better understanding of how surfactin segregation participates in the direct regulation of the signaling process leading to ISR. In this aim, the analysis the PM sub-compartmentalization and the protein trafficking by DRM proteomic should be considered as a tool among others. Another valuable approach to deeply understand the biologically relevance of the dynamic association of proteins to membrane microdomains upon surfactin stimulation would be a two-hybrid screening of the proteins characterized in these domains. By determination of the interaction partners of unknown proteins, the possible functions of these new associated proteins may be inferred and the cascade of events from elicitor perception to ISR more understood.

Furthermore, newly developed imaging techniques such as total internal reflection fluorescence microscopy (TIRFM) or variable-angle epifluorescence microscopy (VAEM) can be successfully applied and allow the visualization of mobile proteins located in and around the plasma membrane with very high signal-to-background ratio (Owen *et al.*, 2007; Konopka and Bednarek, 2008; Spira *et al.*, 2012). A recent study showed TIRFM to also be applicable to study single molecule trafficking of PM-resident proteins in plant cells (Martiniere *et al.*, 2012). These techniques could thus help to analyze domain structures in the PM as well as dynamic compartmentalization of PM in responses to elicitors in more detail in the future.

Significance

For their producing bacteria cells, lipopeptides act as signal driving cannibalism, biofilm formation or other coordinated responses in bacteria subpopulations but also as inhibitor of enzymatic functions in competing microorganisms or as repellant for feeding protozoa (Lopez *et al.*, 2009, Raaijmakers *et al.*, 2010). In the interaction with their hosts, our work shows that surfactin-type lipopeptides from *Bacillus* retain some elicitor activity of plant immunity. More generally, it also illustrates that such compounds may induce some modifications in cell processes of target organisms without causing any detrimental leakage in the plant plasma membrane. This can be extended to other eucaryotic cells since it has been reported that surfactin can interfere with several stages of the immune processes in animal cells, can display antitumor activity and retains some anti-inflammatory properties (Hwang *et al.*, 2007, Kim *et al.*, 2007, Hwang *et al.*, 2005, Takahashi *et al.*, 2006). Molecular perception of *Bacillus* lipopeptides by other organisms may thus be more subtle than expected. As far as it concerns surfactin-type cLPs and plant hosts, our work set new hypothesis to explain these activities. We demonstrate here for the first time that cLPs induce some enrichment or depletion into specific lipid platforms of different proteins. We hypothesize that it may be: (1) the direct consequence of the generation of insertion sites for some proteins in the entire plasma membrane due to specific cLP segregation at lipid phase interfaces; or (2) the result of indirect adjustment of lipid-modifying enzyme activities through an induced rearrangement of the plasma membrane lipid organization. Such enzymes and several of the proteins newly associated into lipid defects or into specific microdomains are able to act as regulator of early defense events observed in tobacco cells and take likely part to the triggering of signaling pathways leading to ISR. It reinforces the current hypothesis of a role for lipid microdomains-driven lateral compartmentalization in plant cell signaling. By contrast with the PAMPs flg22 and cryptogein, for which such similar observations have been obtained, no surfactin receptor protein have been characterized so far. The lipid bilayer of target cells could now be considered as the preferential domain for cyclic lipopeptide perception from where arise subsequent cellular responses. Although their original mechanism of action may not be similar for all members of the class of amphiphilic ISR elicitors, these compounds become more attractive than ever for further development of needful innovative biopesticides.

Material and Methods

Bacterial and plant cell growth

The wild-type *B. amyloliquefaciens* strain S499 was isolated from soil by L. Delcambe (CNPEM, Liège, Belgium) and studied in our laboratory for several years. *B. amyloliquefaciens* CH1, CH2, and AK3 strains were given by R. Borriss (Center of Bacterial Genetic, Biology Institute, Humboldt University of Berlin, Germany). *B. amyloliquefaciens* strains were cultured in aerobic condition in flasks containing optimized medium with the following composition per liter: 7 g of yeast extract, 30 g of casein peptone, 20 g of saccharose, 1.9 g of KH_2PO_4, 0.45 g of $MgSO_4$, 10 mg of citric acid, 3.6 mg of $MnSO_4$-H_2O, 0.014 mg of $ZnSO_4$-$7H_2O$, 0.01 mg of H_3BO_3, 0.005 mg of $FeCl_3$-$6H_2O$, 0.004 mg of $NaMoO_4$, 0.002 mg of KI, and 0.001 mg of $CuSO_4$, adjusted to pH 7 with KOH. All cultures were inoculated with a 16-h-old preculture and incubated at 30°C on a rotary shaker (120 rpm, Multi-Shaker PSU 20). Cellular concentration was measured by optical density at 600 nm.

Tobacco cells (*Nicotiana tabacum* L. cv. Bright Yellow-2) were cultivated in Murashige and Skoog medium (MP Biomedicals, Irvine, CA, U.S.A.) (4.4 g/L) at pH 5.8, completed with saccharose at 30 g/L, KH_2PO_4 at 0.2 g/L, myo-inositol at 50 mg/L, thiamine at 2.5 mg/L, and 2,4-dichlorophenoxyacetic acid at 0.2 mg/L. Tobacco cells were grown at a constant temperature (28°C) in the dark on a rotary shaker (110 rpm, Multi-Shaker PSU 20) and subcultured weekly by the addition of 4 mL of cells to 100 mL of fresh liquid medium.

Experiments were realized using cells in exponential growth phase after 5 to 6 days at a concentration of 0.01 mg fresh weight/mL of culture. Cell fresh weight was obtained by gentle filtration on miracloth paper.

Preparation and analysis of surfactin variants

The mixture of surfactin (95% purity) was obtained from *Bacillus amyloliquefaciens* strain S499 and the various homologues were purified from this mixture as described previously (Jourdan *et al.*, 2009). In all instances, surfactin was used from a methanolic stock solution 1 mg/mL. The methylation of surfactin was carried out by dissolving 1 mg of surfactin in 1 mL of anhydrous methanol with 10 µL of concentrated HCl. The sample was kept at 22°C overnight. After adding an equal volume of distilled water, methylated surfactin was purified by HPLC. Linear derivatives of the C14 surfactin homologue were synthesized in the Laboratory of Industrial Biology of the University of Liège/Gembloux Agro-Bio Tech (Belgium) using the method described by Dufour and associates (Dufour *et al.*, 2005). Peptide-modified surfactin were generated by culturing strain

S499 in a medium containing (i) a mixture of sugars (glucose 34%, fructose 57%, maltose 8%, ribose 0.75% in weight) at a final concentration of 5 g/L; (Tran *et al.*) a mixture of organic acids (citrate 77%, succinate 19%, malate 2%, fumarate 0.5%) at a final concentration of 4.5 g/L and (iii) amino acids at 0.5 g/L with casamino acids used for control replaced by L-Leu, L-Val or L-Ileu. 100 mL of the medium was sterilized in 500 mL flasks and amino acid solution was then added by filter-sterilization. Strain S499 was incubated for 72 h at 37°C under agitation.

Linear, methylated and peptide-modified surfactin were purified by HPLC using the isochratic method described for naturally produced lipopeptides. The molar ratio of the amino acids was determined by analyzing acid hydrolyzed products (HCl 6N at 145°C for 4h under vaccum). Amino acid analysis was performed according to the method developed by Agilent Technologies with online derivatization using o-phthalaldehyde and further resolution on a Zorbax Eclipse AAA column. All surfactin variants used in this study were finally checked for purity/structure and quantified by reversed phase HPLC coupled with mass spectrometry as described in annexe I. Assignment of fragmentation ions was supported by data from previous studies (Kowall *et al.*, 1998, Jenny *et al.*, 1991).

Determination of extracellular pH changes

Extracellular pH variation was monitored with a glass pH electrode (Microprocessor pH Meter 211; Hanna Instruments) in 10 ml of agitated cell culture. Lanthanum chloride and EGTA were added in the specified concentrations to cell suspensions 30 s before addition of surfactin, while DPI and AACOCF3 were added 10 and 30 min, respectively, prior to surfactin addition.

Reactive oxygen species accumulation in tobacco cells

Tobacco cells (*Nicotiana tabacum* L. cv. Bright Yellow-2) were cultivated and prepared for experiments as already described (Jourdan *et al.*, 2009). Intracellular ROS accumulation was monitored with the oxidant-sensing fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Tobacco cells were loaded for 5 min with 25 µM of DCFH-DA. Cells were then treated with 15 µM of surfactin. 100 µL of the suspension was analyzed with a plate fluorometer (Wallac Victor 1420) (λexc 485 nm; λem= 535 nm; measurement time: 0.1s). Tomato roots pictures were obtained by loading 10 µM DCFH-DA for 5 min on roots of 15 days old tobacco plants grown on Petri dishes (MS salt medium 5 times diluted added to 14 g/L agar) and treated with 10 µM surfactin. Pictures were taken with a fluorescent Axioskop2-type microscope (505 nm excitation Filter, Carl Zeiss Jena GmbH, Germany). The production of extracellular ROS was monitored by a chemiluminescence measurement of H_2O_2 from the ferricyanide-catalyzed oxidation of luminol

using a luminometer (TD-20/20 Luminometer, Turner Designs, Fresno, CA, U.S.A.). After treatment with surfactin, a 50 µL aliquot of the cell suspension (final concentration 0.15 g FW/mL) was added to 100 µL of 50 mM phosphate buffer (pH 7.9) and 100 µL of 1.1 mM luminol in phosphate buffer. The reaction was started by addition of 100 µL of freshly prepared 14 mM $K_3[Fe(CN)_6]$ and the signal was integrated over the first 30 s after reaction start.

Quantification of ions fluxes

Five-day-old tobacco suspension cells were equilibrated for 5 h in 10 mM MES, 5 mM sucrose, 0.5 mM $CaSO_4$, and 3 mM KOH, pH 5.7, for cells at approximately 150 mg/ml. Samples were taken every 5 min and then filtrated twice on 0.45- and 0.2-µM cellulose acetate membrane filters. Concentrations of nitrate and chlorure ions were measured by conductimetry after separation by ion chromatography (Dionex-120) using a 4-by-250-mm column (Ionpack AS9-HC) and a 9-mM sodium carbonate flow of 1 ml/min. Potassium ions were detected by flame emission spectroscopy (Eppendorf, Hamburg, Germany) with an air-propane flame. The final composition of the spectral buffer was 0.5% CsCl, 2.5% $Al(NO_3)_3$, and 0.1 N HCl.

Cell mortality determination

A volume of 1.5 mL of treated tobacco suspension cells was filtered (Miracloth pore size 22 to 25 µm; Calbiochem) and resuspended in 1 mL of HEPES medium at pH 5.7. A 0.5% (wt/vol, distilled water) Evans Blue solution (100 µL) was added and the mixture incubated for 10 min at 27°C on a rotary shaker (110 rpm). Then, 800 µL were filtered and washed with 10 mL of water to remove nonfixed dye. The fixed dye was finally solubilized by suspending washed cells in 1 mL of a 50% MeOH/1% sodium dodecyl sulfate (SDS) solution at 55°C for 30 min. The quantification of dead cells was carried out by measuring the absorbance of the colored supernatant at 620 nm (Beckman Coulter AD 340, Indianapolis, IN, U.S.A.). A calibration curve was realized by treating tobacco cells with increasing concentrations of Triton X-100 0.9%. Pictures of cells were realized with an Axioskop2-type microscope (Axiocam camera, Axiovision 3.0 software; Carl Zeiss Jena GmbH, Jena, Germany).

Biotests for induced systemic resistance

ISR assays were performed on tobacco plants grown hydroponically from sterilized seeds in a growth chamber under controlled conditions (26°C) for 5-6 weeks. Plant roots were treated with a methanolic solution of 10 µM surfactin or with the same volume of pure methanol for controls and transferred to a high humidity chamber (19 ± 2°C) for 24 h before leaf-infection with *B. cinerea*.

This was achieved by inoculating the fourth leaf with eight drops of spore suspension following the same method as described (Ongena et al., 2005a). Experiments contained at least 10 plants per treatment and disease incidence was expressed in terms of the percentage of *B. cinerea* lesions that clearly grew out of the inoculum drop zone to produce spreading lesions 4-5 days post-infection.

Kinetics of insertion and distribution of lipopeptides within tobacco cells

At various times following addition of surfactin to tobacco cell suspensions at 0.01 g FW/mL, 150 µL of the suspension were collected and centrifuged for 5 min at 5000 RPM. 100 µL of the supernatant were diluted in the same volume of MeOH and then subjected to HPLC-MS for quantification. At the end of the experiment, remaining suspension was centrifuged. Supernatant was analyzed as before while the cell pellet was resuspended in HEPES medium (same volume as discarded supernatant to keep 0.01g cells/mL) and frozen. Thawed solution was then subjected to ultrasonic bath for 10 min and centrifuged. Supernatant was analyzed as before while cell pellet was resuspended in MeOH (same volume as discarded supernatant to keep 0.01g cells/mL). After vortexing and sonication, this solution was centrifuged and 100 µL of the supernatant was diluted in the same volume of HEPES medium before HPLC-MS quantification.

Vesicle preparation

Palmitoyl oleoyl phosphatidyl choline (POPC), palmitoyl linoleoyl phosphatidyl choline (PLPC), dipalmitoyl oleoyl phosphatidyl choline (DPPC), soybean PC, soybean PE, glucosylceramide, stigmasterol and palmitoyl sphingomyelin (PSM) were purchased from Avanti Polar Lipids (Birmingham, AL). Synthetic β-sitosterol was from Sigma-Aldrich (purity >95%). All other chemicals were from Sigma-Aldrich. These lipid substances were used without further purification. All measurements were made in buffer (150 mM NaCl, 10 mM TRIS, 1 mM EDTA; pH 8.5). Lipid mixtures were dried from a chloroform/methanol (2/1; v/v) solution under reduced pressure in a rotavapory evaporator at 30°C and then kept under vacuum overnight. The lipid film was hydrated in buffer during 1 h at 45°C with vortex mixing applied every 15 min and then subjected to five freeze/thaw cycles. The solution was then sonicated 6 times 2 min at 2 min interval under 117 watts power using a sonicator tip with 3mm diameter (Vibracell 75185). However, LUV mimicking plant root plasma membrane and LUV of different lipid phase composition were prepared by extrusion through two stacked Nuclepore polycarbonate membranes of 100 nm pore size. Our plant root plasma membrane model was based on the work of Berglund A.H. et al. (Berglund *et al.*, 2004). We used LUV with lipid composition in mol%: Soy-Phosphatidylcholine: 40; Soy-

Phosphatidylethanolamine: 34; Glucosylceramide: 13; Stigmasterol: 8; β-Sitosterol: 5. During the experiments, vesicles were stored above their melting temperature to avoid fusion events.

Isothermal titration calorimetry (ITC) assays

ITC was performed with a VP-ITC Microcalorimeter (Microcal, Northampton, USA). The calorimeter cell (Volume of 1.4565 mL) was filled with a 10 µM lipopeptide solution in buffer. The syringe was filled with a suspension of LUV at a lipid concentration of 1 mM and a series of injections was performed (Vinj.: 1–15 µL) at constant time intervals (6 min) at 25°C for vesicles obtained by sonication and 23°C for LUV obtained by extrusion. The solution in the titration cell was stirred at 305 RPM. Prior to each analysis, all solutions were degassed using a sonicator bath. The heats of dilution of vesicles were determined by injecting vesicles in buffer and subtracted from the heats determined in the experiments. Data were processed by software Origin 7 (Originlab, Northampton, USA). For lipid size measurements, the average size of SUV suspension was determined at 23°C by Dynamic Light Scattering (DLS) using a Zetasizer nano ZS (Malvern instruments, UK). The ITC data were evaluated by using the model described by (Heerklotz *et al.*, 2001):

The binding coefficient K is given by the equation:

$$K = \frac{R_b}{C_{D,f}} \quad \text{(Eq. 1)}$$

Where R_b is the surfactant-to-lipid ratio (degree of binding) and $C_{D,f}$ the concentration of Surfactin free in solution. If n_L^o and $n_{D,b}$ are referred to the molar amounts of total lipid in the calorimeter cell and bound surfactin, respectively, and $C_{D,b}$ and C_L^o the concentration of bound surfactin and lipid in the calorimeter cell, R_b can be rewritten as:

$$R_b = \frac{n_{D,b}}{n_L^o} = \frac{C_{D,b}}{C_L^o} \quad \text{(Eq. 2)}$$

The total amount of surfactin in the cell, C_D^o, is represented by the following equation:

$$C_D^o = C_{D,b} + C_{D,f} \quad \text{(Eq. 3)}$$

Combining Eq. 2 and Eq.3, Eq.1 can be written as:

$$C_{D,b} = C_D^o \frac{K C_L^o}{1 + K C_L^o} \quad \text{(Eq. 4)}$$

After ith lipid injections the molar amount of bound Surfactin in the calorimeter cell is $n_{D,b}^{(i)}$ and the cumulative heat released is

$$\sum_{k=1}^{i} \delta h_k = n_{D,b}^{(i)} \Delta H_D^{w \to b} = \Delta H_D^{w \to b} C_{D,b}^{(i)} V_{cell} = \Delta H_D^{w \to b} V_{cell} C_D^o \frac{K C_L^o}{1 + K C_L^o} \quad \text{(Eq. 5)}$$

Where $\Delta H_D^{w \to b}$is the molar enthalpy change corresponding to the transfert of Surfactin from the aqueous phase (w) to the bilayer membrane (b).

Thus K and $\Delta H_D^{w \to b}$can be evaluated simultaneously by a fit of the measured cumulative heat as a function of C_L^o.

Theoretical prediction of the behavior of a molecule in the presence of a lipid monolayer composed of one or more lipid components: the big layer method

This method proceeds in two steps. Firstly the calculation of paired interactions between the molecules; then the construction of a grid of n x n (n between 200 and 600) molecules, taking the molar ratios account.

The first step is derived from the hypermatrix method described elsewhere (Brasseur *et al.*, 1987). The molecules of the systems studied (surfactin, POPC, DPPC, sphingomyelin and cholesterol) are first oriented at the interface, taking their hydrophobic and hydrophilic centres into account (Brasseur, 1986). For each pair of molecules (for example for surfactin/POPC/DPPC system: surfactin/POPC, surfactin/DPPC, surfactin/surfactin, POPC/POPC, DPPC/DPPC, POPC/DPPC), the interaction energies (sum of electrostatic, Van der Waals, hydrophobic energies) are calculated for a large number of positions, resulting from translations and rotations of one molecule toward the other. In this study, molecules undergo 36 rotations around themselves with a precision of 10°. For each of these positions, horizontal and vertical translations are carried out on a distance of 10 and 5 Å with a step of 0.5 and 0.25 Å, respectively. For all those positions, an additional tilt of -10° to +10° by step of 0.5° is further applied. The total number of relative positions tested is thus more than 23 400 000 000 (36x36x21x21x41). For each pair of molecules, the statistical Boltzmann energy is considered. The latter is calculated taking into account a Boltzmann statistics corresponding to the sum of the interaction energy of each relative position tested multiplied by the probability of the position. This interaction energy matrix is then used in the second step.

The second step consists in the construction and minimization of the selected system using the interaction matrix calculated in step 1. In our case, n=200, a grid of 40 000 molecules, initially positioned at random, is constructed and the energy of the system is calculated. The energy of one molecule is equal to the sum of the energies with its 24 closest neighbours in the grid. Random permutations are made and the energy of the new configuration is calculated. By a monte carlo

procedure, this new configuration is kept or not, as a function of the energy difference between the two states. For a grid of 40 000 molecules, one calculation step consists in 40 000 permutations. 50 000 to 100 000 steps are carried out. For the molecules at the border of the grid, the molecules at the opposite border are considered as their closest neighbours, avoiding border limits. The calculations are repeated at least three times independently.

Graphically, each molecule type is represented by a colored point and all the points are represented on the grid. This permits to visually observe the preferential interactions and phase separation between the molecules studied.

Preparation and purity of tobacco plasma membranes

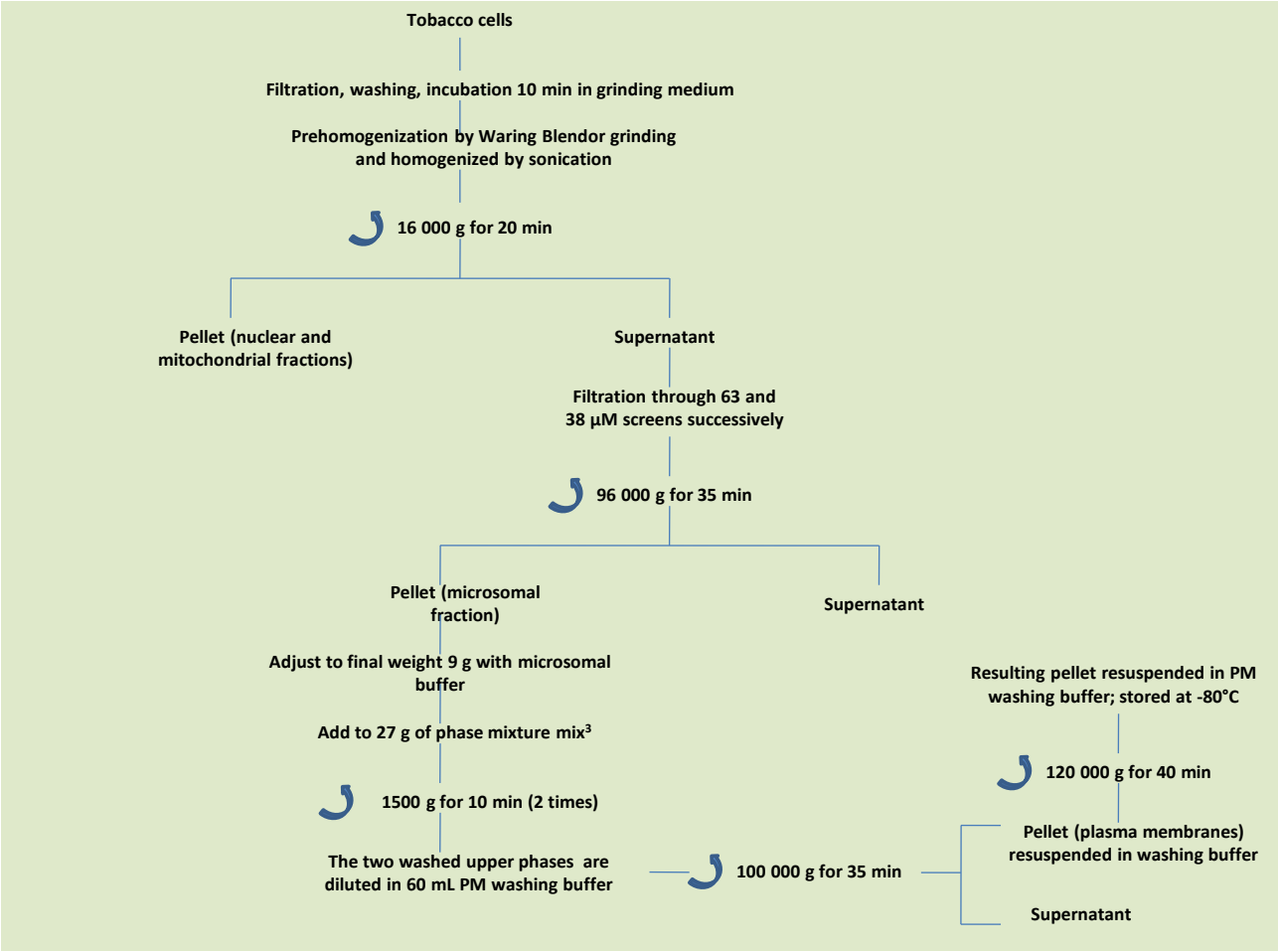


Figure 36 : Procedure of extraction of PM and DRM from tobacco BY-2 cell suspension cultures.

All steps were performed at 4°C. Cells were collected by filtration, and homogenized with a Waring Blendor in grinding medium (50 mM Tris-MES, pH 8.0, 500 mM sucrose, 20 mM EDTA, 10 mM DTT, and 1 mM PMSF). The homogenate was centrifuged at 16 000 g for 20 min. After centrifugation, supernatants were collected, filtered through two successive screens (63 and 38 µm), and centrifuged at 96 000 g for 35 min. This microsomal fraction was purified by partitioning in an

aqueous two-phase system (polyethylene glycol 3350/dextran T-500; 6.4% each) to obtain the PM fraction. Protein content of the plasma membrane preparations was measured according to Bradford's method, using BSA as standard. Protein yield was between 1.0–2.5 mg of protein/100 g fresh weight. Purity was determined by using marker ATPases of PM and other cell compartments. Basal ATPase activity was measured at pH 6.7 and 7.5, vanadate (0.5 mM)-sensitive ATPase activity at pH 6.7 for plasma membrane; azide (0.5 mM)-sensitive ATPase activity at pH 7.5 for mitochondria and nitrate (50 mM)-sensitive ATPase activity at pH 6.7 for tonoplast. P_i concentration was measured with the ATPase assay kit from Innova Biosciences. 100 µL of substrate containing ~5 µg of membrane proteins was added to 100 µL of buffer/reagents at two different pH mix for 30 min. The activity was measured by reading the absorbance at 620 nm.

Isolation of Detergent-Resistant Membranes

Plasma membranes were resuspended in a buffer containing 50 mM Tris-HCl, pH 7.4, 3 mM EDTA, and 1 mM 1,4-dithiothreitol and treated with 1% Triton X-100 (w/v) for 30 min on ice with very gentle shaking every 10 min. Solubilized membranes were recovered after centrifugation at 100 000 g for 45 min, diluted in buffer, and centrifuged again for 4 h at 100 000 g. The pellet was resuspended in buffer, and protein concentrations were determined using the Bradford reagent with BSA as standard.

Proteomic analysis

The 6 samples replicates were all quantified using RCDC kit (BioRad). All the samples were treated in parallel and peptides digests were extracted in acidic conditions and further purified at saturation on C18 ZipTip 5 µg capacity (Millipore). The eluted peptides were finally placed in basic conditions: ammonium formiate 100 mM, adjust at pH 10 with ammonia, and spiked with a commercial mix of 4 proteins peptides digests used as internal standards prior injection (MPDS mix standard E1 or E2 - Waters). The theoretical quantity of peptides analyzed per sample, was set at 2.5 µg of protein equivalent, spiked with 150 fmoles in ADH of MPDS mix standard (Waters). Sample DRMT were spiked with MPDS mix1 and DRMS with MPDS mix2. All samples were injected on the 2D-*nano* Aquity UPLC (Waters) coupled online with the *q*-TOF Synapt HDMSTM G1 system (Waters). The configuration of the 2D-*nano* UPLC system was a reverse phase pH 10 / reverse phase pH3 based 2D separation. The first dimension separation was made on a X-Bridge BEH C18 5 µm column (300 µm _ 50 mm). The trap column Symmetry C18 5 µm (180 µm-20 mm) and analytical column BEH C18 1.7 µm (75 µm-250 mm) (all Waters) were used after an online dilution to lower pH value (pH 3). The samples were loaded at 2 µL/min (20 mM ammonium

formiate, pH 10) on the first column and subsequently eluted in 5 steps (10, 14, 16, 20 and 65% acetonitrile). Each eluted fraction was desalted on the trap column after a 10 times online dilution to pH 3 and subsequently separated on the second analytical column; flow rate 300 nL/min, solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), gradient 0 min, 97% A; 90 min, 60% A. The MS acquisition parameters were: data independent, alternate scanning (MSE) mode, 50_1500 m/z range, ESI +, V optics, scan time 1 s, cone 30 V and lock mass [Glu1]-Fibrinopeptide B ($[M+2H]^{2+}$: 785.84206 m/z). Raw data were processed (deconvoluted, deisotoped) and the protein identification and relative quantification were done using ProteinLynx Global SERVER (PLGS) v2.5. The processing parameters were: MS TOF resolution and the chromatographic peak width were set to automatic, low-/elevated- energy detection threshold to 250/100 counts, identification intensity threshold to 1500 counts and lock mass window to 785.84206 m/z +/- 0.40 Da. Protein identification was performed using the data base extracted from UNIPROT *Nicotiana tabacum*.

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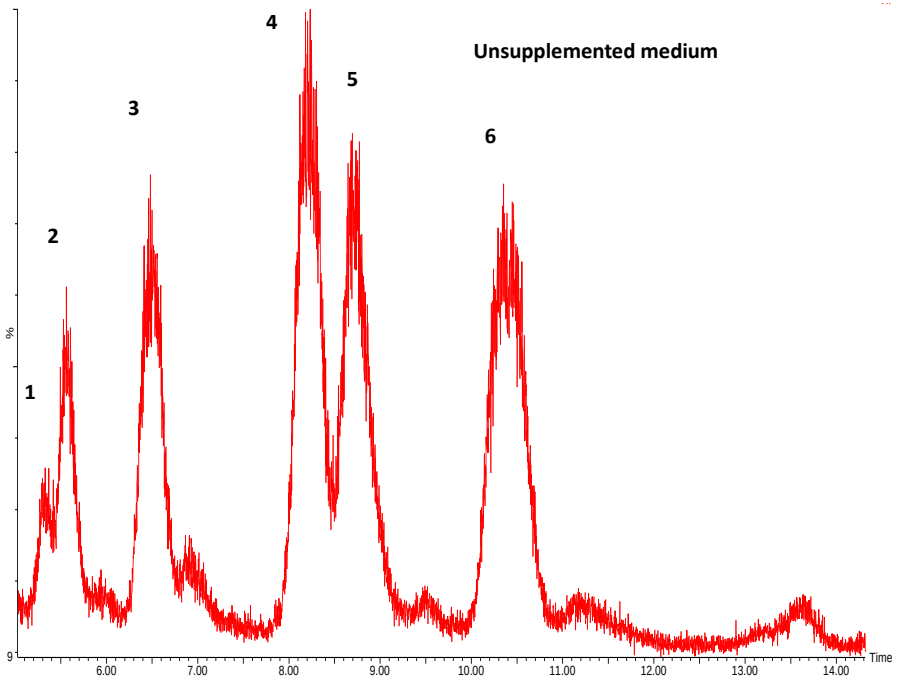
Annexes

I. Characterization of surfactin isoforms



	Amino acid molar ratio				
	Leu	**Val**	**Ileu**	**Glu**	**Asp**
Peak 1	3.9	1.0	0.0	1.0	0.9
Peak 2	4.5	0.8	0.0	1.1	1.2
Peak 3	3.8	1.1	0.0	1.2	0.9
Peak 4	4.1	1.3	0.0	1.2	0.8
Peak 5	3.9	1.1	0.0	0.9	1.3
Peak 6	3.7	0.8	0.0	1.0	1.2

Figure 37 : LC-MS chromatogram of surfactin isoforms from *Bacillus amyloliquefaciens* S499 grown in unsupplemented medium. Table details the amino acid molar ratio within the peptide cycle of surfactin isoforms predicted after acid hydrolysis.

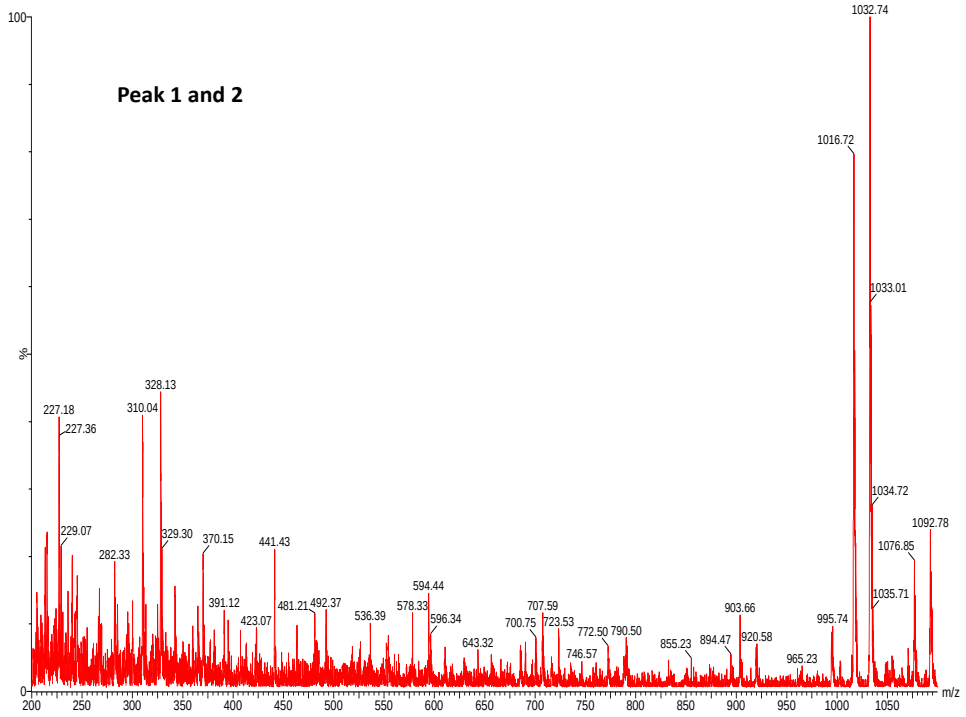


Figure 38 : Fragmentation MS spectrum of compound corresponding to peak 1 and 2 produced in unsupplemented medium. Table details fragment ions giving amino acid sequence information.

m/z	Fragment ion
994.7	$[(CH_3\text{-}(CH_2)_8\text{-CHO-CH2-CO}\rightarrow\text{Glu}\rightarrow\text{Leu}\rightarrow\text{Leu}\rightarrow\text{Val}\rightarrow\text{Asp}\rightarrow\text{Leu}\rightarrow\text{Leu}) + H]^+$
1016.7	$[(CH_3\text{-}(CH_2)_8\text{-CHO-CH2-CO}\rightarrow\text{Glu}\rightarrow\text{Leu}\rightarrow\text{Leu}\rightarrow\text{Val}\rightarrow\text{Asp}\rightarrow\text{Leu}\rightarrow\text{Leu}) + Na]^+$
1032.7	$[(CH_3\text{-}(CH_2)_8\text{-CHO-CH2-CO}\rightarrow\text{Glu}\rightarrow\text{Leu}\rightarrow\text{Leu}\rightarrow\text{Val}\rightarrow\text{Asp}\rightarrow\text{Leu}\rightarrow\text{Leu}) + K]^+$
903.7	1016.7-Leu
790.5	903.7-Leu
707.6	$[(NaOH_2$ Leu-Leu-Asp-Val-Leu-Leu) + $H]^+$
594.4	$[(NaOH_2$ Leu-Leu-Asp-Val-Leu) + $H]^+$
481.2	$[(NaOH_2$ Leu-Leu-Asp-Val) + $H]^+$

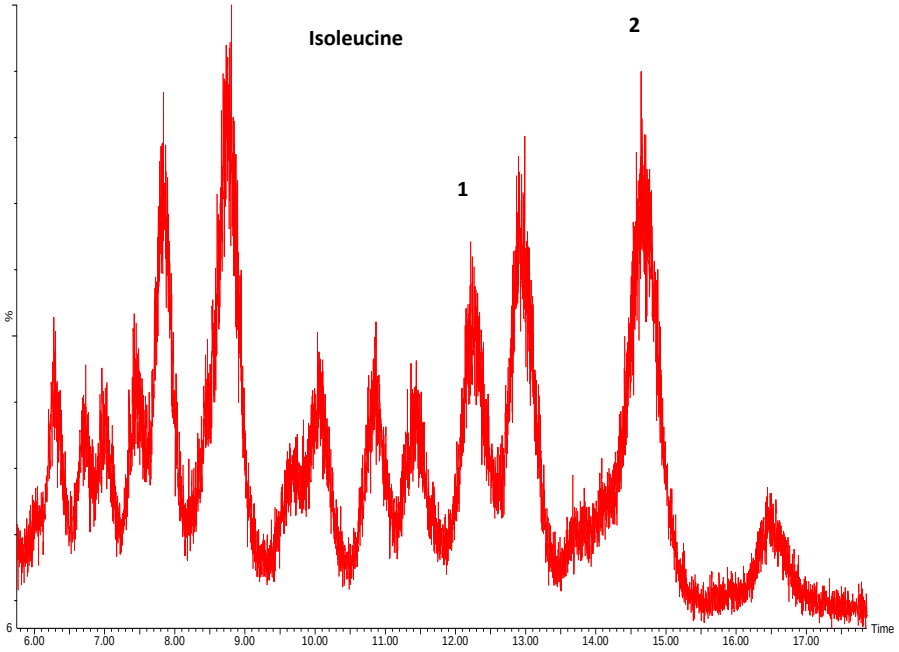


Figure 39 : LC-MS chromatogram of surfactin isoforms from *Bacillus amyloliquefaciens* S499 grown in isoleucine supplemented medium. Table details the amino acid molar ratio within the peptide cycle of surfactin isoforms predicted after acid hydrolysis.

	Amino acid molar ratio				
	Leu	**Val**	**Ileu**	**Glu**	**Asp**
Peak 1	3.6	1.2	1.9	1.3	1.2
Peak 2	3.8	1.2	2.2	1.1	1.4

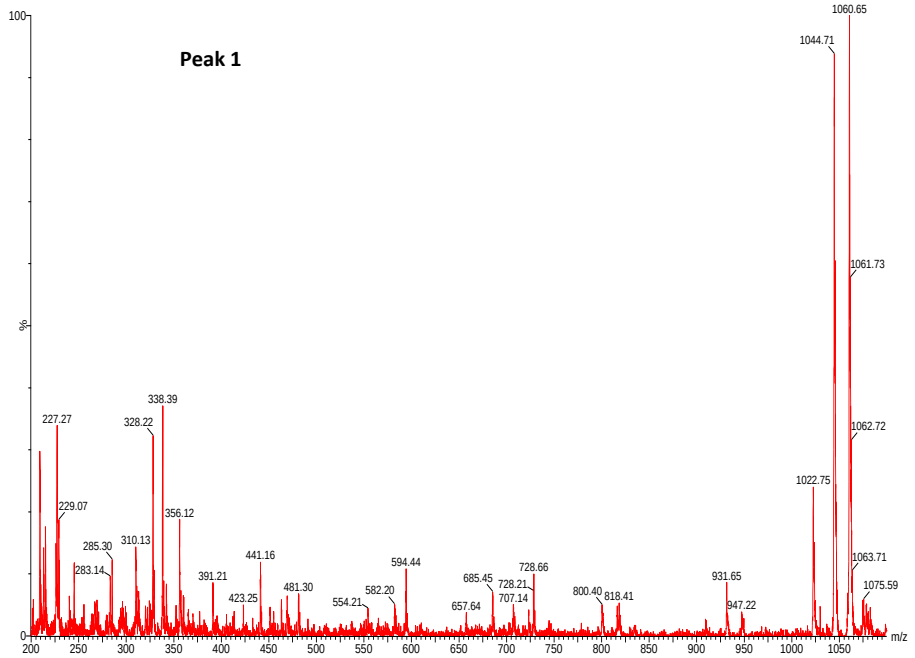


Figure 40 : Fragmentation MS spectrum of compound corresponding to peak 1 produced in isoleucine supplemented medium. Table details fragment ions giving amino acid sequence information.

m/z	Fragment ion
1022.7	$[(CH_3\text{-}(CH_2)_{10}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu/Ileu\rightarrow Leu/Ileu\rightarrow Val\rightarrow Asp\rightarrow Leu/Ileu\rightarrow Leu/Ileu) + H]^+$
1044.7	$[(CH_3\text{-}(CH_2)_{10}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu/Ileu\rightarrow Leu/Ileu\rightarrow Val\rightarrow Asp\rightarrow Leu/Ileu\rightarrow Leu/Ileu) + Na]^+$
1060.7	$[(CH_3\text{-}(CH_2)_{10}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu/Ileu\rightarrow Leu/Ileu\rightarrow Val\rightarrow Asp\rightarrow Leu/Ileu\rightarrow Leu/Ileu) + K]^+$
931.6	1044.7-Leu/Ileu
818.4	931.6-Leu/Ileu
594.4	$[(NaOH_2$ Leu/Ileu-Leu/Ileu-Asp-Val-Leu/Ileu) + $H]^+$
481.3	$[(NaOH_2$ Leu/Ileu-Leu/Ileu-Asp-Val) + $H]^+$

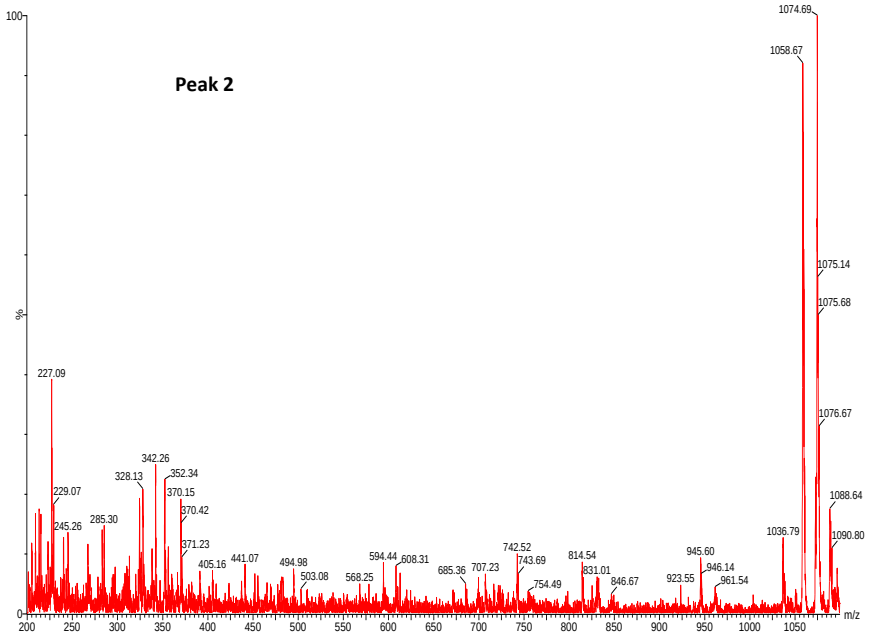


Figure 41 : Fragmentation MS spectrum of compound corresponding to peak 2 produced in isoleucine supplemented medium. Table details fragment ions giving amino acid sequence information.

m/z	Fragment ion
1036.7	$[(CH_3\text{-}(CH_2)_{11}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu/Ileu\rightarrow Leu/Ileu\rightarrow Val\rightarrow Asp\rightarrow Leu/Ileu\rightarrow Leu/Ileu) + H]^+$
1058.8	$[(CH_3\text{-}(CH_2)_{11}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu/Ileu\rightarrow Leu/Ileu\rightarrow Val\rightarrow Asp\rightarrow Leu/Ileu\rightarrow Leu/Ileu) + Na]^+$
1074.7	$[(CH_3\text{-}(CH_2)11\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu/Ileu\rightarrow Leu/Ileu\rightarrow Val\rightarrow Asp\rightarrow Leu/Ileu\rightarrow Leu/Ileu) + K]^+$
945.6	1058.8-Leu/Ileu
832.5	945.6-Leu/Ileu
707.5	$[(NaOH_2$ Leu/Ileu-Leu/Ileu-Asp-Val-Leu/Ileu-Leu/Ileu) + $H]^+$
594.3	$[(NaOH_2$ Leu/Ileu-Leu/Ileu-Asp-Val-Leu/Ileu) + $H]^+$

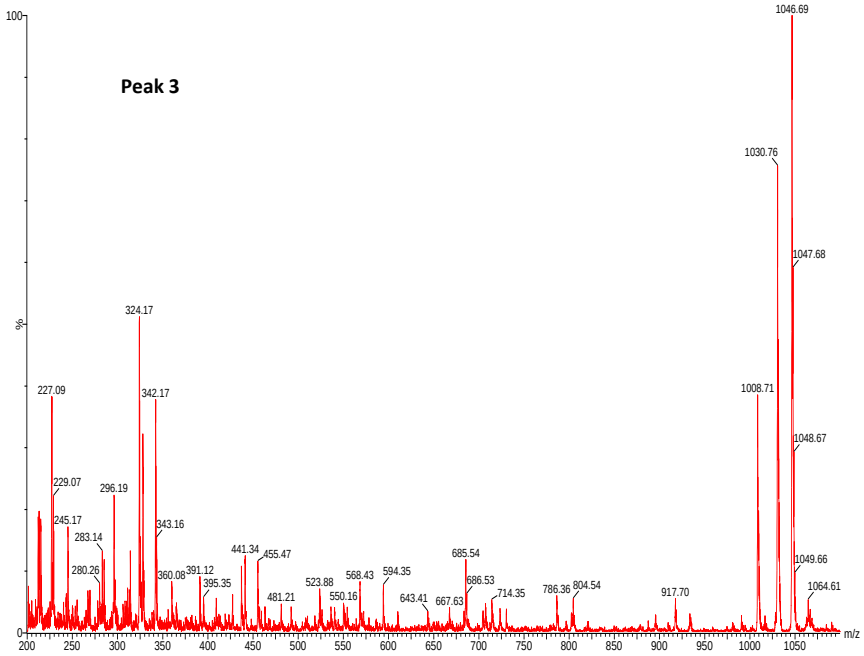


Figure 42 : Fragmentation MS spectrum of compound corresponding to peak 3 produced in unsupplemented medium. Table details fragment ions giving amino acid sequence information.

m/z	Fragment ion
1008.8	$[(CH_3\text{-}(CH_2)_9\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Leu) + H]^+$
1030.7	$[(CH_3\text{-}(CH_2)_9\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Leu) + Na]^+$
1046.7	$[(CH_3\text{-}(CH_2)_9\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Leu) + K]^+$
917.7	1030.7-Leu
804.5	917.7-Leu
594.4	$[(NaOH_2$ Leu-Leu-Asp-Val-Leu) + $H]^+$
481.2	$[(NaOH_2$ Leu-Leu-Asp-Val) + $H]^+$

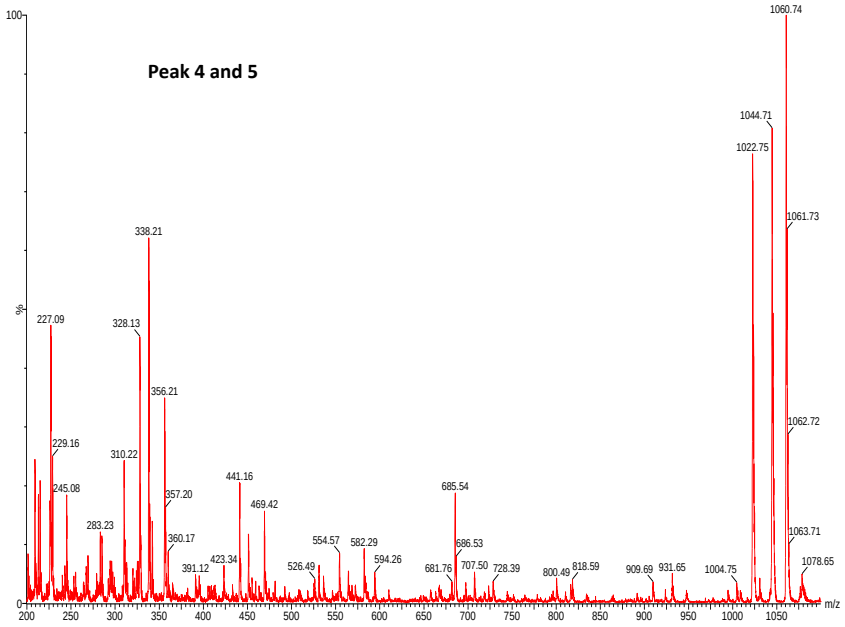


Figure 43 : Fragmentation MS spectrum of compound corresponding to peak 4 and 5 produced in unsupplemented medium. Table details fragment ions giving amino acid sequence information.

m/z	Fragment ion
1022.8	$[(CH_3\text{-}(CH_2)_{10}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Leu) + H]^+$
1044.7	$[(CH_3\text{-}(CH_2)_{10}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Leu) + Na]^+$
1060.7	$[(CH_3\text{-}(CH_2)_{10}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Leu) + K]^+$
931.6	1044.7-Leu
818.6	931.6-Leu
707.5	$[(NaOH_2$ Leu-Leu-Asp-Val-Leu-Leu) + $H]^+$
594.2	$[(NaOH_2$ Leu-Leu-Asp-Val-Leu) + $H]^+$

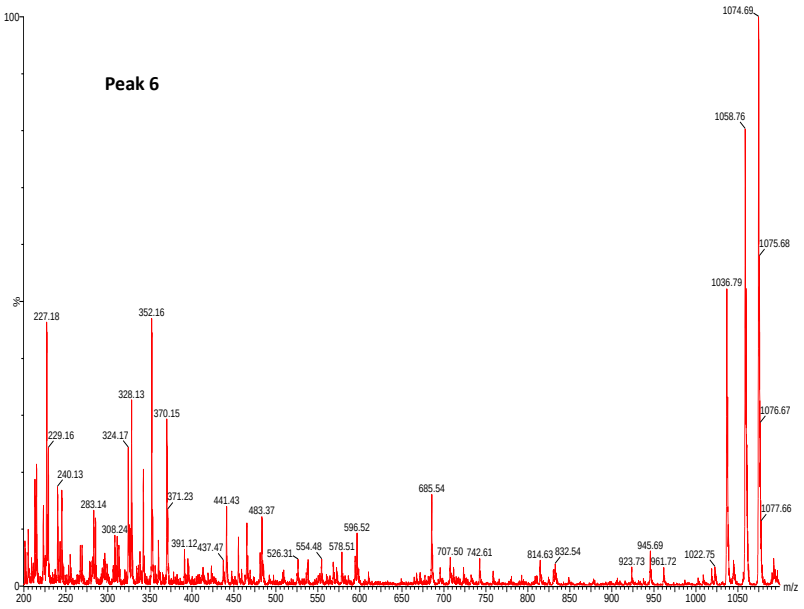


Figure 44 : Fragmentation MS spectrum of compound corresponding to peak 6 produced in unsupplemented medium. Table details fragment ions giving amino acid sequence information.

m/z	Fragment ion
1036.7	$[(CH_3\text{-}(CH_2)_{11}\text{-CHO-}CH_2\text{-CO}\rightarrow\text{Glu}\rightarrow\text{Leu}\rightarrow\text{Leu}\rightarrow\text{Val}\rightarrow\text{Asp}\rightarrow\text{Leu}\rightarrow\text{Leu}) + H]^+$
1058.8	$[(CH_3\text{-}(CH_2)_{11}\text{-CHO-}CH_2\text{-CO}\rightarrow\text{Glu}\rightarrow\text{Leu}\rightarrow\text{Leu}\rightarrow\text{Val}\rightarrow\text{Asp}\rightarrow\text{Leu}\rightarrow\text{Leu}) + Na]^+$
1074.8	$[(CH_3\text{-}(CH_2)_{11}\text{-CHO-}CH_2\text{-CO}\rightarrow\text{Glu}\rightarrow\text{Leu}\rightarrow\text{Leu}\rightarrow\text{Val}\rightarrow\text{Asp}\rightarrow\text{Leu}\rightarrow\text{Leu}) + K]^+$
961.7	1074.8-Leu
945.7	1058.8-Leu
832.5	945.7-Leu
707.5	$[(NaOH_2$ Leu-Leu-Asp-Val-Leu-Leu) + $H]^+$

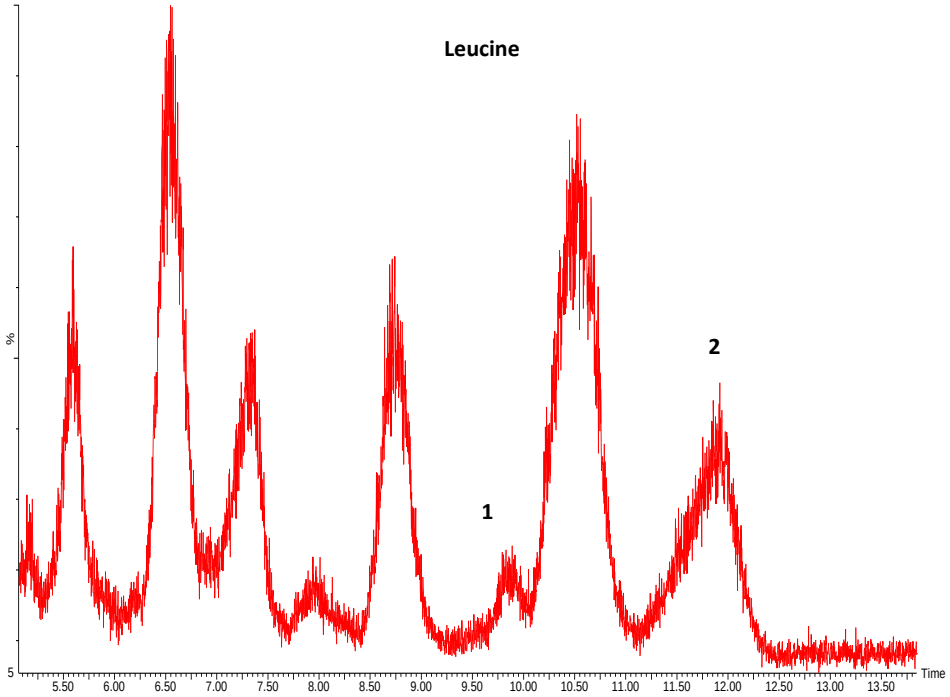


Figure 45 : LC-MS chromatogram of surfactin isoforms from *Bacillus amyloliquefaciens* S499 grown in leucine supplemented medium. Table details the amino acid molar ratio within the peptide cycle of surfactin isoforms predicted after acid hydrolysis.

	Amino acid molar ratio				
	Leu	**Val**	**Ileu**	**Glu**	**Asp**
Peak 1	4.9	0.0	0.0	1.1	1.2
Peak 2	4.8	0.0	0.0	1.2	1.3

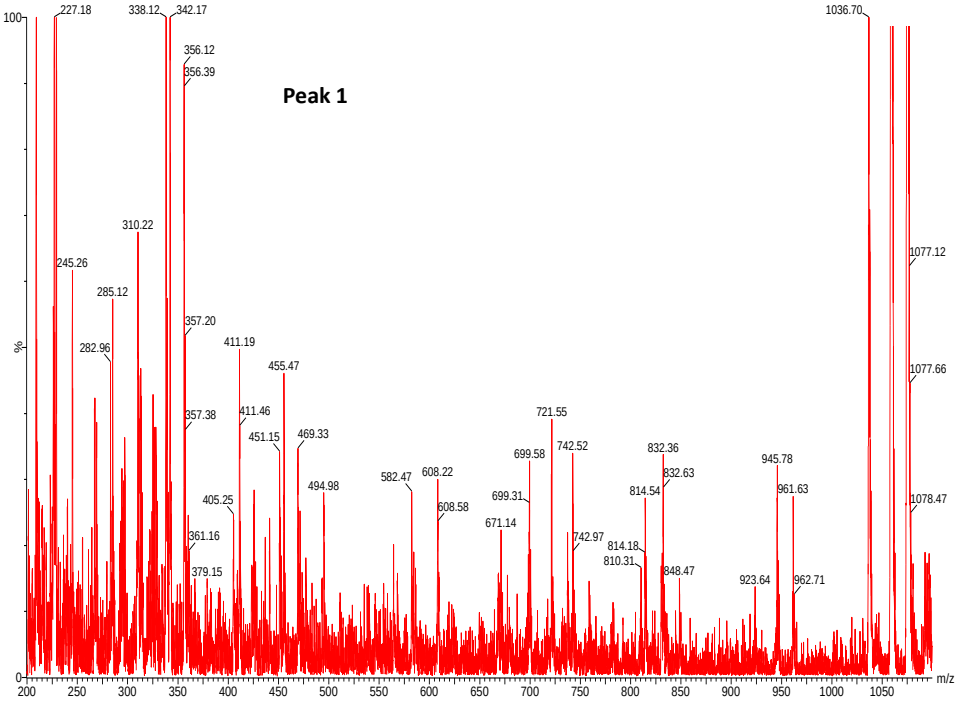


Figure 46 : Fragmentation MS spectrum of compound corresponding to peak 1 produced in leucine supplemented medium. Table details fragment ions giving amino acid sequence information.

m/z	Fragment ion
1036.7	$[(CH_3\text{-}(CH_2)_{10}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Leu\rightarrow Asp\rightarrow Leu\rightarrow Leu) + H]^+$
1074.7	$[(CH_3\text{-}(CH_2)_{10}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Leu\rightarrow Asp\rightarrow Leu\rightarrow Leu) + K]^+$
961.6	1074.7 -Leu
848.5	961.6-Leu
721.5	$[(NaOH_2$ Leu-Leu-Asp-Leu-Leu-Leu) + $H]^+$
608.2	$[(NaOH_2$ Leu-Leu-Asp-Leu-Leu) + $H]^+$

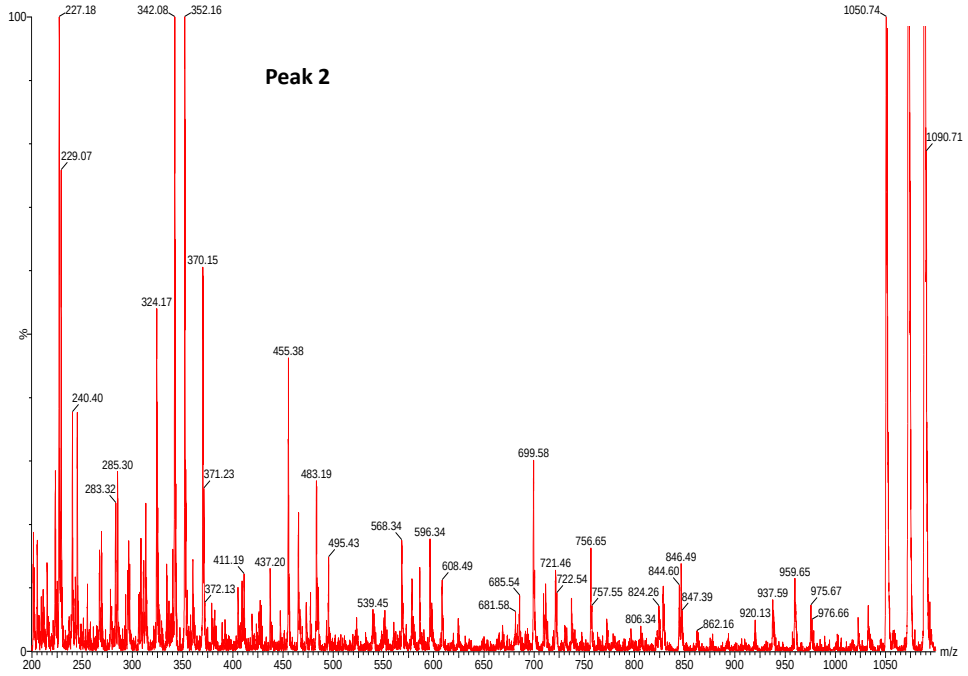


Figure 47 : Fragmentation MS spectrum of compound corresponding to peak 2 produced in leucine supplemented medium. Table details fragment ions giving amino acid sequence information.

m/z	Fragment ion
1050.7	$[(CH_3\text{-}(CH_2)_{11}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Leu\rightarrow Asp\rightarrow Leu\rightarrow Leu) + H]^+$
1072.7	$[(CH_3\text{-}(CH_2)_{11}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Leu\rightarrow Asp\rightarrow Leu\rightarrow Leu) + Na]^+$
959.7	1072.7-Leu
846.5	959.7-Leu
721.5	$[(NaOH_2$ Leu-Leu-Asp-Leu-Leu-Leu) + $H]^+$
495.4	$[(NaOH_2$ Leu-Leu-Asp-Leu) + $H]^+$

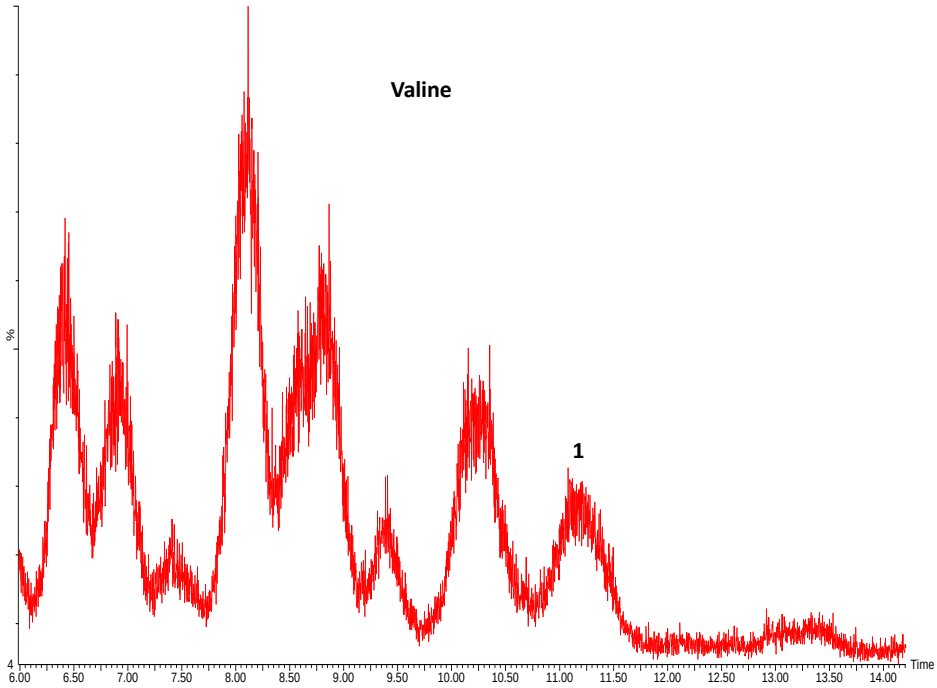


Figure 48 : LC-MS chromatogram of surfactin isoforms from *Bacillus amyloliquefaciens* S499 grown in valine supplemented medium. Table details the amino acid molar ratio within the peptide cycle of surfactin isoforms predicted after acid hydrolysis.

	Amino acid molar ratio				
	Leu	**Val**	**Ileu**	**Glu**	**Asp**
Peak 1	2.9	1.8	0.0	1.2	1.0

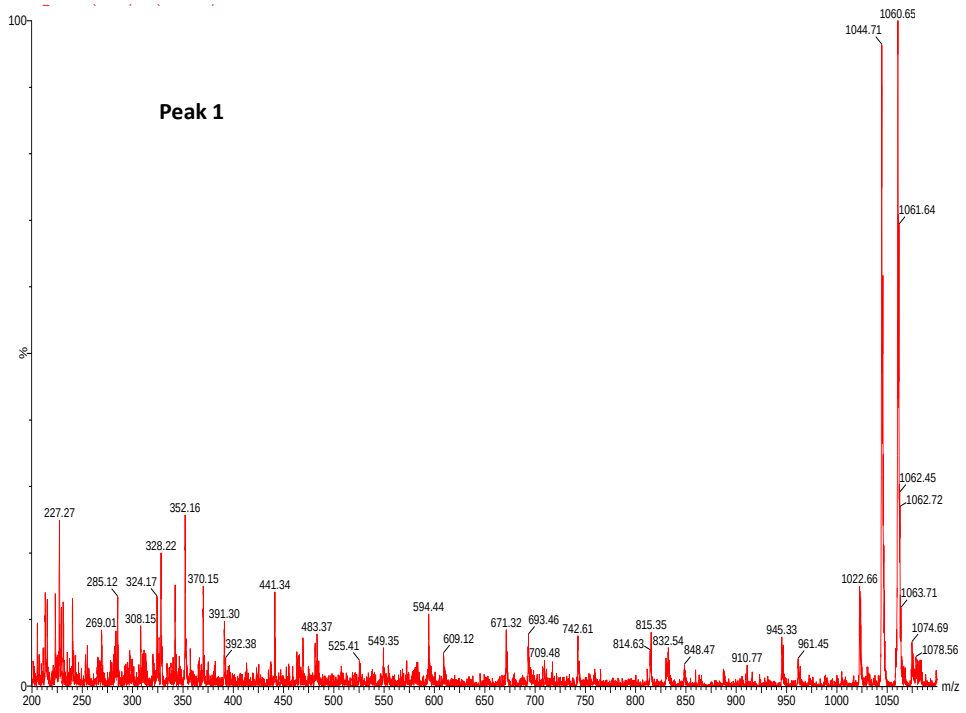


Figure 49 : Fragmentation MS spectrum of compound corresponding to peak 1 produced in valine supplemented medium. Table details fragment ions giving amino acid sequence information.

m/z	Fragment ion
1022.8	$[(CH_3\text{-}(CH_2)_{11}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Val) + H]^+$
1044.7	$[(CH_3\text{-}(CH_2)_{11}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Val) + Na]^+$
1060.7	$[(CH_3\text{-}(CH_2)_{11}\text{-}CHO\text{-}CH_2\text{-}CO\rightarrow Glu\rightarrow Leu\rightarrow Leu\rightarrow Val\rightarrow Asp\rightarrow Leu\rightarrow Val) + K]^+$
945.3	1044.7-Val
832.5	945.3-Leu
693.5	$[(NaOH_2$ Leu-Leu-Asp-Leu-Leu) + H]$^+$
594.4	$[(NaOH_2$ Leu-Leu-Asp-Leu-Leu) + H]$^+$

II. Scientific communications

a. Publications

PAMPs, MAMPs, DAMPs and others: the diversity of elicitors of the plant immunity as promising unexploited biocontrol agents
Publication year : 2012
Author(s): Henry, G., Thonart, P., Ongena, M.
Journal – Impact factor: Biotechnologie, Agronomie, Société et Environnement (BASE) – 0.454
Volume : 16(2)- Pages : 257-268
Digital publishing: http://www.bib.fsagx.ac.be/base/home/

The bacterial lipopeptide surfactin targets the lipid fraction of plasma membrane to trigger immune-related defense response in the host plant
Publication year: 2011
Author(s) : G. Henry, M. Deleu, E. Jourdan, P. Thonart and M. Ongena,
Journal – Impact factor: Cellular Micobiology – 5.625
Volume : 13(11)- Pages : 1824-1837
Digital publishing : http://onlinelibrary.wiley.com/doi/10.1111/j.1462-5822.2011.01664.x/abstract

***Bacillus*-based biocontrol of *fusarium* disease on tomato cultures in Burundi.**
Publication year : 2009
Author(s): Nihorimbere V, Ongena M, Cawoy H, Henry G, Brostaux Y, Kakana P, Thonart P.
Journal – Impact factor: Communications in Agricultural and Applied Biological Sciences. – Impact factor 2012 still computing
Volume : 74(3) - Pages : 645-649.
Digital publishing: -

Insights into the defense-related events occurring in plant cells following perception of surfactin-type lipopeptide from *Bacillus subtilis*
Publication year: 2009
Author(s): E. Jourdan, G. Henry, F. Duby,J., Dommes,J., P. Barthélemy, P. Thonart, and M. Ongena
Journal – Impact factor : Molecular Plant-Microbe Interactions - 4.275
Volume : 22(4) - Pages : 456-468
Digital publishing: http://apsjournals.apsnet.org/loi/mpmi

The roles of cyclic lipopeptides in the biocontrol activity of *Bacillus subtilis*
Publication year : 2009
Author(s): Ongena M., Henry G., and Thonart P.
Journal – Impact factor: Recent Developments in Disease Management -
Volume : 1 - Pages : 59-69
Digital publishing: http://www.springerlink.com/content/l124w3l682732366/

b. Symposium & Congress

The 9th International Congress of Plant Pathology (*ICPP*)
The role of lipopeptides in the biocontrol of *Bacillus subtilis*
Orators(s): M. Ongena, G. Henry, V. Nihorimbere, E. Jourdan, A. Adam and P.Thonart.
Place: Turin
Country: Italy
Date: 24/08/2008
Written intervention (Poster) + oral communication (Ongena M.)

Meeting of the IOBC/WPRS : workshop Molecular Tools for Understanding and Improving Biocontrol
***Bacillus* lipopeptides as MAMPs for non-pathogenic bacteria perception and defense responses elicitation in plant cells**
Orators(s): G. Henry, E. Jourdan, P. Thonart and M. Ongena
Place: Interlaken
Country: Switzerland
Date : 10/09/2008
Written intervention (Poster) + oral communication

XIV International Congress on Molecular Plant-Microbe Interactions
Insights into the plant defense mechanisms induced by *Bacillus* lipopeptides
Orators(s): M. Ongena, G. Henry, A. Adam, E. Jourdan, P. Thonart
Place: Quebec
Country : Canada
Date : 19/07/2009
Digital publishing: http://www.ismpminet.org/meetings/abstracts/2009/c09ma72.asp
Written intervention (Poster) + oral communication (Ongena M.)

XIV International Congress on Molecular Plant-Microbe Interactions
***Bacillus subtilis* cyclic lipopeptides as elicitors: How are they perceived by plant cells?**
Orators(s): G. Henry, E. Jourdan, P. Thonart, M. Ongena
Place : Quebec
Country : Canada
Date : 19/07/2009
Digital publishing: http://www.ismpminet.org/meetings/abstracts/2009/p09ma227.asp
Written intervention (Poster)

13th International Conference on Organized Molecular Films
From cells to model membranes: study of the mechanism of action of surfactin, a bacterial elicitor of defence mechanisms in plants
Orators(s): G. Henry, M. Deleu, M. Ongena
Place: Quebec
Country: Canada
Date: 20 /07/2010
Written intervention (Poster)

63rd International Symposium on Crop Protection
Rhizobacteria-generated cyclic lipopeptides as inducers of plant systemic immunity
Orators(s): G. Henry, P. Thonart and M. Ongena
Place: Ghent

Country: Belgium
Date : 24/05/2011
Written intervention (Poster) + oral communication

11ème édition du Bioforum
Defence responses induced in tobacco cells by *Bacillus* lipopeptides acting as elicitors of systemic resistance in plants
Orators(s): G. Henry, E. Jourdan, M. Ongena and P. Thonart
Place: Liège
Country : Belgium
Date : 11/10/2007
Written intervention (Poster)

FNRS Contact Group Meeting: "From gene to function". Plants and their soil environment
***Bacillus subtilis* cyclic lipopeptides (cLPs) as elicitors: how are they perceived by plant cells?**
Orators(s): G. Henry, E. Jourdan, M. Ongena and P. Thonart
Place: Gembloux
Country: Belgium
Date: 02/04/2009
Written intervention (Poster) + oral communication

Sustainable plant management using biosourced polymers : when chemistry meets plant biology
Molecular Interactions and defense events induced following perpcetion of *Bacillus* lipopeptides by plant cells
Orators(s): G. Henry, P. Thonart and M. Ongena and
Place: Université de Reims Champagne Ardenne
Country: France
Date: 14/10/2009
Written intervention (Poster) + oral communication (Ongena M.)