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# Dialogue moléculaire entre les rhizobactéries et leur hôte végétal : deux nouveaux éliciteurs impliqués dans l'induction de résistance aux pathogènes.

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Dissertation présentée par

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## *Résumé*

Lors du phénomène appelé « résistance systémique induite » (ISR), des rhizobactéries non pathogènes peuvent conférer à la plante un certain degré de protection à des attaques ultérieures par un phytopathogène via la stimulation de mécanismes de défense systémiques. Cette « immunité » s'initie suite à la perception par la plante de molécules dites élicitrices produites par le microorganisme bénéfique. L'objectif de cette thèse est de contribuer à la caractérisation de ce dialogue moléculaire en se basant sur des études antérieures poursuivies au laboratoire qui ont mis en évidence la capacité des souches *Pseudomonas putida* BTP1 et *Bacillus subtilis* S499 (aussi dénommée M4) à induire l'ISR chez leur hôte végétal.

La première partie des recherches sur BTP1 a permis d'isoler en tant qu'éliciteur un nouveau type de composé produit par les *Pseudomonas*. Cet éliciteur a été isolé à partir de surnageant de culture de la bactérie et est constitué d'un noyau benzylamine dont l'azote est alkylé par deux groupements méthyle et un groupement tétradécyle, responsables de l'hydrophobicité relative de la molécule. Sur cette base, ce métabolite a été dénommé NABD (N-alkylated benzylamine derivative). Grâce au traitement des plantes avec le composé purifié ou via l'utilisation de mutants altérés dans sa production, nous avons démontré que le NABD était responsable de l'essentiel de l'activité ISR de BTP1 sur le haricot et le concombre. Dans le cas de la tomate par contre, il semble qu'il ne soit pas le seul éliciteur impliqué. En ce qui concerne *Bacillus subtilis* S499, en utilisant des approches similaires, nous avons pour la première fois montré l'implication de deux lipopeptides, la surfactine et la fengycine, comme agents stimulateurs de l'ISR, permettant ainsi de leur attribuer un nouveau rôle dans le contrôle biologique des maladies des plantes par *Bacillus*. Une étude de la relation structure/activité a été entamée et a révélé un rôle du noyau aromatique dans l'établissement de la résistance systémique par le NABD. Pour la surfactine, l'importance de la longueur de la chaîne alkylée ainsi que la présence des charges sur les acides aminés suggèrent que son activité élicitrice soit liée à ses propriétés amphiphiles.

La détection en quantités significatives des deux types d'éliciteurs dans la rhizosphère des plants traités par les bactéries est également en faveur de leur rôle biologique. Dans le cas de BTP1, nous avons étudié l'influence de certains paramètres physiologiques, nutritionnels et physico-chimiques sur la production du NABD. Les résultats montrent que la production du NABD est dépendante de la présence d'acides aminés dans le milieu, qu'elle est effective à un taux de croissance faible du microorganisme, qu'elle est négativement influencée par le fer mais qu'elle n'est pas inhibée par une restriction en oxygène dissous disponible ou par une

acidification du milieu. Globalement, ces données suggèrent que les conditions physico-chimiques dictant l'état physiologique des cellules bactériennes *in situ* peuvent être propices à une synthèse effective de l'éliciteur au niveau des racines.

Des recherches sur les réponses induites chez l'hôte lors de son interaction avec S499 ont été initiées et montrent une nette augmentation d'activités enzymatiques de la voie des oxylipines (activités lipoxygénase (LOX) et lipide hydroperoxydase) dans les feuilles après infection. Une exploration plus approfondie sur suspensions de cellules de tabac en culture avec la surfactine a montré l'induction de plusieurs autres mécanismes précoces de défenses, comme une alcalinisation du milieu extracellulaire, la production d'espèces oxydantes, l'activation d'enzymes de défense telles que LOX et phenylalanine ammonia lyase (PAL), et une possible réorientation de la voie des phénylpropanoïdes. Ces expérimentations menées avec les lipopeptides de *Bacillus* sont parmi les premières à être réalisées avec des éliciteurs spécifiques de l'ISR. Elles ont abouti à la caractérisation de plusieurs réponses précoces dont certaines sont aussi induites par des molécules isolées d'agents pathogènes. Les lipopeptides pourraient ainsi agir en stressant les cellules de l'hôte via une perturbation temporaire de la membrane externe facilitée par leur propriété amphiphile. L'implication de récepteurs dans la reconnaissance des éliciteurs de BTP1 et S499 ne peut cependant pas être exclue.

En conclusion, les lipopeptides et le NABD élargissent la gamme des éliciteurs bactériens de l'ISR isolés jusqu'à présent et peuvent être considérés comme appartenant à une nouvelle classe d'immuno-stimulateurs des plantes comprenant des molécules amphiphiles.

## *Summary*

In the so-called induced systemic resistance phenomenon (ISR), some non pathogenic rhizobacteria are able to stimulate defence mechanisms in the host plant thereby rendering it less susceptible to subsequent phytopathogen attack. This immunization process is initiated into the plant following the perception of elicitors (or determinants) produced by the beneficial microorganism. Previous studies performed in our laboratory have demonstrated the ability of *Pseudomonas putida* strain BTP1 and *Bacillus subtilis* strain S499 (or M4) at triggering ISR. By using these two rhizobacteria, the global objective of this thesis is to contribute to a better understanding of this molecular dialogue between ISR-inducing bacteria and plant cells.

Our first researches with BTP1 led to the isolation, as an elicitor, of a new compound produced by *Pseudomonas*. It was purified from bacterial culture supernatant and identified as a benzylamine core alkylated with two methyl and one tetradecyl groups, conferring its hydrophobic properties. On the basis of this structure, the BTP1 determinant was called NABD, for N-Alkylated Benzylamine Derivative. By testing the pure molecule or mutant strains altered in its production, we have shown that NABD was mainly responsible for ISR-activity of the BTP1 strain on bean and cucumber while in tomato, another unidentified compound could also be involved. In the case of *Bacillus subtilis* S499, we used similar approaches to demonstrate for the first time that the lipopeptides surfactin and fengycin may act as elicitors to stimulate systemic resistance, thereby attributing them a new role in the biocontrol of plant diseases. Structure/activity studies have revealed the implication of the benzylamine aromatic core in the biological activity of NABD. In the case of surfactin, reduced activity of some homologues indicates that its perception is dictated by structural clues such as the length of the acyl moiety and the presence of charges in the cyclic peptide part.

Also in support to their involvement in ISR triggering, significant quantities of NABD and lipopeptides were recovered from the rhizosphere of bacterized plants. The influence of some physiological and physico-chemical factors on NABD production by BTP1 was further investigated in *in vitro* experiments. Our results show that the molecule is more efficiently produced at low cell growth rate and in the presence of amino acids in the medium but is negatively iron-regulated. Other abiotic factors, such as low oxygen concentration or low pH do not have drastic effects on NABD biosynthesis by the strain. Globally, with regard to specific conditions that the strain undergoes in the rhizosphere environment, it supposes that the nutritional/physiological state of BTP1 cells growing on plant roots is compatible with an effective production of the elicitor.

Plant responses induced following root treatment with *Bacillus subtilis* S499 have also been investigated. Working on whole plants, it first revealed an increase in enzyme activities of

the oxylipin pathway (lipoxygenase (LOX) and lipid-hydroperoxydase) after infection. Further investigations were conducted on cultured tobacco cells and we have shown the induction of some early events, such as extracellular pH alkalinization, reactive oxygen species production, defence enzyme stimulation (LOX and phenylalanine ammonia lyase (PAL), and accumulation of some phenolics from the phenylpropanoid pathway. These experimentations performed with *Bacillus* lipopeptides are the first conducted with ISR-specific elicitors and led to the characterization of early events that can also be triggered by pathogen associated molecules. It is still not clear whether bacterial LPs are recognized by plant cells via specific receptors but the amphiphilic and detergent properties of surfactin strongly suggest that these LPs can interact via a less specific mechanism based on some limited destabilization of the membrane structure.

In conclusion, NABD and lipopeptides studied in this thesis enlarge the range of elicitors from non pathogenic bacteria isolated to date, and may be considered as members of a new class of ISR-inducing compounds of amphiphilic nature.

## *Communications scientifiques*

### *Publications en premier ou co-premier auteur*

§Ongena, M., Jourdan, E., Schäfer, M., Kech, C., Budzikiewicz, H., Luxen, A. and Thonart, P. (2005) Isolation of an N-alkylated benzylamine derivative from *Pseudomonas putida* BTP1 as elicitor of induced systemic resistance in bean. *Molecular Plant-Microbe Interactions* **18**(6): 562-569.

Jourdan, E.[*], Adam, A.[*], Ongena, M., Duby, F., Dommes, J. and Thonart, P. (2005) Resistance induced in cucumber and tomato by a non-pathogenic *Pseudomonas putida* strain. *Parasitica* **61**: 13-22.

Jourdan, E., Ongena, M., Adam, A. and Thonart, P. (2006) Physiological and physico-chemical factors modulating ISR elicitor production by *Pseudomonas putida*. *IOBC/wprs Bulletin* **29**: 45-52.

Jourdan, E., Ongena, M., Adam, A. and Thonart, P. (2007) PGPR-induced systemic resistance: activity of amphiphilic elicitors and structural analogues on different plants species. *IOBC/wprs Bulletin* **30**: 123-126.

§Ongena, M.[*], Jourdan, E.[*], Adam, A., Paquot, M., Brans, A., Joris, B., *et al* (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environmental Microbiology* **9**(4): 1084-1090.

§Ongena, M.[*], Jourdan, E.[*], Adam, A., Schafer, M., Budzikiewicz, H. and Thonart, P. (2008) Amino acids, iron, and growth rate as key factors influencing production of the *Pseudomonas putida* BTP1 benzylamine derivative involved in systemic resistance induction in different plants. *Microbial Ecology* **55**(2): 280-292.

§Jourdan, E.[*], Henry, G.[*], Duby, F., Dommes, J., Thonart, P. and Ongena, M. Surfactin-type lipopeptides as MAMP for non-pathogenic *Bacillus* perception and defence responses elicitation in plant cells. *Molecular Plant-Microbe Interactions* (Submitted).

§Jourdan, E., Ongena, M. and Thonart, P. L'immunisation des plantes par les rhizobactéries en tant que nouvelle arme pour la lutte biologique contre les maladies des cultures. *Cahiers Agriculture* (Submitted).

§Jourdan, E., Ongena, M. and Thonart, P. Caractéristiques moléculaires de l'immunité des plantes induite par les rhizobactéries non pathogènes. *Biotechnologie, Agronomie, Société et Environnement* (Submitted).

### *Publications en co-auteur*

§Ongena, M.[*], Duby, F.[*], Jourdan, E., Beaudry, T., Jadin, V., Dommes, J. and Thonart, P. (2005) *Bacillus subtilis* M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. *Applied Microbiology and Biotechnology* **67**(5): 692-698.

[*] Auteurs ayant contribué de manière égale à l'article.

§ Publications inclues dans cette thèse

***Présentations orales***

International Organization for Biological Control Symposium "Multitrophic interactions in soil", 5-8 June 2005, Wageningen (The Netherlands). "Physiological and physico-chemical factors modulating ISR elicitor production by Pseudomonas putida".

Meeting Ulg-FNRS. "Journée de contact en biologie végétale", 16 march 2006, University of Liège (Belgium). "Elicitation of systemic resistance against microbial diseases induced in plants by non-pathogenic rhizobacteria".

International Organization for Biological Control Symposium "Biological control of fungal and bacterial plant pathogens", 6-10 September 2006, Spa (Belgium). "PGPR-induced systemic resistance: activity of amphiphilic elicitors and structural analogues on different plants species".

Seminar, 21 May 2007, University of Liège (Belgium). "La résistance systémique induite par les PGPR : Activité d'éliciteurs amphiphile et d'analogues de structure sur différentes plantes".

Seminar, 20 December 2007, University of Liège (Belgium). "Les rhizobactéries : une aide à la résistance au stress abiotique chez les plantes? "

***Posters***

Bioforum, 6 December 2004, Liège (Belgium). "Study of a novel elicitor produced by *Pseudomonas putida* BTP1 in the ISR on bean".

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7th International Plant Growth Promoting Rhizobacteria Workshop, 28 may-2 June 2006, Noordwijkerhout (The Netherlands). "Role of lipopeptides in the biological control activity of *Bacillus subtilis*"

Bioforum, 17 may 2006, Liège (Belgium). "New players in the molecular talk associated with plant immunization by non-pathogenic bacteria"

Bioforum, 11 October 2007, Liège (Belgium). "Defence responses induced in tobacco cells by *Bacillus* lipopeptides acting as elicitors of systemic resistance in plants".

***Participation à des réunions scientifiques***

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# Introduction

# *Chapitre I. L'immunisation des plantes par les rhizobactéries en tant que nouvelle arme pour la lutte biologique contre les maladies des cultures*

*Cahiers Agriculture* (Submitted)

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**Résumé**

L'utilisation de certains microorganismes non pathogènes en tant que biopesticides est une technologie émergente et écologiquement compatible considérée comme alternative prometteuse aux pesticides de synthèse. Globalement, l'effet protecteur conféré par ces agents de lutte biologique est basé sur la compétition pour les nutriments essentiels, sur l'activité antagoniste vis-à-vis de la croissance des pathogènes via la production d'antibiotiques ou d'enzymes et/ou sur leur capacité à stimuler des systèmes de défense chez l'hôte végétal. Certaines rhizobactéries non pathogènes peuvent en effet initier l'expression de mécanismes de défense s'établissant dans tous les organes de la plante, lui permettant de mieux se défendre vis-à-vis d'une agression ultérieure. Ce phénomène, dénommé résistance systémique induite, fait appel séquentiellement à la reconnaissance par l'hôte d'éliciteurs produits par l'agent inducteur, à l'émission d'un signal requis pour propager l'état induit de manière systémique et à l'expression de mécanismes de défense *sensu stricto* qui permettent de limiter la pénétration du pathogène dans les tissus de la plante. Parce qu'elle permet de lutter contre une large gamme d'agents pathogènes (bactéries, champignons, virus, nématodes), parce qu'elle ne fait appel qu'au patrimoine génétique naturel de l'hôte végétal sans l'introduction de gènes de résistance étrangers, parce qu'elle n'est pas propice au développement de résistance chez les pathogènes (multiplicité et variété des mécanismes de défense induits), parce que l'augmentation du niveau de résistance s'établit de manière durable, l'exploitation de l'ISR constitue une des voies les plus prometteuses de traitement biologique des cultures en vue de leur protection.

**La lutte biologique comme alternative aux pesticides synthétiques**

Le marché global dédié aux produits phytosanitaires est estimé à 26.7 milliards de dollars US en 2005 (Thakore, 2006). Les pesticides synthétiques dominent ce marché mais un mauvais choix peut mener à une problématique d'écotoxicité de leurs résidus. De plus, leur utilisation irrationnelle peut conduire à une perte d'efficacité due à l'adaptation du microbe pathogène visé et à des effets néfastes sur les populations non cibles partageant l'écosystème. Par ailleurs, l'intérêt du consommateur pour un aliment plus sain et pour des méthodes de gestion des maladies des cultures plus compatibles écologiquement se développe rapidement. Toutes ces raisons, ajoutées aux contraintes de plus en plus élevées qui sont placées pour satisfaire à l'homologation de ces produits chimiques, font que leur marché diminue de manière significative depuis l'an 2000 et devrait suivre la même tendance dans les années à venir. De

nouvelles stratégies telles que le développement des biopesticides ont donc logiquement émergé comme alternatives phytosanitaires écologiquement compatibles.

**Les biopesticides microbiens**

De très larges à beaucoup plus restrictives, plusieurs définitions de la lutte biologique ont été proposées mais aucune n'est adoptée par tous les acteurs concernés. « L'utilisation d'organismes vivants, ou des produits de leurs gènes, pour limiter ou supprimer les activités et les populations de pathogènes » nous parait être un bon compromis pour faire référence à la lutte biologique. Deux des principaux avantages des agents de lutte biologique résident dans leur compatibilité environnementale et dans le fait qu'ils offrent des modes d'action multiples et différents de ceux des pesticides chimiques pour combattre les organismes nuisibles. Utilisés comme compléments ou en alternance avec les pesticides classiques dans une perspective de lutte intégrée, ils permettent de réduire l'apparition de résistances chez les pathogènes cibles. La lutte biologique peut également être employée dans les situations où aucune solution n'est disponible, ou lorsque les pesticides conventionnels ne peuvent pas être utilisés compte tenu de préoccupations éco-toxicologiques liées à l'accumulation potentielle de leurs résidus ou encore lorsque le produit doit être certifié issu de l'agriculture biologique. Parmi les biopesticides, les agents microbiens sont soit des bactéries, des champignons, des levures, et même des virus. Ils sont employés dans le monde entier dans des cultures en champ et en serres pour réduire de multiples maladies sur diverses céréales, légumineuses, fruits, fleurs et plantes d'ornement (Fravel, 2005 ; Paulitz and Bélanger, 2001).

**La stimulation de la capacité défensive des plantes parmi les modes d'action des rhizobactéries protectrices**

Les mécanismes par lesquels les agents de lutte biologique bactériens peuvent prodiguer leur effet protecteur sont multiples et peuvent varier pour un microorganisme donné en fonction du pathosystème (couple hôte végétal/pathogène) sur lequel il est appliqué. Cependant, d'un point de vue global, leur effet bénéfique se fonde sur certains phénomènes qui ne seront pas détaillés ici mais nous référons à quelques excellentes revues comme points de départ pour l'obtention d'une information plus complète (Compant *et al.*, 2005). Brièvement, le pré-requis à toute efficacité biologique d'une souche donnée est son potentiel à coloniser efficacement le système racinaire et la couche environnante du sol appelée rhizosphère. Cela signifie s'imposer par

rapport aux autres populations microbiennes et persister en utilisant les faibles ressources nutritionnelles disponibles dans le microenvironnement. Plusieurs propriétés intrinsèques contribuent au pouvoir colonisateur telles que la mobilité, la faible exigence nutritionnelle, le changement de phase ou la vitesse de croissance (Lugtenberg *et al.*, 2001, Van Den Broek *et al.*, 2003). La microflore bactérienne du sol tire donc profit des aliments sécrétés par les racines mais, en contrepartie, certaines souches influencent avantageusement l'hôte végétal par la stimulation directe de la croissance (fixation de l'azote, solubilisation de phosphate, et production des phytohormones) ou/et en le protégeant contre l'infection par des phytopathogènes. En raison de l'effet global qu'elles procurent, ces souches sont désignées également sous le terme de rhizobacteries promotrices de la croissance des plantes ou PGPRs (Mercado-Blanco and Bakker, 2007).

Une fois installé dans la rhizosphère, le microorganisme d'intérêt peut intervenir favorablement tout d'abord en privant les organismes potentiellement infectieux des ressources nutritives (substrats carbonés, oligoéléments essentiels) et spatiales nécessaires à son développement. Il peut également agir par antagonisme, c'est-à-dire en inhibant directement la croissance des pathogènes par la production d'antibiotiques et/ou d'enzymes lytiques antifongiques. Cette antibiose est probablement le mécanisme le plus connu et peut-être le plus important employé par les agents de biocontrôle pour limiter l'invasion des microbes pathogène dans les tissus végétaux (Raaijmakers *et al.*, 2002). Certaines interactions antagonistes plus spécifiques entre agent bénéfique et infectieux peuvent aussi intervenir comme le parasitisme des hyphes des pathogènes fongiques ou l'inactivation par le PGPR des facteurs de germination et de pathogénicité. Cependant, l'isolement de certaines souches de PGPR protectrices mais n'agissant par aucun de ces mécanismes a permis de mettre en lumière le fait qu'elles puissent agir indirectement en renforçant la plante vis-à-vis d'une attaque ultérieure.

**Le phénomène de la Résistance Systémique Induite (ISR)**

Lorsque leurs barrières physiques et biochimiques constitutives ne suffisent plus à limiter le pouvoir infectieux de pathogènes nécrotiques, les végétaux peuvent faire appel à l'activation d'un spectre complexe de mécanismes de défense cellulaires. Cette activation dépend de l'interaction moléculaire qui s'établit entre la plante et son agresseur générant donc différents types de résistances induites qui peuvent s'exprimer de manière systémique dans les organes autres que celui ayant subit la première infection. Dans ce contexte, la « résistance systémique acquise » (SAR) qui résulte d'une interaction incompatible avec un (micro)organisme pathogène de virulence modérée, compte parmi les phénomènes les plus étudiés (Durrant and

Dong, 2004). Les évènements moléculaires associés à la SAR sont de mieux en mieux connus. Ainsi, la transmission du signal émis suite à la perception de l'agent infectieux repose sur différentes voies dans lesquelles l'acide salicylique, l'acide jasmonique et l'éthylène jouent un rôle crucial (Glazebrook *et al.*, 2003). Cependant, ces voies s'interpénètrent et agissent avec d'autres mécanismes pour former un réseau de régulation modulable permettant à la plante d'initier une réponse défensive spécifique en fonction de la nature du pathogène, qu'il soit virus, bactérie, champignon, insecte ou nématode (De Vos *et al.*, 2005).

L'expression de mécanismes de défense systémiques chez les plantes peut également être initiée suite à l'interaction avec certaines rhizobactéries non pathogènes lors d'un phénomène appelé « résistance systémique induite » (ISR). Tout comme dans le cas de la SAR, ces réactions de défense leur permettent de mieux se défendre vis-à-vis d'une agression ultérieure. L'expression phénotypique du phénomène de l'ISR peut être divisé en quatre étapes principales (Figure 1). Ces étapes sont i) la perception par la plante des molécules bactériennes responsables de l'élicitation du phénomène, ii) la transmission du signal nécessaire à la systémisation du phénomène dans la plante, iii) la mise en alerte de la plante au niveau systémique qui, dans la plupart des cas, n'est pas accompagnée de modifications majeures de l'activité transcriptionnelle avant l'attaque du pathogène et iv) l'expression du ou des mécanisme(s) de défense *sensu stricto* induits permettant de limiter voire inhiber la pénétration du pathogène dans les tissus de l'hôte végétal.

**Un phénomène inductible par de nombreuses bactéries**

Le survol de la littérature de ces dernières années illustre la diversité des microorganismes non pathogènes capables d'induire l'ISR. Quelques ascomycètes comme *Penicilium*, *Trichoderma*, *Fusarium* ou *Phoma* mais également des oomycètes comme *Phythophthora* sont connus pour stimuler l'expression des gènes de défense des plantes. Parmi le royaume bactérien, la liste des espèces décrites comme inductrices de l'ISR a augmenté rapidement au cours des 15 dernières années (Tableau 1). Elle inclut des bactéries gram-positives et en particulier des bacilles comme *Paenibacillus polymyxa*, *Bacillus pumilus*, *B. mycoides*, *B. subtilis*, *B. amyloliquefaciens*, *B. pasteurii*, *B. thuringiensis* ou *B. cereus* (Kloepper *et al.*, 2004). Plusieurs espèces gram-négatives sont également intensivement étudiées dans le contexte de la lutte biologique basée sur l'induction de résistance parmi lesquelles des membres du genre *Pseudomonas* (*P. fluorescens*, *P. putida*, *P. aeruginosa*, *P. aureofaciens*, *P. corrugata*) ou des entérobactéries telles que *Serratia* (*S. marcesens*, *S. plymuthica*) ou *Pantoea agglomerans*. La plupart de ces bactéries vivent librement dans la rhizosphère mais certaines, telle que

*Rhizobium elti* peuvent également pénétrer dans les espaces intercellulaires des tissus racinaires et donc se comporter comme des endophytes (Benhamou *et al.*, 2000, Reitz *et al.*, 2002).

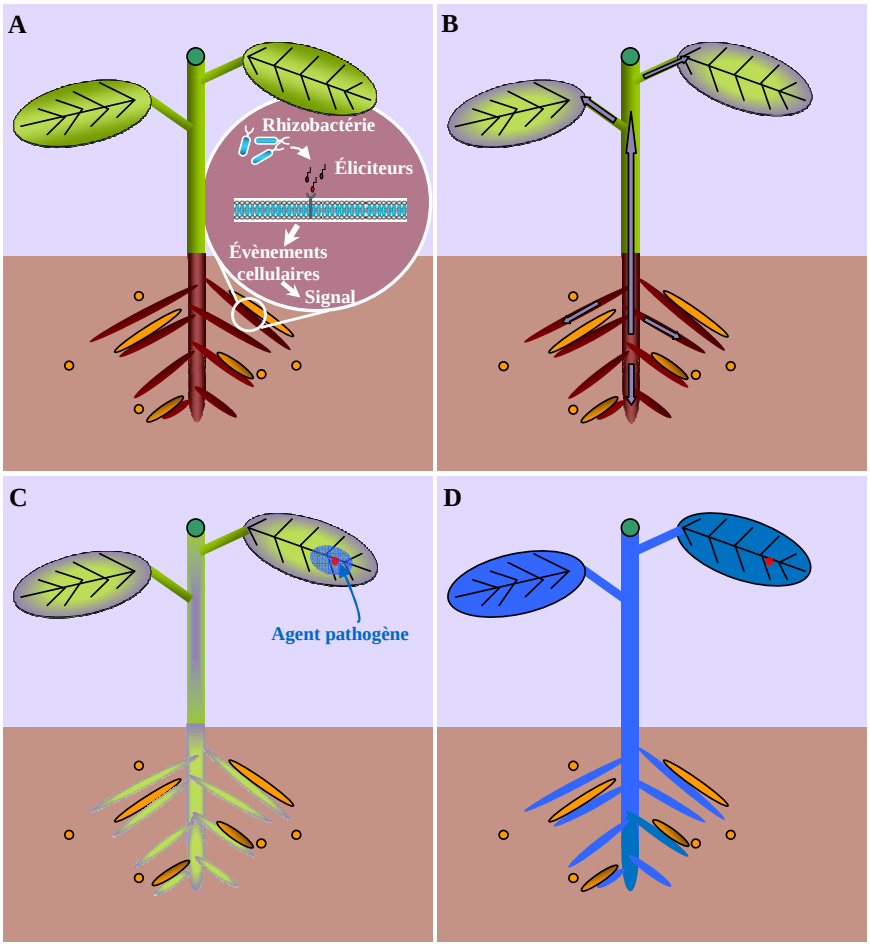


**Figure 1.** Les différentes phases du phénomène d'induction de résistance chez les plantes par les rhizobactéries. La perception de la bactérie par l'hôte végétal via un(des) éliciteur(s) moléculaire(s) est la première étape (A). Suite à ce dialogue moléculaire, il y a émission d'un signal à travers toute la plante menant à un état « induit » systémique alors que la bactérie inductrice ne migre pas (B). Cet état induit n'est que peu perceptible d'un point de vue moléculaire mais permet à la plante de réagir rapidement et de limiter une infection ultérieure d'abord localement autour du site d'attaque (C). Il s'en suit une réaction systémique menant à un renforcement de tous les organes qui permet une certaine résistance vis-à-vis d'une agression future (D).

### Un phénomène stimulable dans de nombreuses plantes pour lutter contre de multiples pathogènes

Il existe de nombreuses spécificités mécanistiques dans les quatre étapes principales du phénomène qui justifient que l'ISR puisse être considérée comme une branche à part entière de

l'immunité systémique des plantes. Heureusement, la spécificité ne se place, d'une manière générale, qu'au niveau moléculaire et l'intérêt d'un point de vue agricole repose notamment sur le fait que de nombreuses plantes soient réceptrices à ce phénomène (Table 1). L'ISR induite par les PGPRs a été tout d'abord démontrée chez les dicotylédones et plus spécifiquement dans les familles des cucurbitacées (concombre, pastèque), solanacées (pomme de terre, tomate, tabac), brassicacées (radis, *Arabidopsis*), fabacées (haricot, pois) et caryophyllacées (oeillet). Cependant, des travaux plus récents ont prouvé que l'ISR peut également être efficace chez certaines plantes monocotylédones, telles que riz, maïs et canne à sucre et dans d'autres cultures telles que l'arachide, le trèfle blanc, le café, le pommier et même le pin, prouvant qu'elle peut se produire dans les gymnospermes.

Par analogie avec la SAR, la protection conférée via l'ISR est également peu spécifique concernant la nature de l'agent infectieux contre lequel elle permet de lutter (Tableau 1). Etant donné la systémicité du phénomène, l'état induit s'exprime dans tous les organes de la plante, des racines aux feuilles et aux fruits. L'ISR est donc efficace non seulement contre les pathogènes du sol comme *Rhizoctonia solani*, *Fusarium oxysporum* ou *Pythium aphanidermatum* mais également contre les dommages causés par les agents pathogènes qui ciblent les parties aériennes. Parmi ces derniers, on peut citer les champignons *Alternaria brassicicola*, *Botrytis cinerea*, *Colletotrichum lagenarium*, les bactéries *Pseudomonas syringae*, *Erwinia amylovora*, *Xanthomonas campestris* et certains virus tel que les virus de la mosaïque du tabac ou du concombre ou le virus de la marbrure de la tomate. L'efficacité de l'ISR induite par les rhizobactéries peut même s'étendre au contrôle des infections causées par plusieurs types de nématodes comme *Globodera pallida* provoquant le kyste chez la pomme de terre, *Heterodera schachtii* chez la betterave sucrière ou *Meloidogyne incognito* actifs sur le coton et la tomate. Selon certaines études, l'induction de résistance systémique peut également être efficace pour la gestion d'infections par certains insectes dans plusieurs types de récoltes.

D'un intérêt crucial pour une perspective appliquée, il apparaît également qu'une bactérie donnée peut déclencher chez une même plante, une résistance contre divers microbes pathogènes (Tableau 1). Par exemple, le traitement du concombre ou de la tomate par un même PGPR (*Pseudomonas fluorescens* 89-B-61 ou *Pseudomonas putida* 89-B-27) permet de diminuer l'incidence des maladies provoquées par deux champignons différents, l'un, *Fusarium oxysporum*, infectant les racines et l'autre, *Colletotrichum orbiculare*, le système foliaire, par deux bactéries, *Pseudomonas syringae* et *Erwinia tracheiphila* et par un coléoptère (Bais *et al.*, 2006, Raupach *et al.*, 1996, Yan *et al.*, 2002). Ces résultats suggèrent que la réponse de la plante puisse être biochimiquement complexe et que des mécanismes de défense multiples puissent être stimulés pour répondre de manière adéquate en fonction de l'envahisseur. Cela ne signifie cependant pas que le potentiel immunisant d'une souche donnée

soit universel et une certaine spécificité concernant le génotype de la plante a été observée. Une même bactérie n'induit pas forcément un niveau de résistance similaire chez différentes espèces végétales et ni même chez différents cultivars de la même plante (Van Loon and Bakker, 2005). L'ISR semble ainsi être lié aux caractéristiques génétiques spécifiques de la rhizobacterie et de la plante hôte.

**Table 1.** Diversité des espèces bactériennes pour lesquelles des souches ont été identifiées en tant qu'agents inducteurs de la résistance systémique chez les plantes. Ce tableau illustre également la diversité des pathosystèmes dans lesquels l'ISR a été impliquée.

| **Espèces** | **Plante hôte/pathogène(s)** |
|---|---|
| ***1- Gram-négatives*** | |
| *Pseudomonas fluorescens* (souche WCS417) | Cacahuète/*Fusarium* ; *Arabidopsis*/*Fusarium*, *Pseudomonas* ; Tomate/*Fusarium* ; Radis/*Alternaria*, *Fusarium*, *Pseudomonas* |
| *P. aeruginosa* (souche 7NSK2) | Haricot/*Botrytis*, *Colletotrichum* ; Tomate/*Botrytis* ; Tabac/virus de la mosaïque |
| *Serratia marcesens* (souche 90-166) | Concombre/*Colletotrichum*, *Pseudomonas* ; Tabac/*Peronospora* ; *Arabidopsis*/*Pseudomonas* ; Concombre/coléoptère ; Pin/*Cronartium* ; Tomate/virus de la mosaïque |
| *S. plymuthica* | Tomate/*Fusarium* |
| *Burkholderia cepacia* | Tabac/*Phytophthora* |
| *Pantoea agglomerans* | Pommier/*Erwinia* |
| *Kluyvera cryocrescens* | Tomate/cucumovirus |
| *Flavomonas oryzihabitans* | Concombre/coléoptère |
| *Rhizobium elti* | Pomme de terre/nématode |
| *Agrobact. radiobacter* | Pomme de terre/nématode |
| ***2- Gram-positives*** | |
| *Bacillus. pumilus* (souche SE34) | Tabac/*Peronospora* ; *Arabidopsis*/*Pseudomonas* ; Concombre/coléoptère ; Tomate/virus de la mosaïque ; Pin/*Cronartium* ; Pois/*Fusarium* ; Tomate/*Fusarium*, *Phytophthora* |
| *B.amyloliquifaciens* (souche IN937) | Tomate/virus de la mosaïque ; Concombre/coléoptère ; *Arabidopsis*/*Erwinia* |
| *B. thuringiensis* | Café/*Hemileia* |
| *B. mycoides* | Betterave sucrière/*Cercospora* |
| *B. pasteurii* | Tabac/*Peronospora* |
| *B. sphaericus* | Pomme de terre/nématode |
| *B. cereus* | Poivre/nématode |
| *Paenibacillus polymyxa* | *Arabidopsis* / *Erwinia* |

**Un phénomène valorisable pour le développement des biopesticides**

Les biopesticides au sens large, avec un marché de 672 millions de dollars, ne représentent en 2005 qu'environ 2,5% des ventes des produits phytosanitaires. Parmi les biopesticides, les produits microbiens sont minoritaires et les produits bactériens autres que ceux à base de *Bacillus thuringiensis* ne comptent que pour une faible partie. Exploiter le potentiel des bactéries reste donc une technologie toujours émergeante. Cependant, avec une croissance annuelle du marché de 10%, les biopesticides, quel que soit le secteur, devraient atteindre plus de 4% du marché phytosanitaire en 2010. Plus de vingt-cinq produits microbiens sont

disponibles commercialement en 2005 aux Etats-Unis et parmi eux, 36% ont été enregistrés au cours des cinq dernières années. Le marché pour le contrôle biologique est donc en progression et l'industrie des biopesticides microbiens est active et en croissance (Thakore, 2006).

Parce qu'elle permet de lutter contre une large gamme d'agents pathogènes, parce qu'elle ne fait appel qu'au patrimoine génétique naturel de l'hôte végétal sans l'introduction de gènes de résistance étrangers, parce qu'elle n'est pas propice au développement de résistance chez les pathogènes (multiplicité et variété des mécanismes de défense induits), parce que l'augmentation du niveau de résistance s'établit de manière durable, l'exploitation de l'ISR constitue une des voies les plus prometteuses de traitement biologique des cultures en vue de leur protection. Plusieurs microorganismes récemment mis sur le marché en tant que biopesticides agissent, du moins en partie, par renforcement de la plante hôte via l'ISR. Cela inclut les champignons *Fusarium oxysporum* souche Fo47 (firme Natural Plant Products) (Fuchs *et al.*, 1997), *Trichoderma Harzianum* T-22 (Binab T®, firme Bio-Innovation ab, Suède) (Bailey and Lumsden, 1998) et *Trichoderma Harzianum* T-39 (Trichodex®, firme Makhteshim Ltd, Israël) (De Meyer *et al.*, 1998) mais aussi plusieurs bactéries dont *Bacillus subtilis* GBO3 (Kodiak®, firme Gustafson, Etats-Unis) (Raupach and Kloepper, 2000) et *B. subtilis* QST 713 (Serenade®, firme Agraquest, Etats-Unis), ou *Pseudomonas chlororaphis* 63-28 (AtEze®, firme EcoSoil, Etats-Unis) (Chen *et al.*, 1999, Chen *et al.*, 2000).

Malgré tous les avantages du phénomène, la protection conférée aux plantes via l'ISR n'est que partielle dans la plupart des cas. Elle doit donc plutôt être considérée comme une approche complémentaire et en aucun cas en tant que stratégie unique pour la gestion des maladies des récoltes. Optimiser l'efficacité de ce phénomène passe notamment par une compréhension optimale des facteurs à la fois physiologiques, biotiques mais aussi environnementaux régulant l'expression des éliciteurs par les bactéries. C'est seulement sur cette base que nous pourrons développer des produits contenant un agent actif dans un état physiologique approprié au moment de l'application et de définir les conditions dans lesquelles ces produits pourront être exploités de manière optimale.

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# *Chapitre II. Caractéristiques moléculaires de l'immunité des plantes induite par les rhizobactéries non pathogènes*

*Biotechnologie, Agronomie, Société et Environnement* (Submitted)

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**Résumé**

La reconnaissance par la plante de certaines bactéries de la rhizosphère peut conduire à une réaction d'immunisation lui permettant de mieux se défendre vis-à-vis d'une attaque ultérieure par un organisme pathogène. Utilisé en combinaison avec d'autres approches phytosanitaires, ce phénomène d'induction de résistance systémique (ISR) par les rhizobactéries est considéré comme une stratégie prometteuse dans la lutte biologique contre les maladies des cultures. Cet article présente les aspects moléculaires intervenant à chacune des trois étapes de ce processus, c'est-à-dire i) la perception des éliciteurs bactériens par les cellules de la plante, ii) la transmission d'un signal nécessaire pour propager l'état induit dans tous les organes de la plante et iii) l'expression des mécanismes de défense proprement dits qui vont limiter ou inhiber la pénétration du pathogène dans les tissus infectés. L'état des connaissances sur l'ISR est exposé en parallèle avec les informations dont on dispose concernant la résistance systémique acquise qui est similaire d'un point de vue phénotypique mais qui est induite suite à une interaction incompatible avec un agent pathogène.

**L'ISR dans l'immunité systémique des plantes**

Lors de l'agression d'une plante par un pathogène, la maladie est souvent considérée comme une exception plutôt qu'une généralité, et plusieurs cas peuvent se présenter. L'interaction incompatible correspond à une incapacité du pathogène à infecter la plante et peut être due à la présence de barrières physiques (cuticule, paroi végétale) et/ou chimiques (composés antimicrobiens) (Nurnberger and Lipka, 2005). La reconnaissance de l'attaque d'un pathogène et une réponse rapide de l'hôte peuvent également restreindre ou stopper l'invasion. Après la pénétration du phytopathogène à travers la paroi végétale ou via une blessure, sa présence peut être révélée grâce à des motifs moléculaires associés aux pathogènes (PAMPs) (Chisholm *et al.*, 2006). La perception de ces motifs par la plante pourra initier une réponse immunitaire de base (PAMP-Triggered Immunity, PTI), afin de limiter l'invasion (Jones and Dangl, 2006). Cependant, si les défenses préexistantes sont inappropriées, que la plante ne réagit pas assez rapidement ou que les voies de défense sont « désactivées », l'interaction entre la plante et le microorganisme sera compatible et suivie par une prolifération du pathogène. En effet, les pathogènes ont appris à masquer leur présence en interférant avec les voies de défense de la plante. Cette désactivation s'effectue via la sécrétion par le pathogène d'effecteurs appelés protéines d'avirulence qui sont, dans le cas des bactéries gram négatives, directement injectés

dans la cellule hôte par un système spécifique (Type Three Secretion System, TTSS) (Abramovitch and Martin, 2004).

En parallèle aux protéines d'avirulence, certaines plantes ont développé des protéines polymorphiques NBS-LRR (Nucleotide Binding Site and Leucine Rich Repeat Domains), codées par les gènes de résistance R (Gómez-Gómez, 2004). Cette interaction préférentiellement intracellulaire entre les produits de gènes d'avirulence de pathogènes (Avr) et de résistance de l'hôte végétal (R) est appelée résistance « gènes pour gènes ». La réponse en découlant est notamment décrite comme l'immunité déclenchée par les effecteurs (Effector-Trigered Immunity, ETI). L'immunité ETI peut être considérée comme une version amplifiée de l'immunité PTI (Garcia-brugger *et al.*, 2006) et est souvent décrite comme menant à une mort cellulaire programmée localisée au niveau des tissus infectés : la réponse hypersensible (HR) (Jones and Dangl, 2006). En plus de l'inhibition directe du phytopathogène ou de son confinement au site d'infection, la HR libérerait des signaux conditionnant les cellules adjacentes à devenir (plus) réactives et activerait des réponses plus étendues (Kombrink and Schmelzer, 2001). C'est le cas de la LAR (Local Aquired Resistance) qui est caractérisée par une augmentation de la résistance des tissus adjacents du site d'infection, et de la SAR (Systemic aquired resistance), développée dans l'ensemble du végétal (Durrant and Dong, 2004). La SAR est généralement activée par des pathogènes nécrotiques, mais peut aussi être induite suite au traitement par des molécules purifiées issues d'autres pathogènes (Cérébrosides, Lipid transfert protéin, oligosaccharide) et par certains composés synthétiques (acide salicylique, acide β-amino-butyrique, acide 2,6-dichloro-isonicotinique ou acibenzolar-S-methyl-benzothiadiazole, BTH) (Sticher *et al.*, 1997). La résistance conférée par ce phénomène persiste dans le temps, et est effective sur une multitude de plantes et contre un large spectre de pathogènes incluant virus, bactéries, champignons ou nématodes.

L'effet protecteur conféré par la SAR est phénotypiquement similaire à un autre phénomène, découvert plus récemment, déclenché suite à l'interaction avec un microorganisme non pathogène. Cette « immunisation » de la plante, ne provoquant pas de symptômes visibles de maladie, est appelée résistance systémique induite (ISR) (Van Loon, Bakker, 2005). L'ISR s'est avérée être induite par des microorganismes variés et plus particulièrement par des rhizobactéries. Celles-ci incluent des bactéries Gram-positif comme *Bacillus pumilus*, *B. subtilis*, *B. amyloquefaciens* et *B. thuringiensis* (Kloepper *et al.*, 2004), ou des bactéries Gram-négatif qui sont les plus étudiées dans le contexte de l'ISR. Bien que des entérobactéries comme *Serratia* (*S. marcesens*, *S. plymuthica*) ou *Pantoea agglomerans* interviennent dans la mise en alerte de la plante hôte, la plupart des souches Gram-négatif impliquées appartiennent au genre *Pseudomonas* (*P. fluorescens*, *P. putida*, *P. aeruginosa*) (Bakker *et al.*, 2007). Comme pour la SAR, l'efficacité de cette immunisation diminue significativement l'impact de

maladies causées par des champignons racinaires (*Fusarium oxysporum*, *Pythium alphanidermatum*) ou aériens (*Botrytis cinerea*, *Alternaria brassicicola*), par des bactéries (*Pseudomonas syringae*, *Erwinia amylovora*, *Xanthomonas campestris*), des virus (*Tobacco mosaic virus*, *Tomato mottle virus*) et par certains nématodes (*Globodera pallida*, *Meloidogyne incognita*) (Ongena and Thonart, 2006).

Dans l'ensemble, les bactéries ayant un impact positif sur la plante par le biais d'un effet protecteur ou via une stimulation de sa croissance ont communément été regroupées sous le terme de rhizobactéries promotrices de la croissance des plantes, ou PGPR (Plant Growth Promoting Rhizobacteria) (Mercado-Blanco and Bakker, 2007).

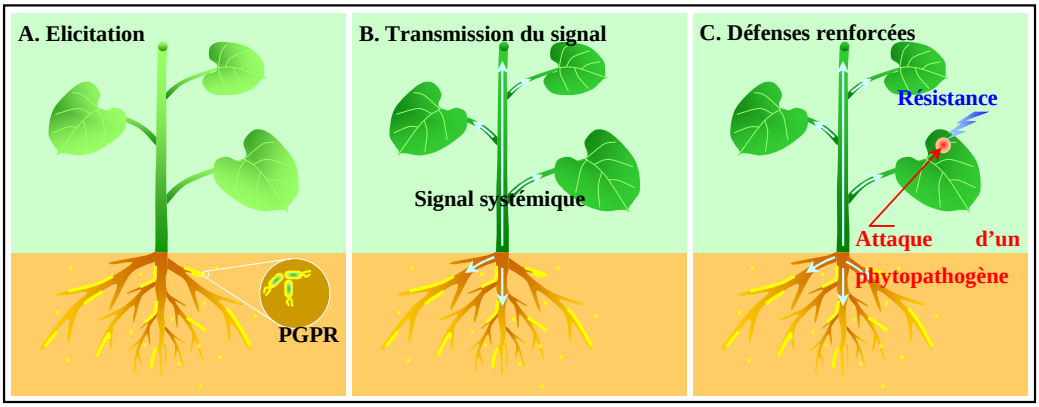


**Figure 1.** La résistance systémique induite chez les plantes par des rhizobactéries. Les trois étapes principales sont l'élicitation (A) : les rhizobactéries ou PGPR (Plant Growth Promoting Rhizobacteria) interagissent avec les racines de l'hôte et produisent des éliciteurs qui sont perçus par la plante. Après la reconnaissance des déterminants, un signal est véhiculé dans l'ensemble de la plante afin de l'alerter (B). Enfin, lors d'une éventuelle attaque par un agent phytopathogène, la plante sera en mesure de répondre plus efficacement à l'agression, lui conférant ainsi une résistance (C).

Contrairement aux réponses mises en place par la plante suite à des attaques par des pathogènes, celles induites par des microorganismes bénéfiques sont moins connues et beaucoup de questions concernant l'ISR restent encore à être élucidées. Néanmoins, l'ISR peut être divisée en trois étapes principales que sont la perception des molécules actives produites par le PGPR ou élicitation; la transmission d'un signal systémique dans la plante et l'expression des mécanismes de défense de l'hôte (Ongena and Thonart, 2006) (Figure 1). Malgré un effet protecteur visiblement similaire, l'ISR semble présenter certaines divergences avec la SAR. Dans les sections suivantes, nous proposons de détailler les différentes étapes intervenant lors de l'ISR induite par des rhizobactéries et lors de la SAR.

**Table 1.** Molécules élicitrices de mécanismes de défense chez les plantes. Les **[termes]** représentent les motifs minima responsables de l'activité biologique de l'éliciteur. Le tableau reprend des éliciteurs présents chez (1) des phytopathogènes, (3) les éliciteurs issus de rhizobactéries capables d'induire l'ISR et (2) les éliciteurs présents chez les deux types de microorganismes. DP, degrés de polymérisation ; Arach., acide arachidonique ; SA, acide salicylique ; BTH, benzo-(1,2,3)-thiadiazole-7-carbothioic S-methyl ester ; INA ; acid 2,6-dichloroisonicotinique.

| Eliciteurs | Microorganismes | Références |
|---|---|---|
| ***1- Eliciteurs isolés de phytopathogènes*** | | |
| Syringoline, Coronatine | Bactérie (*Pseudomonas*) | (Tamogami and Kodama, 2000; Waspi *et al.*, 2001) |
| Ergostérol, Cérébrosides | Composants des membranes des champignons (*Cladosporium, Magnaporthe, Fusarium*) | (Umemura *et al.*, 2004; Rossard *et al.*, 2006) |
| Endopolygalacturonase (Pectinase) **[BcPG1]** | Champignons (*Botrytis, Mucor*) | (Poinssot *et al.*, 2003) |
| Invertase **[peptide N-mannosylaté (8 residues)]** | Levure | (Basse *et al.*, 1993) |
| Transglutaminase **[Peptide Pep-13]** | Oomycètes (*Phytophthora*) | (Brunner *et al.*, 2002) |
| Xylanase **[pentapeptide TKLGE]** | Champignons (*Trichoderma*) | (Ron and Avni, 2004) |
| Cold shock protein **[motif RNP-1]** | Bactérie Gram positif (*Micrococcus*) | (Felix and Boller, 2003) |
| Facteur d'élongation (EF-Tu) **[Elf18 (Fragment N-acetylé terminal de EF-Tu)]** | Bactérie Gram négatif | (Zipfel *et al.*, 2006) |
| Harpine (Hrp N & Z) | Bactérie Gram négatif (*Erwinia, Pseudomonas*) | (Clarke *et al.*, 2005) |
| Necrosis-inducing proteins **[Nep1, Nip, PaNie213]** | Bactérie (*Erwinia*) Oomycètes (*Pythium, Fusarium, Phytophthora*) | (Pemberton and Salmond, 2004) |
| Lipid-transfer proteins **[Elicitins (Cryptogein, Capsicein)]** | Oomycètes (*Phytophthora*) | (Garcia-Brugger *et al.*, 2006) |
| β-megaspermin | Oomycètes (*Phytophthora*) | (Baillieul *et al.*, 2003) |
| Protéines Avr | Champignon (*Cladosporium*) | (Chisholm *et al.*, 2006) |
| Exopolysaccharides **[Polysaccharides]** | Bactérie (*Pantoea*) | (Ortmann *et al.*, 2006) |
| Oligogalacturonides (Fragment de paroi) **[Oligogalacturonides (9 > DP > 20)]** | Fragment de la paroi pectique de la plante | (Aziz *et al.*, 2004) |
| Chitine, oligomère de Chitosan, Fucans sulphatés, Laminarine, Psg (Phytophthora Sojae Glucan) **[β-glucans]** | Oomycètes (*Phytophthora*), Champignon (*Pyricularia*), Composants de la paroi de l'algue brune (*Pelvetia, Laminaria*) | (Klarzynski *et al.*, 2000; Ramonell *et al.*, 2002) |
| Synthétique (SA, BTH, INA, Arach.) | | (Oostendorp *et al.*, 2001) |
| ***2- Eliciteurs communs aux phytopathogènes et aux PGPR*** | | |
| Lipopolysaccharides (LPS) | Bactérie (*Xanthomonads, Pseudomonas, Sinorhizobium*) | (Erbs and Newman, 2003) |
| Flagelline **[peptide Flg22]** | Bactérie (*Pseudomonas, Acidovorax*) | (Gomez-Gomez and Boller, 2002; Meziane *et al.*, 2005) |
| ***3- Eliciteurs isolés de PGPR*** | | |
| Pyoverdines | Bactérie (*Pseudomonas fluorescens*) | (Höfte and Bakker, 2007) |
| Acide Salicylique, Pyochéline | Bactérie (*Pseudomonas fluorescens, putida*) | (Audenaert *et al.*, 2002) |
| 2,4-Diacetylphloroglucinol (DAPG) | Bactérie (*Pseudomonas fluorescens* | (Iavicoli *et al.*, 2003) |
| Pyocyanine | Bactérie (*Pseudomonas aeruginosa*) | (De Vleesschauwer *et al.*, 2006) |
| Massétolide A | Bactérie (*Pseudomonas fluorescens*) | (Tran *et al.*, 2007) |
| Butandiol | Bactérie (*Bacillus subtilis, amyloliquefaciens*) | (Ryu *et al.*, 2004) |
| N-Acyl Homoserine lactone | Bactérie (Serratia liquefaciens) | (Schuhegger *et al.*, 2006) |

**Perception des éliciteurs par les cellules végétales**

*Les éliciteurs de pathogènes*

La reconnaissance mutuelle entre un microorganisme et son hôte est basée sur une « communication » moléculaire, et, en règle générale, les composés capables de déclencher une réponse de l'hôte sont dénommés éliciteurs. Les éliciteurs de phytopathogènes sont constitués des PAMPs, non spécifiques, qui sont reconnus par plusieurs plantes et des composés plus spécifiques, les protéines d'avirulence, qui sont reconnues par des cultivars particuliers (Montesano *et al.*, 2003).

Les PAMPs interviennent dans l'immunité PTI et appartiennent à une large classe de molécules incluant des composés lipidiques, des glycolipides, des polymères de sucres, ou des (glyco-) peptides et protéines (Table 1). Toutefois, les structures des différentes classes de PAMPs sont hautement conservées d'un microorganisme à l'autre. Ces éliciteurs, isolés de virus, bactéries, champignons, oomycètes ou algues marines, sont produits de manière constitutive par le pathogène car ils sont généralement essentiels à son bon fonctionnement (Nurnberger and Lipka, 2005; Chisholm *et al.*, 2006).

D'autre part, bien qu'elles ne soient pas considérées comme des PAMPs, les protéines d'avirulence sont tout de même reprises comme molécules élicitrices de défenses, puisqu'elles sont en mesure de déclencher une réponse hypersensible chez les plantes possédant les gènes de résistance. Chez les plantes sensibles, elles facilitent la pathogénicité, entre autres, en retardant le renforcement de la paroi (formation de callose), en modifiant l'activité de protéines dans la cellule hôte ou en interférant avec la transcription des gènes de défense (Montesano *et al.*, 2003; Chisholm *et al.*, 2006; Jones and Dangl, 2006).

Les récepteurs des PAMPs, les PRRs (Pattern Recognition Receptors) ou RLK (Extracellular receptor-like kinases), n'ont pas encore été tous identifiés. Cependant la plupart de ceux connus sont constitués de trois domaines distincts : un domaine extracellulaire (riche en leucine) interagissant avec le PAMP, un domaine transmembranaire et un domaine kinase, responsable de la transmission du signal (Nurnberger and Kernmerling, 2006).

*Les éliciteurs de l'ISR*

Le paragraphe consacré aux éliciteurs de l'ISR, initialement présent dans l'article soumis, a été enlevé. Le lecteur se reportera au chapitre III qui décrit ces métabolites de manière plus approfondie.

**Premiers évènements cellulaires induits**

Plusieurs recherches sont désormais disponibles sur les événements précoces, ou « Early events », mis en place dans les cellules végétales après la reconnaissance d'un éliciteur. L'utilisation sur suspensions de cellules de la cryptogeine, protéine nécrotique secrétée par *Phytophthora cryptogea*, a permis d'établir un modèle lors de l'interaction avec les PAMPs (Garcia-Brugger *et al.*, 2006). Succinctement, la fixation de l'éliciteur sur son récepteur agirait sur des canaux calciques et, via une cascade d'événements, aboutirait à l'expression de gènes de défense. Une description plus détaillée de ces phénomènes est fournie dans la Figure 2.

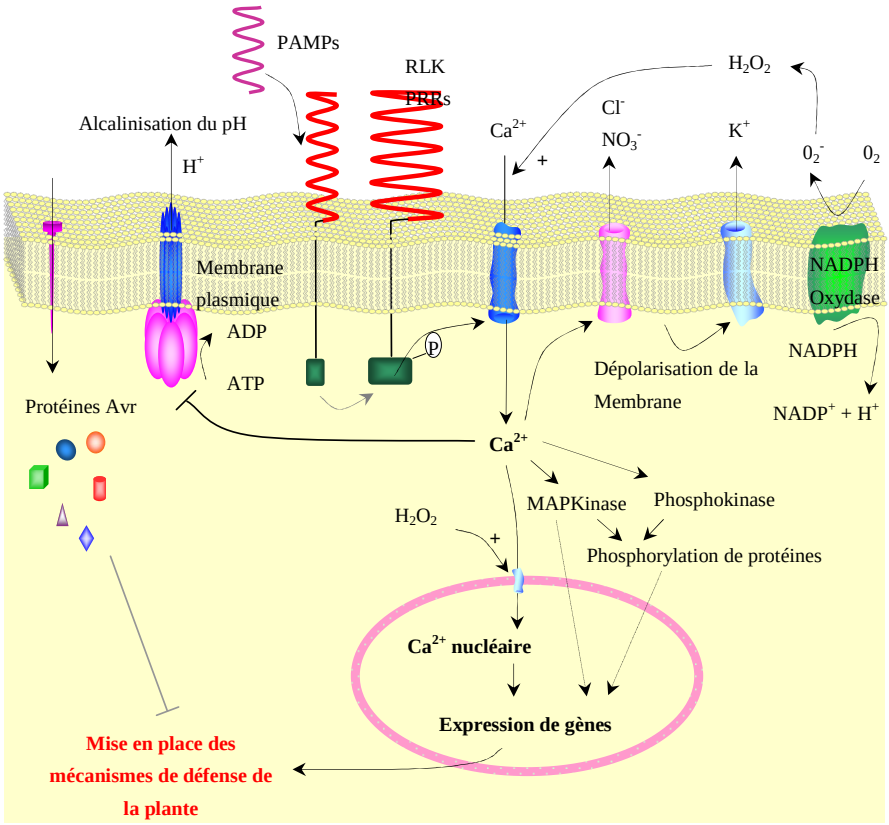


**Figure 2.** Modèle hypothétique des événements précoces basé sur la reconnaissance de la cryptogeine par les cellules de tabac. La reconnaissance du PAMP par son récepteur déclenche un important influx de calcium dans la cellule. L'augmentation de calcium intracellulaire provoque entre autres une alcalinisation du milieu extracellulaire (via l'inhibition des pompes ATPases), une inhibition d'entrée du glucose dans la cellule, un efflux d'anions, une dépolarisation de la membrane plasmique, l'activation de la NADPH oxydase, la production de peroxyde d'hydrogène ($H_2O_2$), et l'activation des MAP kinases. Les MAP kinases ainsi que l'augmentation de la concentration en calcium dans le noyau déclencheraient l'expression de gènes de défense. L'introduction de protéines d'avirulence par des phytopathogènes peut aboutir à une perturbation des voies de défenses, menant au développement de ce dernier et à l'apparition de la maladie.

Les études sur la flagelline ont essentiellement été menées avec des flagelles purifiés à partir de souches pathogènes ou avec des peptides synthétiques. En plus de l'alcalinisation du milieu extracellulaire ou la production d'$H_2O_2$ illustrées par certains auteurs, la flagelline est aussi en mesure de stimuler la production d'éthylène (Felix *et al.*, 1999). En effectuant des travaux sur *Arabidopsis*, d'autres auteurs ont réussi à identifier l'ensemble de la cascade des MAP kinases ainsi que des facteurs de transcription qui étaient activés après la fixation du peptide flg22 sur son récepteur FL2 (Zipfel *et al.*, 2004; Chinchilla *et al.*, 2006). Enfin, à part une diminution de la viabilité des cellules de riz en culture, le traitement par la flagelline ne semble par provoquer de mort cellulaire sur les cultures de cellules de plantes dicotylédones (Che *et al.*, 2000). Il peut donc être envisageable que le récepteur FLS2 puisse aussi reconnaître les flagelles des microorganismes bénéfiques, et déclencher une cascade de réponses de défenses sans pour autant provoquer de dégâts dans les cellules de l'hôte. L'utilisation des LPS de *Burkolderia cepacia*, une souche inductrice de l'ISR, a abouti à l'identification de plusieurs phosphoprotéines chez le tabac qui seraient initialement activées par une protéine G couplée à son récepteur (Gerber *et al.*, 2006). Celles-ci indiquent que les événements précoces induits par les LPS impliquent, entre autre, une signalisation dépendante du $Ca^{2+}$, la régulation d'une $H^+$-ATPase ou la production d'espèces oxydantes, suggérant ainsi que des mécanismes liés à la reconnaissance des LPS soient similaires à ceux déclenchés par les PAMPs.

Hormis les LPs et contrairement aux PAMPs, peu d'études décrivant les événements précoces induits lors de l'interaction avec des éliciteurs spécifiques de l'ISR sont publiées. De Vleesschauwer et ses collaborateurs ont observé que le traitement des racines de riz par le PGPR *Pseudomonas aeruginosa* 7NSK2 induisait une résistance contre *Magnaporthe grisea*, un pathogène hémibiotrophe (De Vleesschauwer *et al.*, 2006). Cet effet protecteur est induit par la pyocyanine produite par la souche et les auteurs ont observé que l'application de l'éliciteur purifié provoquait la génération de peroxyde d'hydrogène dans les cellules de la plante hôte, pouvant inhiber la croissance du pathogène.

La particularité de la flagelline et les LPS comme éliciteurs présents chez des phytopathogènes mais également chez des PGPR révèle la complexité dans l'établissement d'un modèle sur l'ISR. Cependant, il semble que des réponses soient communes lors de la reconnaissance des différents types d'éliciteurs, et que des divergences apparaissent par la suite, menant soit à la mort des cellules en contact avec un éliciteur issu de phytopathogène et à l'établissement d'une réponse de type SAR, soit à l'induction de l'ISR par un éliciteur de PGPR.

**Transmission du signal**

Dans l'état actuel des connaissances, les voies de signalisation de l'état induit chez les plantes sont principalement régulées par trois molécules, l'acide salicylique (AS), l'acide jasmonique (JA) et l'éthylène (ET). Au niveau moléculaire, la SAR est aussi caractérisée par une activation de gènes codant pour des protéines PR (Pathogenesis-related), souvent considérées comme marqueur de cette résistance (Durrant and Dong, 2004). La SAR est associée avec une augmentation de la concentration d'AS endogène à la fois localement au niveau du site d'infection, mais aussi de manière systémique dans les tissus distants. Des expériences réalisées avec des plants de tabac *NahG*, incapables d'accumuler l'AS car produisant une enzyme le dégradant en catéchol inactif, ont démontré que cette molécule était indispensable dans la voie de signalisation (Delaney, 1994). À l'origine, plusieurs hypothèses ont d'ailleurs été émises sur le rôle joué par l'AS en tant que signal systémique dans l'établissement de la SAR. Par la suite, des plants de concombre dont les feuilles, inoculées par *Pseudomonas syringae* puis excisées avant toute accumulation d'AS, ont tout de même développé la SAR, contredisant alors les premiers résultats (Molders *et al.*, 1996). Les hypothèses sont actuellement dirigées vers l'existence d'un composé de nature lipidique qui serait la molécule signal diffusant à travers la plante. Des expériences menées sur des plants mutés au niveau du gène DIR1 développent une résistance locale mais sont cependant incapable d'exprimer la SAR. La séquence de ce gène est proche de celles codant pour des protéines LTPs (lipid tranfer proteins), et pourrait donc être impliquée dans la synthèse et la transmission du signal lipidique circulant. La signalisation semble néanmoins requérir l'intervention d'une protéine se liant à l'AS, SABP2, nécessaire à l'activation d'un facteur protéique, NPR1 (Non-expressor of Pathogenesis-Related Genes1) (Figure 3) (Durrant and Dong, 2004). La fixation de l'AS sur SABP2 provoquerait un changement du potentiel redox dans le milieu intracellulaire. Des oligomères de NPR1 seraient alors convertis en monomères capables de migrer vers le noyau et d'interagir avec des facteurs de transcription, aboutissant à l'expression de gènes PR intervenant dans les défenses de la plante (Fobert and Despres, 2005).

Certaines rhizobactéries bénéfiques sont aussi en mesure d'induire une résistance associée à une accumulation d'AS. Celle-ci fut mise en évidence grâce à l'utilisation de plants *NahG*. Ainsi *Pseudomonas aeruginosa* 7NSK2 n'est pas en mesure d'induire l'ISR chez des plants de tabac et de tomate transformés (Höfte and Bakker, 2007). De même, *Bacillus pumilus* SE34 protège *Arabidopsis* contre *Pseudomonas syringae pv. maculicola* via une signalisation dépendante de l'AS (Ryu *et al.*, 2003). Cependant, dans beaucoup de cas, la résistance induite par les PGPRs est considérée comme étant indépendante de l'AS mais plutôt liée à la perception de l'acide jasmonique (JA) et de l'éthylène (ET). Par exemple, l'effet protecteur

conféré par la rhizobactérie *Pseudomonas fluorescens* WCS417 contre le pathogène *Pseudomonas syringae* est maintenu, même sur des plants d'*Arabidopsis NahG* (Pieterse *et al.*, 2002). Par contre, aucune protection n'est observée sur des plants mutants *jar1* (JA-insensitive) ou *etr1* (Ethylene-insensitive), respectivement déficients dans les voies de réponse à l'acide jasmonique et à l'éthylène. Les auteurs ont donc aussi établi que la voie de transduction de l'ISR chez *Arabidopsis* était basée sur une augmentation de la sensibilité à ces hormones plus qu'à une augmentation de leur concentration, et ont montré que la perception de l'ET intervenait en aval par rapport à celle du JA (Pieterse *et al.*, 2000). Une voie de signalisation dépendante de ces deux hormones a par la suite été observée dans de nombreux cas, comme par exemple chez des plants transgéniques de tomate traités par *Bacillus pumilus* SE34 ou *Pseudomonas fluorescens* 89B61 (Yan *et al.*, 2002). De même, l'absence d'accumulation d'AS suite au traitement de racines de haricot par *Pseudomonas putida* BTP1 suggère également une voie indépendante de la signalisation de type SAR (Ongena *et al.*, 2004).

Toujours dans l'optique d'identifier les voies de signalisation de l'ISR, Pieterse et ses collaborateurs ont observé que la souche WCS417 n'était pas capable d'induire l'ISR sur des plants d'*Arabidopsis* mutés au niveau du gène NPR1 (Pieterse *et al.*, 1998). Ainsi, malgré des voies de signalisation globalement différentes, les résistances induites par un pathogène ou par une rhizobactérie restent intimement liées. Le spectre d'efficacité de la SAR et de l'ISR se chevaucherait partiellement et la protéine NPR1 serait apte à réguler distinctement l'expression de gènes de défense dépendante de l'une ou l'autre voie de signalisation, activées en amont. Ces dernières années, les mécanismes régissant les voies de transduction se sont encore complexifiées. En effet, des recherches réalisées sur *Arabidopsis* ont démontré que la souche *Bacillus subtilis* GB03 pouvait induire l'ISR via une voie indépendante du facteur de transcription NPR1 (Ryu *et al.*, 2004). De même, la souche 7NSK2, précédemment citée pour induire une résistance SA-dépendante, peut déclencher l'ISR chez des plants *Arabidopsis NahG* (Ran *et al.*, 2005). Enfin, les réponses induites chez *Arabidopsis*, préalablement inoculée par diverses rhizobactéries (*Bacillus pumilus*, *Serratia marcescens* et *Pseudomonas fluorescens)* peuvent varier selon la variété du pathogène *Pseudomonas syringae* (Ryu *et al.*, 2003).

**Mécanismes de défense**

La dernière étape importante dans l'établissement des résistances systémiques est l'expression des mécanismes de défense proprement dits qui doivent conférer à la plante la capacité de résister aux agents phytopathogènes. Pour cela, les plantes disposent de plusieurs voies

métaboliques inductibles et donc d'un important panel de molécules afin de lutter contre l'invasion par un phytopathogène.

La production d'espèces oxydantes (poussée oxydative), sous formes d'intermédiaires (Reactive Oxygen Intermediates, ROI), comme l'anion superoxyde, le radical hydroperoxyl, ou le peroxyde d'hydrogène ($H_2O_2$), est une des réponses de la plante les plus précoces suite à la reconnaissance du pathogène, (Kombrink and Schmelzer, 2001). D'autres mécanismes, de nature physique et/ou biochimique comme le renforcement des parois ou la synthèse de protéines de défenses pourront intervenir afin de limiter l'infection.

Les protéines PR (Pathogenesis Related) ont à l'origine été définies comme des protéines s'accumulant à l'intérieur ou à l'extérieur des cellules végétales suite à une interaction avec le pathogène (HammondKosack and Jones, 1996). Toutefois, le terme a été étendu à d'autres protéines également impliquées dans les mécanismes de défenses mais exprimées de façon constitutives, comme la phenylalanine ammonia-lyase (PAL) ou la lipoxygénase (LOX). La PAL est une enzyme de la voie de phénylpropanoïdes aboutissant, entre autres, à la synthèse d'AS, à celle de molécules composant la lignine ou à la synthèse de précurseurs qui seront utilisés par d'autres branches métaboliques pour générer des phytoalexines (voir plus loin) (Dixon *et al.*, 2002). La LOX est impliquée dans la transformation de composés lipidiques et, via la voie des oxylipines, est aussi à l'origine de molécules signal comme l'acide jasmonique ou de composés fongitoxiques (Shah, 2005). Ces enzymes permettent donc la production de métabolites directement toxiques pour le pathogène mais pourraient également ralentir sa progression indirectement en favorisant la mort cellulaire via des dommages sur les membranes des cellules (HammondKosack and Jones, 1996). La plupart des protéines PR font parties de familles conservées chez les différentes espèces de plante. Elles sont induites par l'accumulation d'AS, jasmonique ou éthylène, et certaines possèdent en elle-même des propriétés antimicrobiennes comme les chitinases et les glucanases (Van Loon *et al.*, 2006). Dans les phénomènes d'induction de résistance, la protéine PR1 est souvent considérée comme un marqueur spécifique de la SAR. Cependant, plusieurs exemples de rhizobactéries induisant l'expression de protéines PR sont désormais disponibles (Park and Kloepper, 2000; Tjamos *et al.*, 2005). Chez le haricot, *Pseudomonas putida* BTP1 accroît l'activité LOX dans les feuilles infectées par *Botrytis cinere*a aboutissant à une concentration plus élevée en Z-3-hexenal, un composé fongitoxique (Ongena *et al.*, 2004). La même souche peut aussi retarder l'infection de plants de concombre par *Pythium aphanidermatum* grâce à l'accumulation de phytoalexines (Ongena *et al.*, 2000). Ces dernières sont des composés antimicrobiens, lipophiles et de faible poids moléculaire qui s'accumulent rapidement au niveau du site d'infection. Les phytoalexines ont des structures variées et dérivent de diverses voies métaboliques comme celles menant aux phénylpropanoides, flavonoides, isoflavonoides ou encore sesquiterpènes et

polyketides (Hammerschmidt, 1999). Dans la majorité des cas, leur synthèse nécessite la participation de plusieurs enzymes, suggérant une coordination dans les voies de signalisation des cellules infectées. Leur participation dans l'immunisation conférée par des PGPR a initialement été remarquée chez l'œillet (Van Peer *et al.*, 1991) et le haricot (Zdor and Anderson, 1992) mais les exemples d'induction de phytoalexines dans le contexte de l'ISR restent assez rares.

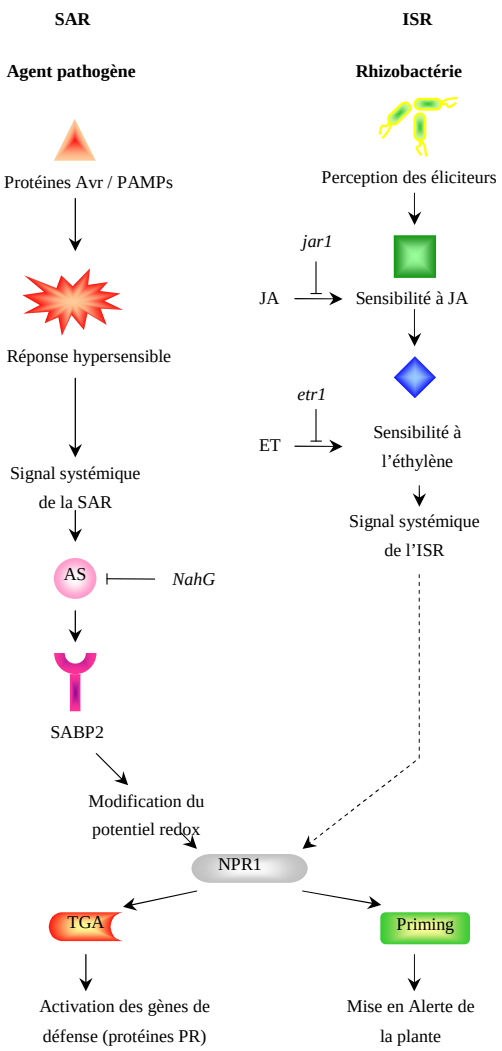


**Figure 3.** Modèle proposé des voies de transduction impliquées dans la résistance systémique induite par un pathogène (SAR) et une rhizobactérie bénéfique (ISR). La reconnaissance de protéines d'avirulence, et éventuellement de PAMPs (Pathogen associated molecular patterns), provoque une réponse hypersensible (HR) au niveau du site d'infection. Un signal est ensuite transmis à l'ensemble de la plante via une voie dépendante de l'acide salicylique (AS). Celui-ci activerait la protéine SABP2 (SA-binding protein) et provoquerait une modification du potentiel redox dans le cytoplasme. La protéine NPR1 (Non-expressor Pathogenesis-Related Genes) subirait alors un changement de conformation. Des oligomères de NPR1 migreraient dans le noyau où ils s'associeraient avec le facteur de transcription TGA pour initier la transcription des gènes de défense. Dans le cas de l'ISR induite par des rhizobactéries non pathogènes, la reconnaissance des éliciteur activerait dans la plupart des cas une voie de transduction dépendante de la sensibilité à l'acide jasmonique (JA) et à l'éthylène (ET). Celle-ci a été mise en évidence via l'étude de mutants (*jar1* et *etr1*) qui sont respectivement insensibles au jasmonate et à l'éthylène, et incapables de développer l'ISR. La voie de signalisation de L'ISR converge vers celle de la SAR au niveau de la protéine NPR1, également indispensable pour le développement de l'ISR, et aboutirait à une mise en alerte de la plante, ou priming.

Enfin, les plantes peuvent faire face à l'invasion d'un pathogène en ralentissant sa pénétration dans les tissus végétaux. Ce retardement est assuré par l'épaississement des parois

par apposition de lignine ou de callose (un polymère de β-1,3 glucan difficilement dégradable), sous la forme d'une excroissance intracellulaire, ou papille, au niveau du site d'infection (Huckelhoven, 2007). Par ailleurs, les précurseurs de la lignine ainsi que les radicaux libres produits pendant les réactions de polymérisation peuvent affecter la plasticité de la membrane ou inactiver les enzymes de pathogènes. Parmi les quelques exemples, nous pouvons citer *Bacillus pumilus* SE34, qui agit sur le pois contre *Fusarium oxysporum* (Benhamou *et al.*, 1996), ou *Pseudomonas fluorescens* 63-28 et *Bacillus amylofacien* EXTN-1, qui renforcent le concombre respectivement contre *Pythium aphanidermatum* et *Colletotrichum orbiculare* (Chen *et al.*, 2000; Jeun *et al.*, 2004).

**Mise en alerte de la plante**

Dans le contexte des réactions de défense systémiques, l'interaction avec le microorganisme inducteur ne mène pas toujours directement au renforcement du végétal. Durant ces dernières années, un concept basé sur la mise en alerte des plantes ou potentialisation (priming) semble s'être généralisé. Cet état induit n'est pas très perceptible d'un point de vue moléculaire tant que l'agression par le pathogène n'est pas ressentie. Il permet cependant à l'hôte végétal de mettre en place de manière plus rapide et plus violente les différents mécanismes de défenses et ainsi de répondre de manière plus efficace à une éventuelle agression par des pathogènes ou des insectes mais aussi à divers stress abiotiques (Conrath *et al.*, 2006). Ce mécanisme permet à la plante de se « préparer » à une infection future sans pour autant engendrer d'investissements lourds au niveau des ressources métaboliques et énergétiques (au détriment des processus cellulaires fondamentaux) alors qu'aucune agression ne doit encore être combattue (Van Hulten *et al.*, 2006). La première mise en évidence du priming dans le cadre de l'ISR a été démontrée sur l'œillet inoculé par une souche de *Pseudomonas*. Van Peer et ses collaborateurs (Van Peer *et al.*, 1991) ont observé une accumulation de composés antifongiques significativement plus rapide chez les plants traités par la bactérie après l'infection par *Fusarium oxysporum* alors qu'aucune différence n'avait pu être détectée avant l'introduction du pathogène. Le même phénomène apparaît chez le pois traité par *Bacillus pumilus* SE34, où un dépôt rapide de callose et de matériel phénolique sur les parois au niveau du site de pénétration n'est observé qu'après l'ajout du pathogène *Fusarium oxysporium* (Benhamou *et al.*, 1996).

En parallèle aux analyses biochimiques, les techniques d'analyse d'expression de gènes ont aussi contribué à la mise en évidence du priming. Dans l'étude du transcriptome d'*Arabidopsis* après élicitation par *P. fluorescens* WCS417, Verhagen et ses collaborateurs (Verhagen *et al.*,

2004) n'ont visualisé que très peu de changements dans les transcripts génétiques, tant au niveau des cellules racinaires que dans les tissus foliaires. Par contre, une nette augmentation dans l'expression de certains gènes au niveau des feuilles était mesurable dès l'infection par le phytopathogène *P. syringae* pv. tomato DC3000. Toujours au niveau génétique, des analyses d'hybridation soustractive sur le concombre ont également démontré que l'expression de certains gènes induits par la bactérie bénéfique *Pseudomonas chororophis* O6 ne devenait apparente qu'après l'infection des feuilles par *Corynespora cassiicola* (Kim *et al.*, 2004). Dans certains cas cependant, une expression génétique spécifiquement induite suite à la perception du PGPR a été rapportée. Par exemple, des travaux réalisés avec la souche *Bacillus subtilis* S499 sur le concombre et la tomate ont montré que l'ISR était tout de même associée avec des changements au niveau transcriptionnel avant infection par *Pythium aphanidermatum* (Ongena *et al.*, 2005b). Le priming peut aussi intervenir dans le cas de la SAR ou plus généralement dans le cadre de la résistance aux pathogènes (champignons, insectes) et même aux stress abiotiques, mais il n'y est pas invariablement associé comme cela semble être le cas pour l'ISR.

**Conclusion**

Les différentes approches en génétique, biologie et biochimie ont permis de mieux comprendre les interactions entre plantes et microorganismes dans le contexte de l'immunisation de l'hôte végétal. Certaines de ces interactions peuvent être néfastes ou bénéfiques et respectivement conduire à l'établissement de la SAR, dans le cas de phytopathogènes; ou induire l'ISR, dans le cas de PGPR. Cependant, les réactions permettant une discrimination par la plante entre éliciteur de l'ISR et PAMP ne sont à ce jour pas connus. Certains mécanismes semblent communs, suggérant un lien étroit dans la reconnaissance d'un microorganisme, pathogène ou non. Ce rapprochement intervient également lors de la transmission du signal systémique, puisque le facteur de transcription NPR1 est nécessaire dans l'induction de l'ISR ou de la SAR. En plus des voies de signalisation ou des réponses de défense mises en place, l'ISR chez une plante donnée semble aussi être influencée par la nature de la rhizobactérie inductrice et par la nature du pathogène. En effet, des études présentées sur *Arabidopsis* avec *Bacillus pumilus* révèlent l'implication de deux voies distinctes suivant le pathovar utilisé. De même, la capacité d'une souche de PGPR à induire l'ISR varie en fonction des phytosystèmes sur lesquels elle est testée. Une meilleure connaissance, d'un point de vue moléculaire, de toutes les étapes du phénomène est nécessaire pour mieux comprendre la combinaison des facteurs qui sont à l'origine de cette spécificité. Les PGPR apparaissent néanmoins comme un atout majeur dans

le développement du végétal. En effet, en plus de l'activité stimulatrice de croissance de certaines souches, il semble que les PGPR puissent « mettre en veille » les défenses de leur hôte sans pour autant les « allumer », conférant ainsi une spécificité nuancée au phénomène d'ISR.

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## *Chapitre III. Eliciteurs de l'ISR : les clés de l'immunisation des plantes par les rhizobactéries.*

**Introduction**

L'environnement en contact avec les racines des plantes, la rhizosphère, est le lieu d'une multitude d'interactions. En effet, outre différents facteurs du sol comme l'acidité, la teneur en eau, la température ou la disponibilité en minéraux, le développement du végétal peut aussi être influencé par la présence d'organismes vivants, et notamment de microorganismes. Certains champignons ou bactéries pathogènes auront un effet néfaste sur la plante, et seront la cause de maladies, alors que d'autres procureront un effet bénéfique. En dehors des relations strictement symbiotiques, cet effet s'exprime soit via la stimulation de sa croissance (assimilation de nutriment, production de phytohormones), soit en conférant une protection contre les agressions des phytopathogènes. C'est le cas des rhizobactéries promotrices de la croissance des plantes (Plant Growth Promoting Rhizobacteria, PGPR). Elles colonisent le système racinaire des plantes et puisent dans les exsudats les ressources nécessaires pour leur métabolisme (Van Loon, 2007).

Même si la monopolisation de l'espace et des nutriments présents dans le sol et/ou dans les exsudats racinaires intervient dans le phénomène, la protection conférée chez leur hôte par les PGPR est souvent due à un mécanisme d'antibiose, ou inhibition de la croissance des agents pathogènes via la sécrétion de composés antibiotiques (Mercado-Blanco and Bakker, 2007). Cependant, un nouveau mode de protection a été mis en évidence durant les dernières décennies. Elle est basée sur un rehaussement induit par le PGPR, des capacités défensives de la plante face à une agression par un phytopathogène. Définie comme la résistance systémique induite (ISR) (Van Loon *et al.*, 1998), « l'immunisation » de la plante découle de la reconnaissance par les cellules racinaires de métabolites particuliers secrétés par le PGPR : les éliciteurs de l'ISR. Aujourd'hui de plus en plus de travaux de recherches relatent le potentiel de certaines souches à induire l'ISR chez leur hôte. Dans certains cas, les éliciteurs responsables de l'activité des souches ont été identifiés. Ces molécules, aussi appelés déterminants, sont de natures variées et pouvaient jusqu'il y a peu être divisées selon trois classes : des composants de surface cellulaire, des métabolites à activité chélatrice du fer et des molécules à activité antibiotique (Van Loon and Bakker, 2005; Ongena and Thonart, 2006). Ces dernières années, plusieurs autres éliciteurs inclassables dans ces trois catégories ont également été isolés. Nous proposons dans ce chapitre de résumer les différents composés connus qui sont impliqués dans cette « communication » particulière entre les PGPR et la plante, menant à la mise en place de l'ISR.

**Les composants de surface cellulaire**

Les lipopolysaccharides (LPS) et les flagelles comptent parmi les composants de surface cellulaires les plus souvent impliqués dans l'induction de mécanismes de défenses chez les plantes. Ils constituent des éléments importants pour les bactéries Gram-negatives car ils interviennent soit dans leur mobilité, et donc leur capacité à se déplacer vers des environnements propices à leur croissance, soit dans l'adhésion sur les racines (Persello-Cartieaux *et al.*, 2003). Les LPS peuvent aussi contribuer à la survie de la bactérie en agissant comme une barrière contre la diffusion de toxines en provenance de l'hôte (Newman, 2002).

Les LPS sont exposés sur la membrane externe de la bactérie et sont constitués d'une partie lipidique (lipide A), d'un noyau oligosaccharidique et d'une partie O-polysaccharide (antigène O) (Figure 1) (Erbs and Newman, 2003). Leur implication dans le phénomène d'ISR a été démontrée sur divers pathosystèmes grâce à l'utilisation de LPS purifiés, d'extraits d'enveloppe de cellules, ou via l'utilisation de mutants possédant des motifs antigéniques modifiés. Les premières démonstrations de leur activité biologique ont été réalisées par Van Peer et ses collaborateurs, qui ont testé la capacité de *Pseudomonas fluorescens* WCS417 à diminuer l'impact de la maladie provoquée par *Fusarium oxysporum* (Van Peer *et al.*, 1991; Van Peer and Schippers, 1992). Concernant les Brassicacée, les LPS des souches de *Pseudomonas fluorescens* (WCS374 et WCS417) et *Pseudomonas putida* (WCS358) stimulent l'ISR chez le radis et *Arabidopsis* contre *Fusarium oxysporum* (Leeman *et al.*, 1995; Van Wees *et al.*, 1997). La souche WCS358 est également en mesure de protéger *Arabidopsis thaliana* et le haricot, une fabacée, contre des pathogènes foliaires comme *Pseudomonas syringae* ou *Botrytis cinerea* (Meziane *et al.*, 2005). Enfin, des effets similaires ont été observés sur les systèmes « rhizobactérie/plante/pathogènes » tels que *Burkholderia cepacia*/tabac/*Phytophthora nicotiana*, *Pseudomonas* /tomate/*Fusarium oxysporum* et *Botrytis cinerea*, et *Rhizobium etli*/pomme de terre/cyst nematode *Globodera pallida* (Duijff *et al.*, 1997; Reitz *et al.*, 2000; Coventry and Dubery, 2001). Dans de nombreux cas, les LPS possédant une modification de la chaîne antigénique O ne sont pas en mesure de déclencher l'ISR chez la plante hôte, suggérant le rôle de cette sous structure dans la reconnaissance par la plante (Leeman *et al.*, 1995; Meziane *et al.*, 2005). Cependant, dans le cas de la stimulation de résistance contre le nématode *Globodera pallida* chez la pomme de terre inoculée par *Rhizobium etli*, il semblerait que l'activité des LPS soit dépendante du noyau oligosaccharide (Reitz *et al.*, 2002). Actuellement, peu informations sont disponibles sur la manière dont les LPS sont présentés à la plante ni comment celle-ci les reconnaît. Des auteurs suggèrent que les LPS soient perçus lorsqu'ils font partie intégrante de la membrane bactérienne ou sous la forme

de micelles (Beveridge, 1999). D'autre part, à notre connaissance aucun récepteur aux LPS ou de complexes de liaison ont été identifiés chez les plantes (Gerber *et al.*, 2004).

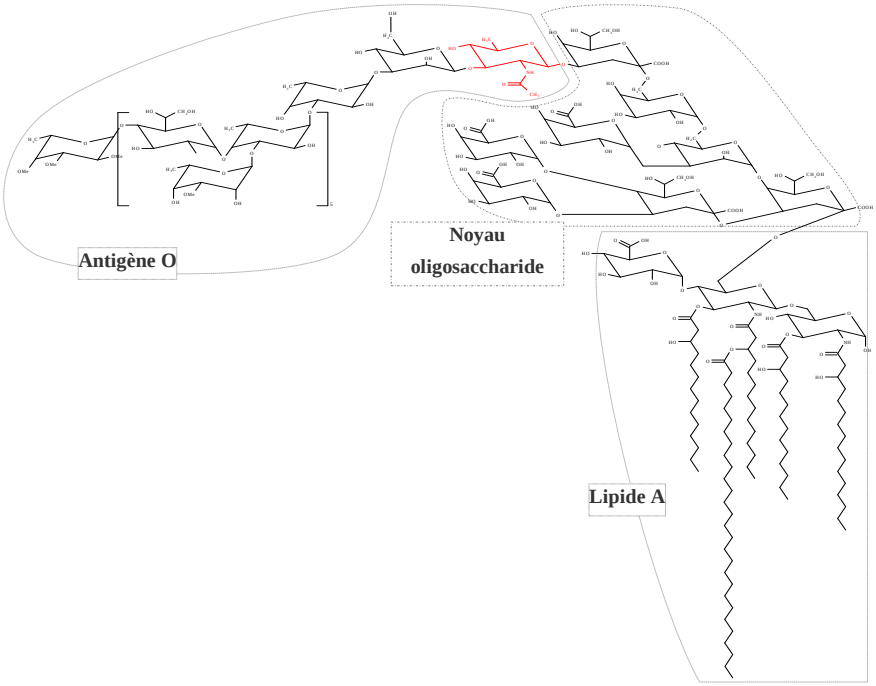



**Figure 1.** Exemple de structure de lipopolysaccharides (LPS) de *Rhizobium elti*. Les LPS sont constitués d'une partie lipidique (lipide A), d'un noyau oligosaccharidique et d'une partie O-polysaccharide (antigène O)

Les flagelles sont des organites constitués de plusieurs milliers de sous-unités d'une protéine, la flagelline. La partie N-terminal de la séquence protéique comporte 22 acides aminés (flg 22) relativement conservés chez certaines bactéries, dont 15 d'entres eux (flg 15) sont identiques chez certaines espèces (Figure 2). L'implication des flagelles dans le phénomène d'ISR a été démontrée grâce à *Pseudomonas putida* WCS358, qui est capable de protéger *Arabidopsis* de la maladie causée par *Pseudomonas syringae* (Meziane *et al.*, 2005). Paradoxalement, les travaux menés par les auteurs révèlent que les flagelles de WCS358 ne semblent pas aptes à stimuler l'ISR chez la tomate et le haricot respectivement infectés par *Botrytis cinerea* et *Colletotrichum lindemuthianum*, suggérant des activités différentes selon le pathosystème étudié. D'après des recherches menées avec le pathogène *Pseudomonas aeruginosa*, le peptide flg22 serait responsable de la perception des flagelles bactériens par les cellules de la plante (Felix *et al.*, 1999). La reconnaissance du peptide se ferait via FLS2, un récepteur transmembranaire de type LRR-kinase (Gómez-Gómez, 2004; Nurnberger and Kernmerling, 2006). Le peptide flg22, voire flg15, serait donc la structure active de la

flagelline et pourrait s'accumuler dans la rhizosphère lors de l'assemblage ou de la dégradation du flagelle, puis diffuser à travers la paroi végétale et être reconnue par la plante (Gomez-Gomez and Boller, 2002; Meziane *et al.*, 2005), En effet, le récepteur FLS2 est exprimé au niveau des racines d'*Arabidopsis* et la séquence d'acides aminés du domaine conservé N-terminal flg15 de la flagelline de *Pseudomonas putida* est identique à celle de flg22 (Felix *et al.*, 1999; Chinchilla *et al.*, 2006). D'autre part, un récepteur similaire à FLS2 a également été identifié chez la tomate, qui malgré cela, ne semble pas être réactive à la flagelline de *Pseudomonas putida* WCS358. Il est alors envisageable que les mécanismes de défenses induits suite à la reconnaissance du peptide par la tomate ne soient pas effectifs contre *Botrytis cinerea* (Meziane *et al.*, 2005). Enfin, chez les autres plantes, la présence d'un tel récepteur reste encore inconnue.

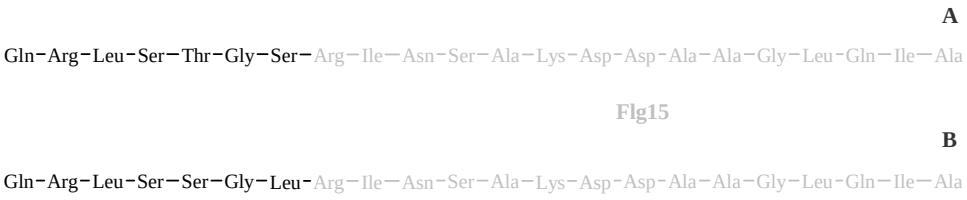



**Figure 2.** Alignement de séquences N-terminales de flagellines.
**A.** Séquence d'acides aminés de flg22 constituant la flagelline du pathogène *Pseudomonas aeruginosa* pv tabaci.
**B.** Séquence d'acides aminés N-terminal de la flagelline de *Pseudomonas putida*.

## Les sidérophores

### *Les pyoverdines*

Malgré son abondance dans le sol, le fer représente un micro-élément difficilement disponible pour les microorganismes de la rhizosphère. En effet, les ions ferriques ($Fe^{3+}$), nécessaires pour la machinerie cellulaire, ne sont présents dans le sol qu'à des concentrations avoisinant $10^{-18}$ M. Les microorganismes du sol ont donc développé une stratégie afin de l'assimiler via la production de molécules chélatrices du fer, les sidérophores (Mercado-Blanco and Bakker, 2007). Les pyoverdines (ou pseudobactines), principale classe de sidérophores produits par les *Pseudomonas* fluorescents, présentent la même structure générale : une séquence peptidique de 6-12 acides aminés, souvent spécifique de la souche productrice, liée à un chromophore, impliqué dans la chélation (Figure 3) (Budzikiewicz, 2004). La compétition pour le fer et l'induction de l'ISR par les sidérophores des PGPR sont d'ailleurs bien décrits dans un article paru récemment (Höfte and Bakker, 2007). Les pyoverdines de *Pseudomonas fluorescens*

WCS358 peuvent induire l'ISR chez *Arabidopsis*, le haricot, la tomate et l'eucalyptus (Meziane *et al.*, 2005; Ran *et al.*, 2005b) et ceux secrétés par WCS374 diminuent, via l'ISR, l'impact de la maladie causée par *Fusarium* sur le radis (Leeman *et al.*, 1996). Le potentiel éliciteur des pyoverdines ne se restreint pas à protéger les plantes contre des bactéries et des champignons. En effet, le développement du virus TNV (Tobacco Necrosis Virus) est limité sur des plants de tabac traités par des pyoverdines de *Pseudomonas fluorescens* CHAO (Maurhofer *et al.*, 1994). Enfin, Press et ses collaborateurs ont observé que *Serratia marcescens* 90-166 pouvait induire une résistance contre des champignons, virus et bactéries pathogènes du concombre, comme *Colletotrichum orbiculare*, *Fusarium oxysporum*, le virus de la mosaique du concombre, *Pseudomonas syringae* et *Erwinia tracheiphila*. La diminution de l'effet protecteur de la souche est corrélée avec un accroissement de la concentration en fer dans le sol, ce qui, d'une manière générale, inhibe la synthèse des sidérophores. Durant leur travaux, ils ont mis en évidence que l'ISR déclenchée chez le concombre était dépendante de la production d'un sidérophore de type catéchol (Press *et al.*, 2001).

Cependant, certaines expériences réalisées avec des souches productrices de pyoverdines ont démontré que ces composés n'étaient pas toujours impliqués dans la stimulation de l'ISR. C'est le cas de *Pseudomonas putida* BTP1, dont les pyoverdines sont inaptes à déclencher une réaction de défense chez le haricot et le concombre (Ongena *et al.*, 2000; Ongena *et al.*, 2002). De même, les pyoverdines des souches WCS358 et WCS417 sont inaptes à induire l'ISR chez le radis (Leeman *et al.*, 1996) et celles produites par 7NSK2 ne sont pas responsables de l'activité élicitrice de la souche sur la tomate contre *Botrytis cinerea* (Audenaert *et al.*, 2002).

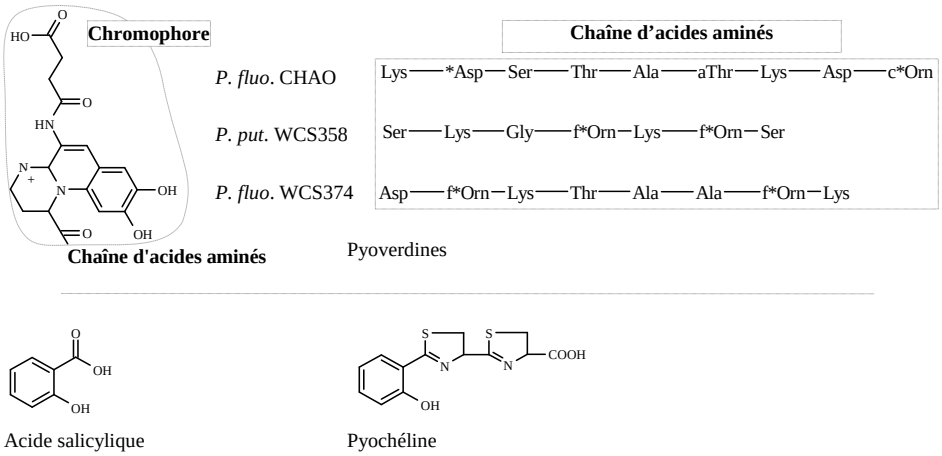



**Figure 3.** Structures de molécules de type sidérophore identifiées en tant qu'éliciteurs de la résistance systémique induite par les rhizobactéries. Les pyoverdines sont constituées d'un chromophore lié à une séquence d'acides aminés. *Acides aminés hydroxylés.

*L'acide salicylique*

L'acide salicylique est synthétisé à partir de l'isochorismate synthase et de la pyruvate lyase. Il intervient comme précurseur de la pyochéline, composé possédant également une activité chélatrice du fer. Le rôle de l'acide salicylique (AS) dans l'induction de résistance systémique a été mis en évidence sur le haricot et la tomate infectés par *Botrytis cinerea* grâce à *Pseudomonas aeruginosa* 7NSK2 et ses mutants non producteurs de pyoverdine, d'AS et/ou de pyochéline (De Meyer and Höfte, 1997; De Meyer *et al.*, 1999b). Par la suite, la même souche s'est avérée active sur le tabac contre le virus de la mosaïque (De Meyer *et al.*, 1999a) et tout dernièrement sur le riz contre *Rhizoctonia solani* (De Vleesschauwer, *unpublished results*). L'utilisation de la souche KMPCH, mutant de 7NSK2 ne produisant que de l'AS, a aussi montré son activité inductrice sur le haricot contre *Colletotrichum lindemuthianum* (Bigirimana and Höfte, 2002). Le rôle de l'AS en tant qu'éliciteur de la résistance systémique induite par des rhizobactéries bénéfiques a aussi été étudié via d'autres approches. Des expériences entreprises avec *Pseudomonas fluorescens* P3, une souche initialement non productrice d'AS et rendue active par l'insertion d'un gène de biosynthèse de l'acide salicylique, ont aussi contribué à la démonstration de l'activité élicitrice de l'AS dans l'ISR chez le tabac contre le virus de la nécrose du tabac (Maurhofer *et al.*, 1998). Par ailleurs, l'application d'AS exogène est aussi capable de stimuler des mécanismes de résistance systémique chez le radis (Leeman *et al.*, 1996) et de façon plus générale, chez les plantes (Sticher *et al.*, 1997).

Mais d'autres travaux suggèrent au contraire que l'AS produit par certaines rhizobactéries n'est pas responsable de l'effet protecteur observé (Press *et al.*, 1997; Ran *et al.*, 2005a). En effet, l'AS produit par la rhizobactérie pourrait être un précurseur de biosynthèse comme dans le cas du catéchol produit par *Serratia marcescens*, précédemment cité (Press *et al.*, 2001). Une conversion de l'AS pourrait également avoir lieu au niveau des racines de la tomate traitée par *Pseudomonas aeruginosa* 7NSK2. La présence de cystéine rejetée dans les exsudats racinaires favoriserait la production par la souche de pyochéline, qui elle-même peut intervenir dans l'induction de résistance de la plante contre *Botrytis cinerea* (voir plus bas) (Audenaert *et al.*, 2002).

**Les antibiotiques**

La production d'antibiotiques chez *Pseudomonas* est décrite comme un important mode d'action dans la suppression des maladies par antibiose (Haas and Défago, 2005). Cependant,

plusieurs études illustrent le potentiel de certains de ces composés à également induire une réponse de type ISR chez les plantes. C'est le cas du 2,4-diacetylphoroglucinol (DAPG), produit par *Pseudomonas fluorescens* CHA0 qui protège *Arabidopsis thaliana* de *Peronospora parasitica* (Iavicoli *et al.*, 2003). Toujours chez *Arabidopsis*, la rhizobactérie *Pseudomonas fluorescens* Q2-87 est également apte à protéger son hôte contre *Pseudomonas syringae* via l'ISR induite par le DAPG (Weller *et al.*, 2004). Enfin, l'action élicitrice de cet antibiotique s'est également avérée efficace contre le nématode *Meloidogyne javanica*, responsable de dégâts sur les racines de tomate (Siddiqui and Shaukat, 2003).

La pyocyanine, composé de la famille des phénazines produit par *Pseudomonas aeruginosa* 7NSK2, est un autre exemple d'antibiotique ayant une activité élicitrice de la résistance chez les végétaux. De récents résultats chez le riz décrivent l'effet à la fois positif et négatif de la pyocyanine produite par la souche 7NSK2 selon le type de pathogène impliqué. En effet, la pyocyanine induit l'ISR contre le pathogène hémibiotrophe *Magnaporte grisea* mais accentue la susceptibilité de la plante au pathogène nécrotrophe *Rhizoctonia solani* (De Vleesschauwer *et al.*, 2006). Chez la tomate, la pyocyanine agirait en synergie avec la pyochéline pour protéger la plante contre *Botrytis cinerea* via l'ISR (Audenaert *et al.*, 2002).

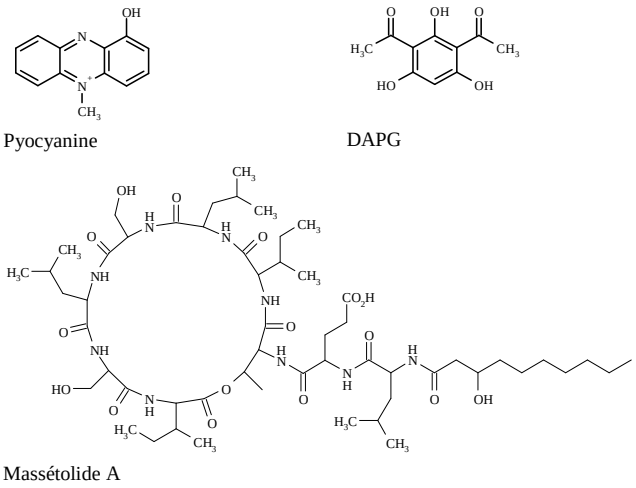



**Figure 4.** Structures d'antibiotiques identifiés en tant qu'éliciteurs de la résistance systémique induite.

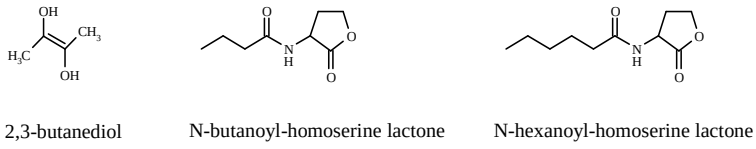
### Nouveaux déterminants de l'ISR

Les bactéries Gram-négatives ne sont pas les seules rhizobactéries impliquées dans le phénomène d'ISR. En effet, ces dix dernières années, une quantité croissante de souches de

*Bacillus* ont été identifiées (Kloepper *et al.*, 2004) pour leur aptitude élicitrice sur des plantes aussi variées que la tomate, le haricot le café ou le concombre.

Romeiro et ses collaborateurs ont isolé des macromolécules produites par *Bacillus cereus* UFV-101 et capables de déclencher l'ISR chez la tomate contre plusieurs pathogènes, champignons ou bactéries. Ces molécules n'ont à ce jour pas été identifiées et leurs natures ne sont pas connues, cependant, selon les auteurs, elles forment une substance unique ou appartiennent à un même groupe, et possèdent une masse moléculaire de 12 kDa (Romeiro *et al.*, 2005).

D'autre part, des composés organiques volatibles, dont le 2,3-butandiol, issus des souches *Bacillus subtilis* GB03 et *Bacillus amylofaciens* IN937a, confèrent aux plantules d'*Arabidopsis* une protection contre le pathogène *Erwinia carotovora* (Ryu *et al.*, 2004). Le même pathogène se voit également être inhibé chez le tabac traité par le 2,3-butandiol seul, ou par *Pseudomonas chlororaphis* O6, une autre souche productrice. Toutefois dans le phytosystème *Pseudomonas chlororaphis* O6/tabac/*Pseudomonas syringae*, même si la souche est active, il semble que le composé volatil ne soit pas impliqué, suggérant une spécificité vis-à-vis du pathogène (Han *et al.*, 2006). Par ailleurs, il est intéressant de noter que le 2,3-butandiol est aussi en mesure de stimuler de manière significative la croissance de son hôte, conférant à la souche O6 une double compétence.




2,3-butanediol N-butanoyl-homoserine lactone N-hexanoyl-homoserine lactone

**Figure 4.** Structures de déterminants de l'ISR récemment caractérisés.

Pour finir, d'après les travaux menés par Schuhegger et ses collaborateurs, des molécules intervenant dans la communication entre les microorganismes, ou quorum sensing, seraient également capables de stimuler le phénomène d'ISR. En effet, les N-acyl-L-homoserine lactones (AHL) de *Serratia liquefaciens* MG1 et *Pseudomonas putida* IsoF sont responsables de la mise en place de l'ISR chez la tomate contre le champignon pathogène *Alternaria alternata* (Schuhegger *et al.*, 2006).

Enfin, nous devons également citer l'action de la massetolide A, un lipopeptide cyclique produit par *Pseudomonas fluorescens* SS101 qui stimule la résistance de la tomate contre *Phytophthora infestans* (Tran *et al.*, 2007).

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# Objectifs

D’un point de vue biochimique et moléculaire, l’induction de résistance chez les plantes par certaines rhizobactéries non pathogènes (ISR) se différentie des autres réactions de défense systémiques (chapitre II et III). L’ISR peut donc être considérée comme composante à part entière de la réaction immunitaire chez les végétaux et elle en est probablement la branche la plus récemment mise à jour. Ce phénomène représente donc un des aspects les plus fascinants des interactions plante-microorganismes mais aussi l’un des plus méconnus. En examinant attentivement le paysage scientifique à l’entame de cette thèse, il est clair que l’intérêt des chercheurs concernés par ce domaine était principalement dirigé vers l’étude de la signalisation et/ou des mécanismes de défense induits dans les tissus de l’hôte végétale (chapitre II). En comparaison, peu de recherches sont consacrées à mieux comprendre la première étape, pourtant cruciale, qui est la production des éliciteurs bactériens et leur perception par les cellules de la plante. Le sujet général de notre travail de thèse est donc de contribuer à l’étude du dialogue moléculaire qui s’établit entre les bactéries et le végétal dans le cadre de l’ISR.

Dans ce contexte, plusieurs études antérieures poursuivies au laboratoire ont mis en évidence la capacité de la souche *Pseudomonas putida* BTP1 à protéger le haricot et le concombre contre *Botrytis cinerea* et *Pythium aphanidermatum* via l’induction de l’ISR (Ongena *et al.*, 2002; Ongena *et al.*, 2000).. Cependant, les rares éliciteurs initialement connus pour déclencher ce type de phénomène, comme les composants de surface cellulaire, les pyoverdines ou l’acide salicylique, n’étaient pas impliqués ou non produits par la souche BTP1. D’autre part, des résultats prometteurs ont également été obtenus avec une autre rhizobactérie étudiée au laboratoire, *Bacillus subtilis* S499 (aussi dénommée M4). Alors qu’elle était à l’origine essentiellement étudiée pour son effet protecteur via des mécanismes d’antibiose, des expériences préliminaires laissaient également entrevoir le potentiel de la souche S499 à induire l’ISR.

Les objectifs de cette thèse ont donc naturellement découlé de ces acquis. Les deux souches ont ainsi été utilisées comme modèles sur lesquels se sont basées toutes nos investigations. Les objectifs et la démarche poursuivie avec les deux organismes est globalement similaire : i) confirmer l’activité inductrice de résistance systémique, ii) identifier le ou les métabolites responsables de cette propriété, iii) évaluer la spécificité d’action de ces molécules par rapport au pathosystème testé, iv) étudier leur production en fonction de certains paramètres de la rhizosphère, v) tenter de comprendre comment elles sont perçues par les cellules végétales, et pré-caractériser les évènements cellulaires précoces qu’elles sont susceptibles d’y engendrer.

Durant les six prochains chapitres, nous présenterons ainsi l’ensemble des résultats qui ont découlés de nos recherches. Les investigations réalisées sur *Pseudomonas putida* seront

développées dans les chapitres IV à VI et seront suivies par le fruit des travaux réalisés avec *Bacillus subtilis* dans les trois derniers chapitres (VII à IX).

Dans le premier chapitre consacré à *Pseudomonas putida* BTP1 (Chapitre IV), nous présenterons l'identification de son éliciteur, le NABD. L'activité biologique de ce nouveau composé mise en évidence sur haricot sera ensuite investiguée sur d'autres plantes de famille différentes afin d'en dégager une possible spécificité (chapitre V). D'autre part, à travers son exsudation racinaire, la plante influence la rhizosphère et indirectement la distribution et le comportement des populations microbiennes. Dans le chapitre V, nous nous sommes donc également intéressés à l'influence de certains paramètres liés à la rhizosphère sur la production du NABD par la souche lorsque celle-ci se développe dans ce microenvironnement. Enfin, la section consacrée à *Pseudomonas putida* BTP1 se clôturera par le chapitre VI, dans lequel nous avons développé une approche originale, via de la chimie organique, afin de synthétiser tout une gamme de dérivés structuraux du NABD dans l'objectif d'identifier les sous-structures d'importance pour son activité biologique.

L'activité de la souche *Bacillus subtilis* M4, aussi dénommée S499, en tant qu'inductrice de l'ISR dans deux pathosystèmes est tout d'abord démontrée dans le chapitre VII. La recherche et l'identification des éliciteurs de la souche impliqués dans le phénomène d'immunisation fait l'objet du chapitre VIII, ainsi que leur action sur plusieurs plantes. Dans la dernière partie du travail, nous avons étudié plus en profondeur leur interaction avec les cellules végétales en utilisant des cultures cellulaires de tabac. L'objectif double était de mieux connaitre les sous-structures impliquées dans la perception de ces élicteurs et la cascade d'évènements précoces qui en résulte (chapitre IX).

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# Résultats

# *Chapitre IV. Isolation of an N-alkylated benzylamine derivative from* **Pseudomonas putida** *BTP1 as elicitor of induced systemic resistance in bean*

*Molecular Plant-Microbe Interactions* **18**(6): 562-569

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**Résumé**

Des recherches préalables à cette thèse ont démontré que la souche *Pseudomonas putida* BTP1 était en mesure d'induire une réduction significative des maladies sur concombre et haricot respectivement provoquées par *Colletotrichum lagenarium* et *Botrytis cinerea* via l'ISR, et que les métabolites connus jusqu'alors pour être actifs en tant qu'éliciteurs ne semblaient pas impliqués ce cas.

Le premier objectif de nos recherches fut donc d'isoler et d'identifier l'éliciteur responsable de l'activité protectrice de la souche. Pour cela, dans ce chapitre, nous nous sommes focalisés sur l'ISR induite chez le haricot. L'éliciteur bactérien a été isolé à partir de surnageant de la souche cultivée un milieu limitant en fer via une succession d'étapes d'extraction et de purification par chromatographie. Il en a résulté l'obtention d'une fraction HPLC contenant un unique composé actif parmi la panoplie des produits sécrétés par la bactérie dans ces conditions de culture.

La comparaison des protections conférées chez le haricot contre *Botrytis cinerea* par le traitement préalable des racines avec la rhizobactérie, le surnageant de culture semi purifié et l'éliciteur pur suggèrent fortement que ce composé soit responsable de l'essentiel de l'activité inductrice de l'ISR de la souche, du moins dans ce phytosystème. Ces résultats ont par la suite été confirmés par plusieurs approches. Un extrait de surnageant reconstitué sans la fraction HPLC contenant cette molécule n'a présenté aucun effet protecteur sur la plante. De même, le surnageant de BTP1 cultivée dans des conditions non limitantes en fer ne contient pas le composé et est également inapte à induire l'ISR chez la plante. Enfin, le test de la souche mutante M1 (et de son surnageant) affectée dans la biosynthèse du NABD et des pyoverdines n'induit aucune diminution significative de la maladie. Par contre, une résistance de la plante contre le phytopathogène est déclenchée lors du traitement par le mutant M3, uniquement affecté dans la production de pyoverdines. Ces expériences ont aussi confirmé que les pyoverdines synthétisées par BTP1 n'étaient pas impliquées dans l'ISR, pas plus que d'autres métabolites.

Par ailleurs, même si une variabilité importante dans les concentrations mesurées d'une expérience à l'autre a été notée, des quantités significatives de NABD ont pu être extraites de la rhizosphère des plants de haricot traités avec BTP1. Bien qu'on n'ait pas pu démontrer par ces dosages que le NABD s'accumule en quantités suffisantes pour induire l'ISR, ces résultats prouvent néanmoins que l'éliciteur est produit par la bactérie lorsqu'elle se développe sur les racines.

Une première identification structurale a été réalisée essentiellement sur base d'analyses en LC-MS-MS. Les données obtenues indiquent que le composé actif possède une structure

constituée d'un noyau benzylamine dont l'azote serait alkylé par des groupements tridécyle, éthyle et méthyle responsables de l'hydrophobicité relative de la molécule. Sur cette base, ce métabolite a été dénommé NABD (N-alkylated benzylamine derivative) ayant pour formule $C_6H_5–CH_2–N^+(CH_3)(CH_2CH_3)–C_{13}H_{27}$.

L'isolement du NABD a ainsi permis d'élargir et de diversifier la gamme relativement restreinte des déterminants microbiens de l'ISR connus jusqu'alors et apparaît aussi comme un nouveau type de molécule isolée chez les *Pseudomonas*.

**Abstract**

Root treatment of *Phaseolus vulgaris* with the nonpathogenic *Pseudomonas putida* BTP1 led to significant reduction of the disease caused by the pathogen *Botrytis cinerea* on leaves. The molecular determinant of *P. putida* BTP1 mainly responsible for the induced systemic resistance (ISR) was isolated from cell-free culture fluid after growth of the strain in the iron-poor casamino acid medium. Mass spectrometry analyses performed on both the bacterial product and synthetic analogues revealed a polyalkylated benzylamine structure, with the quaternary ammonium substituted by methyl, ethyl, and $C_{13}$ aliphatic groups responsible for the relative hydrophobicity of the molecule. The specific involvement of the N-alkylated benzylamine derivative (NABD) in ISR elicitation was first evidenced by testing the purified compound that mimicked the protective effect afforded by crude supernatant samples. The evidence was supported by the loss of elicitor activity of mutants impaired in NABD biosynthesis. Our experiments also showed that other iron-regulated metabolites secreted by the strain are not involved in ISR stimulation. Thus, these results indicate a wider variety of *Pseudomonas* determinants for ISR than reported to date.

**Introduction**

Among the biological control alternatives to the use of chemical pesticides, the application of nonpathogenic soil bacteria living in association with plant roots is of increasing interest (Walsh *et al.*, 2001; Zahir *et al.*, 2004). In many cases, treatment with some of these plant growth-promoting rhizobacteria (PGPR) was associated with reduced plant diseases in greenhouse and field experiments. PGPR can exert their beneficial effect on plants by inhibiting pathogen growth via several mechanisms, such as niche exclusion, competition for nutrients, siderophore-mediated competition for iron, synthesis of antibiotics, and production

of extracellular enzymes (Haas and Keel, 2003; Ramamoorthy *et al.*, 2001; Whipps, 2001). The isolation of some rhizobacteria lacking the ability to exert any direct antagonistic activity toward pathogens sheds new light on the diversity of their modes of action and suggested that such strains may activate host defense systems (Van Loon *et al.*, 1998). Because bacterial treatment at the root level confers durable protection against foliar or systemic pathogens, the PGPRmediated resistance is phenotypically similar to the well-studied systemic acquired resistance (SAR) activated after a first infection by an incompatible necrotizing pathogen (Durrant and Dong, 2004; Sticher *et al.*, 1997). This effect of rhizobacteria is referred to as induced systemic resistance (ISR) (Kloepper *et al.*, 1992). ISR is effective against a broad range of fungal, bacterial, and viral diseases, as well as against some insect and nematode pests, and ISR-based disease control assays were performed successfully under field conditions (Wei *et al.*, 1996; Zehnder *et al.*, 2001).

Until recently, PGPR determinants responsible for ISR elicitation could be divided into two classes: cell surface components, such as flagella or outer membrane lipopolysaccharides, and iron-regulated metabolites with siderophore activity (Van Loon *et al.*, 1998). The elicitor activity of lipopolysaccharides or, more specifically, of their O-antigenic side chain, was identified for *Pseudomonas fluorescens* on carnation (Van Peer and Schippers, 1992), radish (Leeman *et al.*, 1995), and tomato (Duijff *et al.*, 1997) and also for *Rhizobium etli* on potato (Reitz *et al.*, 2002). The composition of lipopolysaccharides from pseudomonads is strain specific and their eliciting activity seems to be dependent on the isolate. Pyoverdines typically synthesized by fluorescent *Pseudomonas* spp. (Budzikiewicz, 2004) were suggested as siderophore-type ISR elicitors. In soil, efficient production of pyoverdines with very high affinity for ferric ions can restrict fungal pathogen growth and thereby limit disease incidence by making this element unavailable (Loper and Buyer, 1991). From experiments involving pyoverdine-nonproducing mutants (Maurhofer *et al.*, 1994) or addition of pure pyoverdines (Leeman *et al.*, 1996), these compounds also were suggested as potential ISR elicitors; however, this hypothesis is controversial and probably cannot be applied commonly to other *Pseudomonas* strains (Van Loon *et al.*, 1998). Investigations conducted by Press and associates (2001) suggest that the expression of genes involved in the biosynthesis of an unidentified catechol siderophore is associated with ISR induced by *Serratia marcescens* 90-166 in cucumber. 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; 1999) and *P. fluorescens* P3, which was converted to an ISR-inducing strain by the insertion of SA biosynthetic genes (Maurhofer *et al.*, 1998). 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 a precursor or intermediate in the biosynthesis of other siderophores, such as pyochelin in *P. aeruginosa* (Serino *et al.*, 1997) or pseudomonine produced by *P. fluorescens* (Mercado-Blanco *et al.*, 2001). No ISR-eliciting activity was clearly demonstrated for the latter compound; however, a role for pyochelin acting synergistically with the phenazine pyocyanin was reported for ISR triggered in tomato by *P. aeruginosa* 7NSK2 (Audenaert *et al.*, 2002). Another antibiotic from *Pseudomonas* spp., 2,4-diacetylphloroglucinol (DAPG), also retains some ability to stimulate defense-related reactions in the host plant (Iavicoli *et al.*, 2003). In general, the mechanisms involved in rhizobacteria-mediated ISR appear to vary among bacterial strains or pathosystems and much remains to be discovered about the nature and variety of bacterial determinants responsible for the elicitation of defense mechanisms.

We previously have reported the ISR-mediated protective effect of *P. putida* BTP1 on cucumber against root rot caused by *Pythium aphanidermatum* (Ongena *et al.*, 1999; 2000). The strain also was effective at reducing disease incidence caused by *Botrytis cinerea* on bean plants (Ongena *et al.*, 2002). Most of the resistance-eliciting activity of the strain was retained in a specific fraction of the supernatant extract obtained after growth in an iron-limited medium. The objective of the current study was to identify the specific agent to which systemic resistance-inducing activity can be attributed.

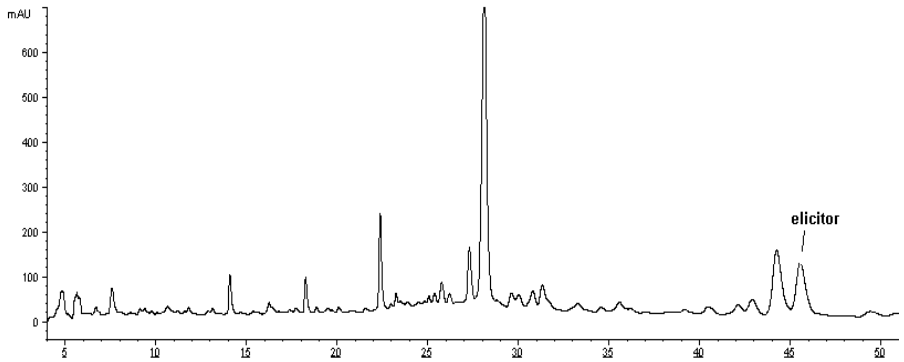


**Figure 1.** Typical chromatogram obtained for the purification/analysis of the elicitor produced by *P. putida* BTP1 by reverse-phase high-performance liquid chromatography from casamino acids medium supernatant extract.

## Results

### *Isolation of the ISR determinant from* Pseudomonas putida *BTP1*

In a previous study, we showed that the ISR-eliciting potential of *Pseudomonas putida* BTP1 was retained mostly by hydrophobic material secreted in the medium upon growth of the strain in the iron-poor casamino acids medium (CAA) (Ongena *et al.*, 2002). The active ingredient

was purified as described below (discussed below) to the single peak in the high-performance liquid chromatography (HPLC) profile (Figure 1). When tested at a concentration of 0.2 µM in the nutrient solution, the compound induced a 24% reduction in the number of spreading lesions caused by the leaf pathogen *B. cinerea* on hydroponically grown bean plants (Figure 2). This protection level was similar to the level observed by testing crude supernatant samples (28%) diluted in a way to add the elicitor at the same final concentration in contact with the roots. Treatment of bean roots with a 10-fold higher concentration of the elicitor did not result in enhanced disease biocontrol level and no protection was provided by applying the elicitor at a concentration as low as 2 nM (data not shown). The material eluted before and after the elicitor during HPLC purification was concentrated and also tested as a single sample. No marked resistance was induced in bean by this treatment (data not shown). Upon addition of ferric chloride to the CAA medium at final concentrations of 100 and 200 µM, production of the elicitor by BTP1 was reduced to values of 1.9 ± 0.28 and 0.7 ± 0.15 ng/$10^8$ CFU, respectively; whereas, without ferric chloride, BTP1 produced 75.5 ± 10.1 ng/$10^8$ CFU of elicitor. None of the resulting culture supernatant extracts, after addition of ferric chloride, showed resistance-inducing activity (Figure 2).

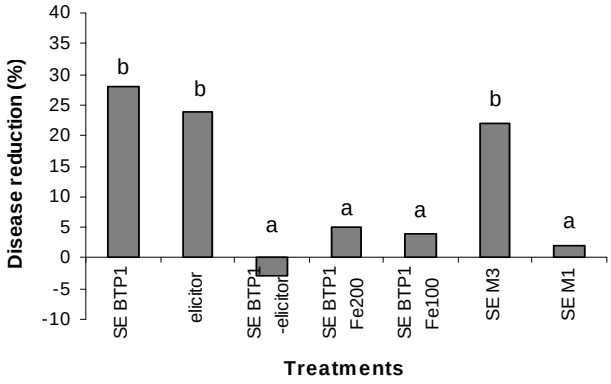


**Figure 2.** Resistance induced in bean plants by the purified elicitor or by various culture extracts from *Pseudomonas putida* BTP1 or its derivatives. Treatments are as follows: ethyl acetate extracts of supernatants (SE) obtained after growth of BTP1, M1, and M3 (SE BTP1, SE M1, and SE M3, respectively) in casamino acids medium (CAA). These extracts containing the elicitor were added to the nutrient solution to obtain a 0.2-µM final concentration of the active compound; SE BTP1-elicitor, supernatant sample corresponding to the SE BTP1 extract but without semi-preparative high-performance liquid chromatography fractions containing the elicitor; elicitor, active compound tested in a pure form at a concentration of 0.2 µM; SE BTP1 Fe200 and SE BTP1 Fe100, ethyl acetate extract of supernatant obtained after growth of BTP1 in CAA supplemented with FeCl3 at final concentrations of 200 and 100 µM. The reduction in the number of *Botrytis cinerea* spreading lesions is relative to the number of lesions counted in control plants. Data are means of three independent experiments except for assays with SE BTP1 Fe200 and SE BTP1 Fe100 treatments that were performed twice. The homogeneity of variances was tested by analysis of variance and data from experiments with the same set-up were pooled for analysis because interactions between experiment and treatment were not significant at $P$=0.05. Means from the different treatments were compared by the method of Newman and Keuls (least significant difference at $\alpha$=0.05). Values indicated with "a" are not statistically different from the control.

*ISR activity of mutants from* P. putida *BTP1*

Additional evidence for the ISR-eliciting potential of the active fraction arose from the evaluation of protective levels triggered by treatment of bean with BTP1 derivatives obtained by chemical mutagenesis. The two mutants designated as M3 and M1 originally were selected for their reductions in pyoverdine biosynthesis. The growth of both M1 and M3 is also markedly reduced in iron-restricted media (King's B medium [KB] or CAA supplemented with the chelating agent ethylenediamine-di-(*o*-hydroxyphenylacetate [EDDHA]) compared with the wild-type BTP1 and fully restored in iron-rich media (data not shown), suggesting that growth of M1 and M3 are reduced when pyoverdines are necessary to supply the cells with iron. Although pyoverdine synthesis is repressed in both isolates, M3 is still effective at producing the ISR determinant at a level similar to BTP1 (Table 1). ISR experiments performed in soil with live cells revealed a complete loss of the protective activity by the M1 derivative. At the same time, M3 retained the ability to induce ISR (Table 1). Bacterial concentrations in the rhizosphere were estimated at $4.6 \pm 2.5$ and $7.2 \pm 2.2 \times 10^5$ CFU/g of root fresh weight for M1 and M3, respectively. Mutant cells were not detected in leaf samples with a detection limit of approximately 100 CFU/g of leaf, suggesting that bacteria did not migrate through the plants.

**Table 1.** Quantification of elicitor and pyoverdine produced by *Pseudomonas putida* BTP1 and two derivatives in relation with resistance induced in bean plants

| Isolate | Pyoverdine (µg/$10^8$ CFU)[y] | Elicitor (ng/$10^8$ CFU)[y] | Disease reduction (%)[z] |
|---|---|---|---|
| BTP1 | 21.2 ± 4.53 | 70.3 ± 14 | 39 b |
| M3 | 4.1 ± 2.21 | 62.8 ± 8.4 | 32 b |
| M1 | 1.2 ± 0.17 | 7.9 ± 3.1 | 6 a |

y Data obtained from 72-h growth of the isolates in casamino acids medium. Each value is the mean of five repetitions.

z Expressed as the reduction in *Botrytis cinerea* spreading lesions relative to the number of spreading lesions in control plants treated with sterile distilled water. Data are means of two independent experiments. The homogeneity of variances was tested by analysis of variance and data from experiments with the same set-up were pooled for analysis because interactions between experiment and treatment were not significant at $P = 0.05$. Means from the different treatments were compared by the method of Newman and Keuls (least significant difference at $\alpha = 0.05$). Values followed by "a" are not statistically different from the control.

The different ISR-eliciting potential of M1 and M3 was examined further by comparing the activity of the corresponding supernatant extracts obtained after their growth in the CAA medium. Crude elicitor extracts from M3 resulted in an enhanced resistance of bean plants to *B. cinerea*; extracts prepared from the M1 culture failed to confer resistance (Figure 2). Other than the strong reduction of peaks corresponding to pyoverdine and ISR elicitor (in the case of M1), no significant change was observed in HPLC profiles upon analysis of 10-fold

concentrated culture supernatant of M1 or M3 compared with BTP1 (detection at 214, 280, and 375 nm; data not shown).

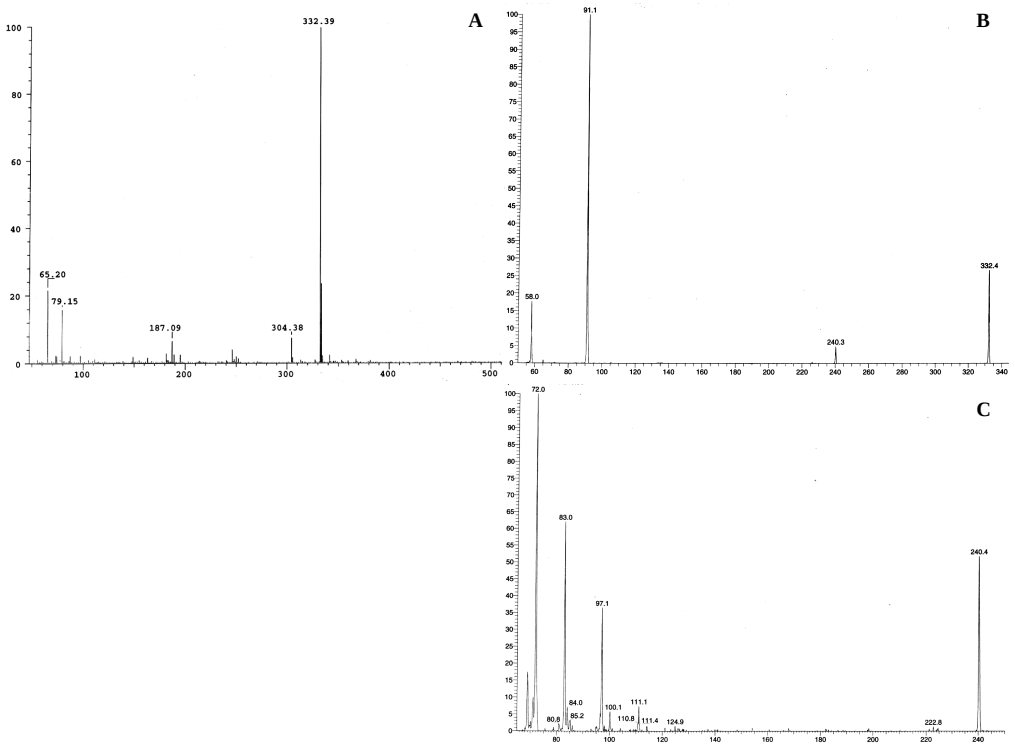


**Figure 3. A.** Electrospray ionization–mass spectrometry analysis spectrum obtained for the analysis of the pure induced systemic resistance determinant from *Pseudomonas putida* BTP1.
**B.** Ion trap octapole collision activation (CA)-spectrum of the molecular ion at *m/z* 332.4.
**C.** CA-spectrum of the main ion at *m/z* 240.3 generated upon fragmentation of the molecular ion.

*Structural characterization of the ISR determinant*

Electrospray ionization–mass spectrometry (ESI-MS) analysis of the active fraction showed two molecular ions at m/z 304.301 and 332.332 (as determined by exact mass measurements), corresponding to $C_{21}H_{38}N$ (calculated 304.3004) and $C_{23}H_{42}N$ (calculated 332.3317), respectively (Figure 3A). Analysis of a solution of the material in $CH_3OD$ revealed no mass change of the molecular ions, indicating the absence of exchangeable protons. Further structural information was obtained by collision-induced fragmentation of the molecular ions in the octapole and the ion trap region of the mass spectrometer. From the major component with $M^+$ *m/z* 332 in the octapole region, three fragments are obtained: *m/z* 91, characteristic for a benzyl group responsible for the UV absorption peak at 276 nm; *m/z* 240 (i.e., $[M - 92]^+$);

and m/z 58 (Figure 3B). Fragmentation in the ion trap gave only m/z 240. Subsequent collision activation (CA) fragmentation of the selected *m/z* 240 in the ion trap (Figure 3C) gave *m/z* 72 as most abundant fragment, and a series of alkenyl ions, *m/z* 69, 83, 97, 111, and 125, indicating the presence of an alkyl chain. CA fragmentation of the component with $M^+$ *m/z* 304 gave the same pattern, indicating that the structural difference lay only in the length of the alkyl chain (two $CH_2$ groups shorter).

Tentative structures for the major compound can be proposed from the fragmentation analysis (Figure 4). Based on the method described by Salvatore and associates (2002), structural analogues were chemically synthesized by successive *N*-alkylation of commercial phenethylamine or benzylamine and a final acido-basic reaction yielding a methylated product. MS spectra of the model compounds (Figure 4A and B) were compared with the microbial product. The formation of *m/z* 91 and the loss of 92u suggest that the benzyl group is connected to N ($C_6H_5$-$CH_2$-N) because Figure 4A upon CA gives *m/z* 105 ($C_6H_5$-$CH_2CH_2$), whereas Figure 4B gives *m/z* 91. The formation of *m/z* 58 ($C_3H_8N$) and *m/z* 72 ($C_4H_{10}N$) products could be explained by the typical amine fragmentations according to the scheme represented in Figure 5. From these results, the structure $M^+$ represented was established for the elicitor that we named *N*-alkylated benzylamine derivative (NABD).

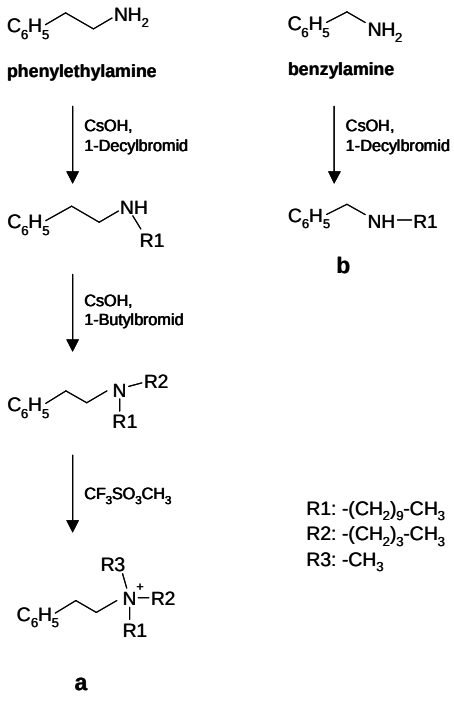


**Figure 4.** Organic synthesis of the quaternary ammonium starting from phenylethylamine (**a**) and of the mono *N*-alkyl intermediate starting from benzylamine (**b**). It involves the formation of *N*-decyl and *N*-decyl-*N*-butyl intermediates via two chemoselective reactions catalyzed by CsOH and a last acido-basic reaction yielding the final methylated product.

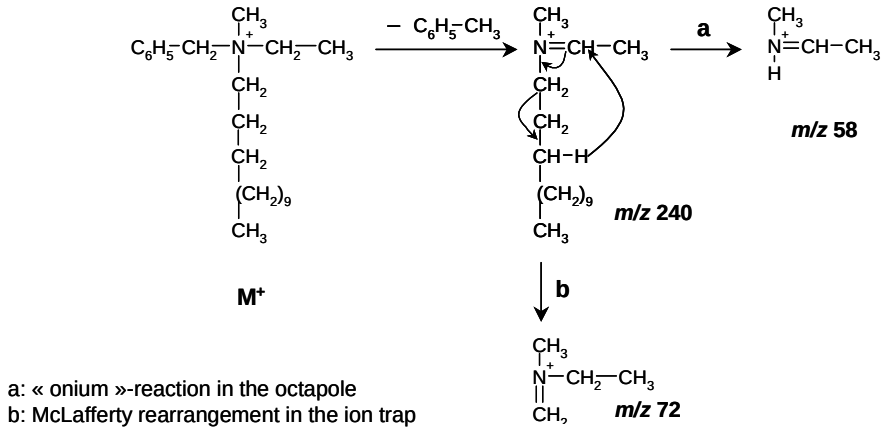


**Figure 5.** Intramolecular reactions generating the major fragments observed upon electrospray ionization–mass spectrometry analysis of the induced systemic resistance elicitor from *Pseudomonas putida* BTP1 ($M^+$).

## Discussion

This work describes the isolation and identification of a metabolite secreted by *P. putida* BTP1 that is specifically involved in the induction of systemic resistance in bean against leaf infection by *B. cinerea*. The elicitor properties were established on the basis of the following observations: i) treatment of bean roots with the pure compound NABD mimicked the protective effect of crude culture supernatant, ii) NABD-depleted culture broth did not induce resistance, iii) addition of $Fe^{3+}$ to the medium led to a loss of ISR-eliciting activity and NABD production, iv) the ISR determinant did not inhibit the growth of the pathogen at the concentrations tested in the ISR assays (data not shown), and v) a BTP1 derivative M1 with reduced NABD synthesis also was impaired in its efficacy to stimulate ISR. Despite a strongly reduced pyoverdine production rate, this mutant and the other one used in this study (M3) retain some root-colonizing ability to a level that should be sufficient to trigger ISR (Raaijmakers *et al.*, 1995). On the other hand, previous in vitro antagonism assays revealed that both *P. putida* BTP1 and the mutant M3 do not produce any antifungal compound (Ongena *et al.*, 1999). We also have verified that strain M1 did not inhibit mycelial growth of *Pythium aphanidermatum, Fusarium oxysporum,* or *B. cinerea* on any of the media tested (Ongena, *unpublished results*). Thus, M1 and M3 seemingly are not affected in the production of other metabolic products that could be specifically involved in biocontrol, such as antibiotics or other ISR elicitors, compared with the wild type.

Further research is required to determine the mechanism of ISR induction by NABD. The specific accumulation of antifungal compounds has been observed in infected leaves following treatment with NABD (Ongena, *unpublished results*). Additional investigations are required to

identify these fungitoxic plant metabolites; however, ISR stimulation by NABD may be related to phytoalexin accumulation, an important defense mechanism involved in the pathogen-induced SAR (Hammerschmidt, 1999). Increased protection by BTP1 already was associated with the stimulation of a systemic response based on the accumulation of antifungal compounds in cucumber (Ongena *et al.*, 2000) and tomato (Ongena, Jourdan, Giger, and Thonart, *unpublished results*). Thus, NABD produced by strain BTP1 may be active on different plant species for the control of diseases caused by various pathogens.

Other *Pseudomonas* metabolites previously reported as ISR elicitors obviously are not involved in the beneficial effect triggered by BTP1. The strain has developed an efficient pyoverdine-mediated iron acquisition system (Jacques *et al.*, 1995; Ongena *et al.*, 2001); however, these chromopeptides do not play any role because previous results showed that pyoverdine-enriched extracts failed to induce resistance (Ongena *et al.*, 2002). Moreover, ISR assays from this study revealed that the BTP1 derivative M3 strongly repressed in pyoverdine synthesis; however, with conserved elicitor production, it still retained almost all the protective effect developed by the wild type. In experiments performed on cucumber with the same strain, several lines of evidence also indicated that pyoverdines had little influence on the protective properties of BTP1 related to plant defense induction (Ongena *et al.*, 1999). SA production by strain BTP1 is much lower than that observed for PGPR (considered to be efficient producers), and its methyl ester form or pyochelin were not detected after growth of the strain in appropriate medium. Moreover, heat-killed BTP1 cells were fully impaired in providing any protective effect in the same pathosystem, suggesting that no heat-stable cell-envelop components, such as lipopolysaccharides, play a major role in the ISR elicitation process (Ongena *et al.*, 2002). Recent studies have shown that some bacterial products such as pyocyanin and 2,4-diacetylphloroglucinol, which are known for their antibiotic activities, also retain some ability to stimulate defense-related reactions in the host plant (Audenaert *et al.*, 2002; Iavicoli *et al.*, 2003). However, we already reported that BTP1 could not inhibit pathogen growth and did not produce or excrete any fungitoxic compounds either in vitro on several media or upon growth on roots (Ongena *et al.*, 1999). Thus, ISR induction by the strain is unlikely to be related to the production of any antibiotic molecule with plant defense-stimulating activity. All these observations strongly suggest that NABD isolated in this work retains most of the elicitor activity among the metabolites produced by the strain.

In situ detection of active metabolites produced by biocontrol bacteria has been described as a strategy that complements the indirect evidence provided by other approaches about their specific involvement in the protective effect developed by the strain. Several attempts were made to detect the eliciting compound in the rhizosphere of bean plants treated with BTP1. Results were highly variable; however, in two independent experiments, amounts of 5 to 8 ng/g

of root fresh weight were measured, suggesting an efficient production of the elicitor by the bacterium growing on roots. This concentration is in the range of production levels observed in situ for other ISR-eliciting compounds, such as SA (Audenaert *et al.*, 2002; De Meyer *et al.*, 1999; Maurhofer *et al.*, 1998), and for a wide variety of antibiotics involved in the biocontrol of soil diseases provided by other fluorescent pseudomonads (Raaijmakers *et al.*, 2002). Although it does not address the question of whether the amount of elicitor is sufficient to activate the plant in situ, it suggests that NABD production by BTP1 has occurred in its ecological niche.

From a structural point of view, biological compounds with plant defense-eliciting properties vary widely in nature. They may be surface components such as β-glucans, chitin-derived oligomers, or pectin fragments; oligogalacturonides released from the fungal pathogen or the host plant, or flagellin- or glycine-rich proteins; peptides such as syringolin isolated from bacterial pathogens; unsaturated fatty acids; or volatiles produced by *B. subtilis* (Ebel and Mithöfer, 1998; Fliegmann *et al.*, 2003; Klarzynski *et al.*, 2003; Kuć, 2001; Nürnberger and Scheel, 2001; Ryu *et al.*, 2004; Tanaka *et al.*, 2003; Waspï *et al.*, 1998). Certain synthetic chemicals, such as 2,6-dichloro isonicotinic acid, benzothiadiazole and its derivative acibenzolar-S-methyl, or β-amino butyric acid, also can be very effective in the induction of SAR when applied to plants (Ostendorp *et al.*, 2001). However, none of these compounds is structurally related to NABD produced by BTP1. The ISR determinant synthesized by BTP1 consists of a benzylamine moiety with the quaternary ammonium substituted by methyl, ethyl, and the long $C_{13}$ alkyl groups responsible for the relative hydrophobicity of the molecule. Phenyl-derived amines belong to the group of biogenic amines which might be produced by bacteria following decarboxylation of free amino acids (Silla Santos, 1996). However, to our knowledge, a compound with the structure established for ISR elicitor synthesized by BTP1 hitherto has not been described among *Pseudomonas* products. Forthcoming work will be devoted to the identification of biosynthesis genes and the study of environmental factors and intracellular pathways regulating NABD production. In parallel, chemical synthesis of various structural analogues will allow evaluation of critical features essential for the ISR-eliciting activity.

## Materials and methods

*Microbial strains*

*Pseudomonas putida* BTP1, isolated from barley roots, was originally selected for its specific features regarding pyoverdine mediated iron transport (Jacques *et al.*, 1995; Ongena *et al.*, 2001). All strains were maintained on *Pseudomonas* agar F medium (KB; Becton, Dickinson, and Company, Le pont de Claix, France) at 4ºC before experimental use, and stored at – 80ºC in cryotubes according to the

manufacturer's recommendations (Microbank; Prolab Diagnostic, Richmond Hill, Canada) for long-term conservation. The fungal pathogen *B. cinerea* was provided by M. Höfte (Ghent University) (De Meyer *et al.* 1997). *B. cinerea* was routinely grown to sporulation on an oatbased medium (oatmeal, 25 g/L; agar, 12 g/L) at room temperature. For long-term storage, spores were conserved in 40% glycerol at –70ºC.

*Isolation and characterization of chemical mutants*
Mutants M1 and M3 were obtained from BTP1 after chemical mutagenesis with *N*-methyl-N′-nitro-*N*-nitrosoguanidine (NTG) following a procedure described previously (Ongena *et al.* 1999). Briefly, the chemical mutagen was added to exponentially growing cells for 15 min under constant agitation. After washing and dilution, cell suspensions were plated onto chrome azurol S agar medium (CAS) and incubated at 30°C for 48 h. Out of approximately 2,500 colonies, 21 isolates were selected for their inability to discolor CAS and, thus, for their siderophore-negative phenotype. M1 and M3 then were selected from this short library on the basis of their different phenotypes regarding ISR elicitor production, which was evaluated after growth in CAA (Cornélis *et al.* 1992) as described below. The two mutants were further examined for several traits. In vitro growth of M1 and M3 were compared with the wild type in liquid media with various iron content: LB (caseine hydrolysate at 10 g/L, yeast extract at 5 g/L, and NaCl at 5 g/L), CAA medium, CAA + EDDHA at 300 mg/L, CAA + $FeCl_3$ $6H_2O$ at 1 g/L, KB medium, and KB + $FeCl_3$ $6H_2O$ at 1 g/L. Flasks were incubated under agitation (150 rpm) at 30°C for 48 h. Biomass concentration and pyoverdine production rates were determined spectrophotometrically as described below. The production of SA was tested by using the method reported by Ongena and associates (2002). Mutant M1 also was tested on various media for its ability to inhibit the growth of some phytopathogens in petri plates by following the method described by Ongena and associates (1999) for BTP1 and M3. Root colonization by mutants was evaluated on 20-day-old plants. Root samples (1 g) were collected randomly from three plants in two independent experiments. They were washed briefly with sterile distilled water to remove most of the soil. They were homogenized in 10 mL of sterile peptone water (bacto-peptone at 1 g/L, NaCl at 9 g/L, and 0.02% [vol/vol] Tween 80) and 1 g of glass beads (0.18 mm in diameter). Serial dilutions were plated on King's B medium and counts of M1 and M3 colonies were made after 24 h of incubation at 30ºC on the basis of their typical morphology (white, smooth, and viscous).

*Elicitor purification*
*P. putida* BTP1 was grown in 3-liter Erlenmeyer flasks (agitated at 150 rpm) containing 1.5 L of CAA medium for 72 h at 30ºC. Cells were eliminated by centrifugation at 13,500 × *g* for 15 min. The lyophilized supernatant was dissolved in distilled water (150-fold concentrated), adjusted to pH 6 with 0.1 M HCl, and extracted three times with a doubled volume of ethyl acetate. The organic phase containing the elicitor was evaporated to dryness under vacuum and the residue was solubilized in 10% MeOH. This solution (15 mL) was applied to an Alltech C18 SPE column (Alltech Associates, Inc., Lokeren, Belgium) (10 g) previously activated by washing with 20 mL of methanol and 20 mL of acetonitrile (ACN) and equilibrated with 40 mL of MilliQ water. The column was extensively washed with water (80 mL) to remove nonadsorbed material and successively rinsed with 20 mL of 20, 40, and 60% ACN to eliminate contaminating compounds that were moderately fixed to the matrix. The elicitor was eluted from the cartridge with 20 mL of 80% ACN and the corresponding fraction was lyophilized. The residue was dissolved in 1 mL of 100% MeOH and the elicitor further purified by reverse-phase HPLC (Hewlett-Packard 1100 series apparatus). Repeated injections of 100-µL aliquots of the methanolic extract were realized on a semi-preparative Chromspher 5 C-18 column (250 by 10 mm, 5-µm packing, Chrompack [Varian-Chrompack, Middelburg, The Netherlands]). Elution was performed with a gradient of pure ACN (solvent B) in 0.1% acetic acid (in milliQ water) as follows: (time in min:% B:flow rate in mL/min) 0:20:1, 2.5:20:4, 20:43:4, 60:43:4, 61:95:4, 66:95:4, 67:20:4, and 73:20:4. Samples were monitored spectrophotometrically at 280 nm and 2-mL samples were collected automatically over the entire run time. Fractions containing peaks of interest were pooled and evaporated in a Speed Vac concentrator SC100 (Savant, Global Medical Instrumentation, Inc., Ramsay, MN, U.S.A.). The pure elicitor was conserved in this lyophilized form at –20ºC until use. The compound was collected from multiple HPLC runs to yield approximately 5 mg of pure material. This

material was used for ISR bioassays and for structural characterization, and also served to relate HPLC peak areas to absolute amounts determined by weighing the pure powder.

*Quantification of elicitor and pyoverdine produced by the strains*
Bacterial cell densities obtained at the end of the cultures were determined by measuring the optical density at 540 nm, knowing that a value of 1 corresponds to $4.5 \times 10^8$ CFU/mL. BTP1 pyoverdine concentrations in the cell-free supernatants were calculated on the basis of the absorbance at 400 nm using a specific molar extinction coefficient ($\varepsilon_{400nm}$, pH 7) of 2.75 $10^4$ mol/L/cm (Jacques *et al.*, 1995). Elicitor production by the different strains was determined from 10-mL samples of culture supernatant collected after growth for 72 h in the CAA medium. Supernatant samples were loaded on small C18 solid-phase extraction cartridges (900 mg; Alltech) after activation and conditioning of the cartridge by flushing with 5 mL of ACN and 10 mL of water. After loading, the packing bed was washed successively with 10 mL of water and 5 mL of 40% ACN. The remaining material containing the elicitor then was desorbed with 2 mL of 100% CAN and evaporated to dryness in a Speed Vac apparatus, and the residue was resolubilized in 80 µl of methanol. This solution (50 µL) was analyzed by reversed-phase HPLC (HP 1100 series system) on a LiChrospher 100 RP C-18 column (250 by 4.6 mm, 5-µm packing; Merck, Darmstadt, Germany) using the same solvent system as described above and at a constant flow rate of 1 ml/min. The molecule of interest was eluted with a gradient of acetonitrile as follows: (time in min:% acetonitrile) 0:20, 2.5:20, 20:47, 60:47, 61:95, and 65:95. Amounts were calculated on the basis of the corresponding peak area at 280 nm.

*Assays for induced resistance with bacteria*
ISR assays were performed with bean (*Phaseolus vulgaris*) cv. Prelude following the method described by Ongena and associates (2002). Briefly, sterilized seed were dipped for 10 min in the bacterial suspension at a concentration of $4 \times 10^8$ CFU/mL prior to sowing. Bean plants then were grown in potting soil previously mixed with BTP1, M1, or M3 cells to a final concentration of $3 \times 10^7$ CFU/g. Additionally, 20 mL of a bacterial suspension at $10^8$ CFU/mL was added as a drench to the roots of each plant, 7 days after sowing. For control plants, seed were soaked for 10 min in NaCl 0.85% prior to sowing and 7-day-old bean plants were watered with 20 mL of the same solution. After approximately 20 days, plants were transferred to a high-humidity chamber (19 ± 2°C) for 24 h before leaf infection with *B. cinerea*. This was achieved by inoculating each primary leaf with eight 7-µl drops of a suspension at $5 \times 10^6$ spores/mL prepared as described (Ongena *et al.*, 2002). Disease incidence was scored every day and was expressed in terms of the percentage of *B. cinerea* lesions that clearly grew out of the inoculum drop zone to produce spreading lesions. Experiments contained 15 plants per treatment and were repeated three times with each bacterial isolate. For disease reduction evaluation, the homogeneity of variances was tested by analysis of variance and data from experiments with the same set-up were pooled for analysis when interaction between experiment and treatment was not significant at $P = 0.05$. Means from the different treatments were compared by the method of Newman and Keuls (least significant difference at $\alpha = 0.05$; Statistica software).

*Assays for induced resistance with supernatant samples and purified elicitor*
These assays were realized with bean plants grown under hydroponic conditions as described previously (Ongena *et al.*, 2002). Sterilized seed were germinated in perlite and 1-weekold seedlings were removed carefully and transferred to test tubes previously filled with 45 mL of nutrient solution. Six days after transfer, plants were treated by adding 1 mL of either a methanolic solution of material extracted with ethyl acetate from various culture supernatants (SE) or a methanolic solution of the pure elicitor at a final concentration of 0.2 µM in the nutrient solution (established after weighing the purified compound and on the basis of the molecular weight determined). SE samples were prepared from lyophilized material corresponding to 300 mL of cell-free culture fluid. The resulting powder was resuspended in 20 mL of distilled water and the solution was adjusted to pH 6.5 with 0.05 M HCl before extraction with ethyl acetate as described above. The residue was solubilized in an adequate volume of 50% methanol. Elicitor-depleted SE samples were prepared by submitting ethyl acetate extracts to semi-preparative HPLC. Fractions collected all over the runs except the one containing the elicitor were pooled and the resulting solution was lyophilized and the residue resolubilized in 50% methanol so as to test the remaining constituents at the same concentration as the elicitor. In all cases,

control plants were treated with 1 mL of pure methanol. The volume of nutrient solution in the tubes was adjusted daily with sterile distilled water during the experimental time. Approximately 14 days after treatment, plants were placed in the high-humidity chamber and infected with the pathogen *B. cinerea* as mentioned above, except that six infection sites were created on each first leaf. In every experiment, 20 plants were used per treatment and disease reductions were calculated from results obtained in two independent experiments.

*Mass spectrometry analyses*

Mass spectrometry investigations of the elicitor were performed with a Finnigan-MAT 900ST instrument equipped with an electrospray ion source (ESI-MS). Collision activation of mass-selected ions was performed either in the octapole region or in the ion trap. In the octapole, consecutive fragmentation processes are possible; whereas, in the ion trap, all fragments must stem from the selected precursor ion. Samples were dissolved in $CH_3OD$.

*Chemical synthesis*

The first two steps for the synthesis of quaternary ammonium starting from phenylethylamine were realized based on the methodology described by Salvatore and associates (2002) with few adaptations. The use of CsOH ensured a highly chemoselective reaction favoring mono-*N*-alkylation of the primary amine over dialkylation. Typically, 1-bromodecane (1.2 equivalent) was added to anhydrous *N*,*N*-dimethylformamide containing phenylethylamine (1 equivalent), cesium hydroxide monohydrate (2 equivalent), and activated powdered molecular sieves. The reaction was allowed to proceed for 48 h at room temperature and yielded mostly *N*-decyl-phenylethylamine (89%) compared with polyalkylated products. The desired secondary amine was purified from the crude mixture as described, with final precipitation obtained by adding ethyl acetate and incubating overnight at 0°C. Purity and structure of this first synthetic intermediate were confirmed by ESI-MS and nuclear magnetic resonance (data not shown). The second step leading to the *N*-decyl-*N*-butyl intermediate was performed in the same way (addition of 1-bromobutane), except that CsOH was not added in excess. This reaction yielded almost exclusively the expected product (98%), which was further purified using the same procedure. The *N*-decyl-*N*-butyl-phenylethylamine served as substrate from the last methylation step, which resulted from an acido-basic reaction performed as follows. The tertiary amine (200 mg, 1 equivalent) was dissolved in 2 mL of anhydrous diethyl ether under inert atmosphere. Methyl-trifluoromethanesulfonate (350 mg, 1.75 equivalent) was added to the solution and the resulting mixture was stirred at 0°C for 2 h until the formation of a white precipitate. This precipitate, containing the expected quaternary ammonium salt in an almost pure form (94.7% as estimated by mass spectrometry), was recovered by filtration, washed successively with 3 mL of cold dichloromethane and the same volume of cold diethyl ether, and dried in vacuo. Synthesis of *N*-alkyl derivative starting from benzylamine strictly followed the procedure described above, with the exception that CsOH was not added in excess.

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## *Chapitre V.* *Amino acids, iron, and growth rate as key factors influencing production of the* **Pseudomonas putida** *BTP1 benzylamine derivative involved in systemic resistance induction in different plants*

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**Résumé**

Dans le chapitre précédent, nous avons décrit la purification de l'éliciteur produit par *Pseudomonas putida* BTP1 et avons démontré qu'il était le composé principal dans l'induction de l'ISR chez le haricot. Ici, nous apportons tout d'abord des informations complémentaires concernant la structure du NABD ainsi que sa capacité à induire une résistance chez d'autres familles végétales. Des analyses complémentaires en spectrométrie de masse et la comparaison des spectres générés par le NABD et plusieurs dérivés synthétiques nous ont guidé vers une légère révision de la structure de l'éliciteur, à savoir, une alkylation de l'ammonium quaternaire par un groupement tetradécyle et deux groupements méthyle $C_6H_5–CH_2–N^+(CH_3)_2–C_{14}H_{29}$.

Nous nous sommes ensuite intéressés à la spécificité de l'éliciteur vis-à-vis de la plante. En effet, *Pseudomonas putida* BTP1 est en mesure d'induire l'ISR chez le haricot, le concombre et la tomate, cependant, pour les deux dernières plantes, l'implication du NABD n'avait pas été prouvée. De plus, toujours dans le domaine de la résistance induite chez les plantes par des rhizobactéries, certaines études révèlent que le phénomène n'est pas toujours déclenché par le même composé. Suite au traitement des plantes avec des cellules vivantes et le composé pur, nous avons démontré que le NABD était le principal éliciteur chez le haricot et le concombre, mais que, chez la tomate, des taux de protection intermédiaires suggéraient qu'un autre composé produit par la souche devait probablement intervenir. De plus, grâce au traitement des racines avec la benzylamine, nous avons aussi montré l'importance du noyau aromatique de la molécule dans l'induction du phénomène d'ISR.

D'un point de vue pratique, une preuve supplémentaire de l'implication du NABD passe par la vérification de sa production lorsque les bactéries se développent sur les racines. Cependant, le(s) gène(s) de biosynthèse du NABD nous est(sont) encore inconnu(s), empêchant à ce stade, de générer dans BTP1 un système rapporteur afin de visualiser la distribution des populations microbiennes associées aux racines qui expriment les gènes de biosynthèse et de quantifier cette expression. Nous avons donc développé une approche *in vitro* afin d'étudier l'influence de certains paramètres physiologiques, nutritionnels et physico-chimiques sur la production du NABD par *P. putida* BTP1. Les résultats présentés dans ce chapitre illustrent que la cinétique de production du NABD est typique d'un métabolite secondaire. En exploitant la technologie du chémostat, nous avons aussi montré que sa synthèse n'est effective qu'à un taux de croissance faible du microorganisme. La production du NABD par la rhizobactérie est aussi négativement influencée par certains oligo-éléments, dont le fer et le cuivre. La source de carbone utilisée par BTP1 apparaît comme un autre élément important dans la production de l'éliciteur puisqu'elle est étroitement conditionnée par la

présence d'acides aminés dans le milieu. D'ailleurs, toujours grâce au chémostat, un fort accroissement de la production du NABD a été observé après addition de phénylalanine dans le milieu de culture sous forme de pulse. L'étude d'autres paramètres abiotiques montre également qu'une restriction en oxygène dissous disponible ou une acidification du milieu n'ont pas d'effet négatif drastique sur le taux de production du NABD. Enfin, des expériences préliminaires présentées comme résultats additionnels avec des biosenseurs sensibles aux N-acyl-homosérine lactones, ne semblent pas suggérer l'implication du quorum-sensing dans le système de régulation de la biosynthèse du NABD.

Globalement, ces résultats viennent en appui de la détection de quantités significatives de NABD dans la rhizosphère (chapitre précédent) et montrent que les conditions nutritionnelles et physico-chimiques dictant l'état physiologique des cellules bactériennes *in situ* peuvent être propices à une synthèse effective de l'éliciteur au niveau des racines.

**Abstract**

The biological control bacterium *Pseudomonas putida* BTP1 exerts its protective effect mostly by inducing an enhanced state of resistance in the host plant against pathogen attack [induced systemic resistance (ISR)]. We previously reported that a specific compound derived from benzylamine may be involved in the elicitation of the ISR phenomenon by this *Pseudomonas* strain. In this article, we provide further information about the *N*,*N*-dimethyl-*N*-tetradecyl-*N*-benzylammonium structure of this determinant for ISR and show that the benzylamine moiety may be important for perception of the molecule by root cells of different plant species. We also investigated some regulatory aspects of elicitor production with the global aim to better understand how in situ expression of these ISR elicitors can be modulated by physiological and environmental factors. The biosynthesis is clearly related to secondary metabolism, and chemostat experiments showed that the molecule is more efficiently produced at low cell growth rate. Interestingly, the presence of free amino acids in the environment is necessary for optimal production, and a specific positive effect of phenylalanine was evidenced in pulsed continuous cultures. The influence of other abiotic factors, such as mineral content, oxygen concentration, or pH, on elicitor production is also reported and discussed with respect to the specific conditions that the producing strain undergoes in the rhizosphere environment.

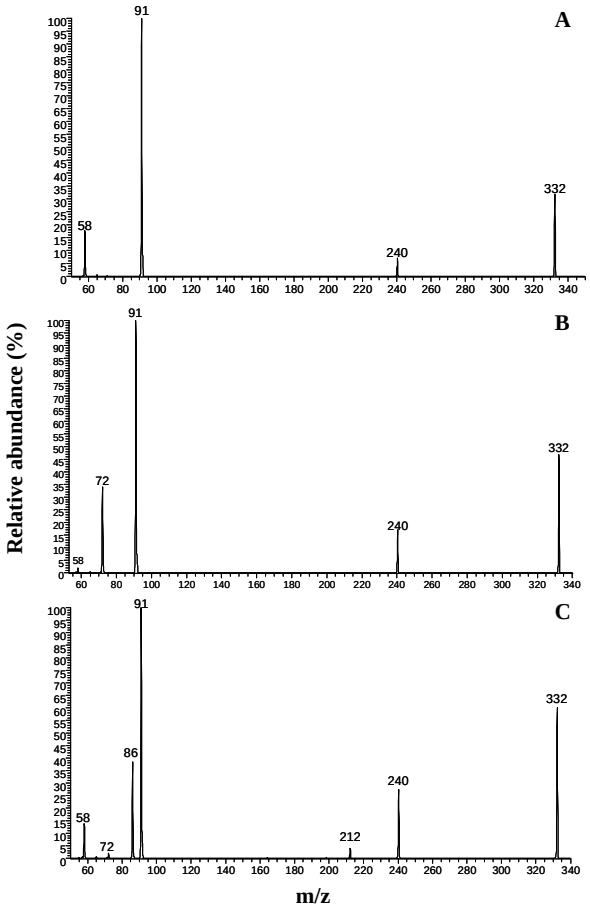
**Introduction**

The use of specific microbial inoculants provides an environmentally friendly means to control plant pathogens and thus represents a promising alternative to limit the widespread and extensive application of synthetic chemicals for crop protection (Bent, 2006; Fravel, 2005). Biocontrol agents antagonize pathogens by competing for niche and nutrients or by producing low molecular weight antimicrobial compounds or extracellular lytic enzymes. Besides these antagonistic activities, some beneficial bacteria may exert their protective effect indirectly through the stimulation of inducible plant defence mechanisms that render the host more resistant to further pathogen ingress (Haas and Défago, 2005). Induction of enhanced defensive capacity can be systemic as root treatment with such bacteria was shown to trigger protective effects on aboveground plant parts. This phenomenon has been termed Induced Systemic Resistance (ISR), and is effective against a broad range of diseases (Bent, 2006; Van Loon *et al.*, 1998; Kloepper *et al.*, 1992). ISR is phenotypically similar to the well-studied Systemic Acquired Resistance (SAR) activated after a first infection by an incompatible necrotising pathogen (Durrant and Dong, 2004). However, the signal transduction pathway and the molecular basis underlying the rhizobacteria-mediated ISR differ in many aspects from the pathogen-induced SAR (Glazebrook *et al.*, 2003; Pieterse *et al.*, 2003).

Some *Bacillus* spp. strains are able to stimulate plant defence responses (Kloepper *et al.*, 2004) but most of the ISR-inducing microbes identified so far are gram-negative bacteria and more particularly species belonging to the *Pseudomonas* and *Serratia* groups (Bakker *et al.*, 2007; Bent, 2006). In comparison with SAR, little is known about the molecular events associated with ISR both regarding the defence mechanisms induced in plant tissues and the elicitation process by non-pathogenic microbes. Elicitor structures identified so far can be classified as cell surface components, iron-regulated metabolites, antibiotics and volatiles. Among the first class, lipopolysaccharides or, more specifically their O-antigenic side chain, were identified as ISR elicitors for *Pseudomonas fluorescens* on carnation (Van Peer and Schippers, 1992), radish (Leeman *et al.*, 1995) and tomato (Duijff *et al.*, 1997) but also for *Rhizobium etli* on potato (Reitz *et al.*, 2002). The composition of lipopolysaccharides from pseudomonads is strain-specific and their eliciting activity seems to depend on the isolate. Bacterial flagellins are protein structural components of flagella that can also be perceived by plant cells and act as elicitors of defence responses in *Arabidopsis* (Meziane *et al.*, 2005; Gomez-Gomez and Boller, 2002). Among the iron-regulated products, pyoverdines typically synthesized by fluorescent *Pseudomonas* (Budzikiewicz, 2004) were suggested as ISR elicitors on the basis of experiments using a non-producing mutant that lost its activity (Maurhofer *et al.*, 1994) or by treating plants with pure compounds (Leeman *et al.*, 1996). Salicylic acid (SA)

is also produced by 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.*, 1999; De Meyer and Höfte, 1997) and *Pseudomonas fluorescens* P3, which was converted to an ISR-inducing strain by the insertion of SA biosynthetic genes (Maurhofer *et al.*, 1998). Nevertheless, several reports showed that SA production by other strains was not associated with ISR (Press *et al.*, 1997; Leeman *et al.*, 1996). Other investigations suggest that the expression of genes involved in the biosynthesis of an unidentified catechol siderophore is also associated with ISR induced by *Serratia marcescens* strain 90-166 in cucumber (Press *et al.*, 2001). SA is also a precursor or intermediate in the biosynthesis of other siderophores such as pyochelin in *Pseudomonas aeruginosa* (Serino *et al.*, 1997). A role for this latter compound acting synergistically with the antibiotic pyocyanin was reported for ISR triggered in tomato by *P. aeruginosa* 7NSK2 (Audenaert *et al.*, 2002). Pyocyanin from 7NSK2 was also recently reported as elicitor of systemic resistance in rice (De Vleesschauwer *et al.*, 2006). 2,4-diacetylphloroglucinol is another antibiotic from *Pseudomonas* that also retains some ability to stimulate defence-related reactions in the host plant (Iavicoli *et al.*, 2003). Finally, a fourth class can be represented by bacterial volatile compounds since 2,3-butanediol produced by *Bacillus* was reported to trigger ISR in *Arabidopsis* (Ryu *et al.*, 2004).

In general, the mechanisms involved in rhizobacteria-mediated ISR thus appear to vary among bacterial strains or pathosystems and much remains to be discovered about the nature and regulation of bacterial determinants responsible for the elicitation of plant defence mechanisms. In the case of *Pseudomonas putida* strain BTP1, the molecule mainly responsible for resistance induction in bean was recently characterized as a *N*-alkylated benzylamine derivative (NABD) (Ongena *et al.*, 2005). In the study presented here, we first aimed to evaluate the specificity of NABD-mediated ISR regarding the host plant species by comparing the protective effect of BTP1 cells and of the pure elicitor in three different pathosystems. The second objective of this work was to provide some insights into the regulatory aspects of the production of NABD. Facing the lack of knowledge about the genetic background responsible for its synthesis, we used liquid culture screening and took advantage of the chemostat culture technology to test the effect of specific substrates and physico-chemical factors. This was done with respect to the physiological, nutritional and abiotic conditions that the producing strain undergoes in the rhizosphere environment.



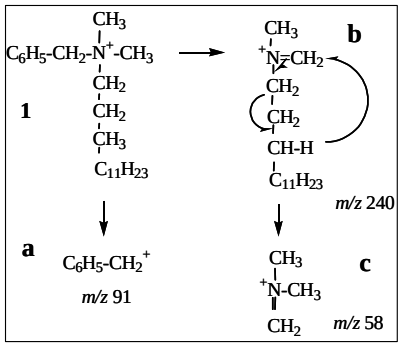




**Figure 1**. ESI-Octapol-MS/MS spectra obtained for the analyses of the precursor ion [M]+ at m/z 332 ($C_{23}H_{42}N^+$) corresponding to commercial *N*-benzyl-*N*,N-dimethyl-N-n-tetradecyl ammonium (a), and to chemically synthesized *N*-benzyl-*N*-ethyl-N-methyl-N-n-tridecyl-ammonium (b) and N-benzyl-N-methyl-N-n-propyl-N-n-dodecyl ammonium (c). Intramolecular reactions and major daughter ions generated upon fragmentation are detailed in the box.

## Results

N,N-*dimethyl*-N-*tetradecyl*-N-*benzylammonium as ISR elicitor from* P. putida *BTP1*

From previous mass spectrometry analyses, we deduced that the active molecule isolated from BTP1 culture supernatant consisted of a benzylamine moiety with the quaternary ammonium substituted by methyl, ethyl, and $C_{13}$ aliphatic groups (Ongena *et al.*, 2005). However, subsequent electrospray ionization tandem mass spectrometry (ESI-MS/MS) experiments revealed the possible formation of atypical amine fragments in such molecular species. In this work, identification of the three alkyl substituents in the natural compound arose from comparative analyses of spectra obtained for the chemically synthesized analog products $C_6H_5–CH_2–N^+(CH_3)(CH_2CH_3)–C_{13}H_{27}$ and $C_6H_5–CH_2–N^+(CH_3)(CH_2CH_2CH_3)–C_{12}H_{25}$ and for the commercial compound $C_6H_5–CH_2–N^+(CH_3)_2–C_{14}H_{29}$ (BDTA) (Figure 1). The *N*-benzyl-*N*,*N*-dimethyl-*N*-*n*-tetradecyl ammonium ion 1 (*m/z* 332) yields three fragments upon collision

activation, viz. *m/z* 91 (a; $C_6H_5CH_2^+$), *m/z* 240 (b; $[M–C_6H_5–CH_3]^+$), and *m/z* 58 (c) formed by McLafferty decomposition (Figure 1 box) (Budzikiewicz and Schäfer, 2005). The isomeric *N*-benzyl-*N*-ethyl-*N*-methyl-*N*-*n*-tridecyl ammonium ion fragments in an analogous way: the McLafferty elimination product is shifted to *m/z* 72 due to the replacement of one methyl by an ethyl group (Figure 1b). The presence of a *n*-propyl group in the *N*-benzyl-*N*-methyl-*N*-*n*-propyl-*N*-*n*-dodecyl ammonium ion gives rise to competitive losses upon collision activation. The main process is again McLafferty elimination from the longer chain resulting now in a fragment *m/z* 86, but by an analogous mechanism, $C_2H_4$ can be lost by degradation of the *n*-propyl group giving the ion *m/z* 212. In addition, *m/z* 86 can eliminate $C_2H_4$ by a second McLafferty process to give *m/z* 58 (Figure 1c). ESI-MS/MS analysis of the *P. putida* BTP1 elicitor under the same conditions yielded a fragmentation pattern identical to 1 both in the nature of the fragments generated (no traces of *m/z* 72 nor *m/z* 86 were observed) and in the relative abundance of these ions (Ongena *et al.*, 2005). We thus unambiguously concluded in favor of a *N*-benzyl-*N*,*N*-dimethyl-*N*-*n*-tetradecyl ammonium structure for the ISR determinant synthesized by *P. putida* BTP1. Thus, it only differs from the previously published structure by the presence of a methyl group instead of the ethyl substituent and consequently a $C_{14}$ alkyl chain instead of $C_{13}$.

*ISR activity of the elicitor on different plants*

To verify that the synthetic molecule (BDTA) and the natural product (NABD) retain similar biological activities, both compounds were tested *in vivo* on bean for the ability to protect plants via induction of systemic resistance. Molecules were tested on 2-week-old plants treated at the root level and infected on leaves with *B. cinerea* to avoid any direct antagonism between the inducing agent and the phytopathogen. Botrytis infection was assessed on the basis of the percentage of spreading lesions formation during thefirst 4 days after inoculation. Pooled results from threeindependent experiments showed statistically similar disease reduction levels of 26 and 28% in plants treated respectivelywith synthetic BDTA and natural NABD, confirming that both compounds retained a similar ISR eliciting potential.

ISR assays were then performed with three different pathosystems with the aim to evaluate the elicitor activity of NABD on various plant species (Figure 2). In the bean/*Botrytis* pathosystem described above, root treatment with NABD provided a similar disease reduction as the one afforded by living cells of the producting BTP1 strain. Data from three experiments on cucumber plants infected with the anthracnose-causing agent *C. lagenarium* revealed that both treatments also induced similar significant disease reductions of approximately 20%.

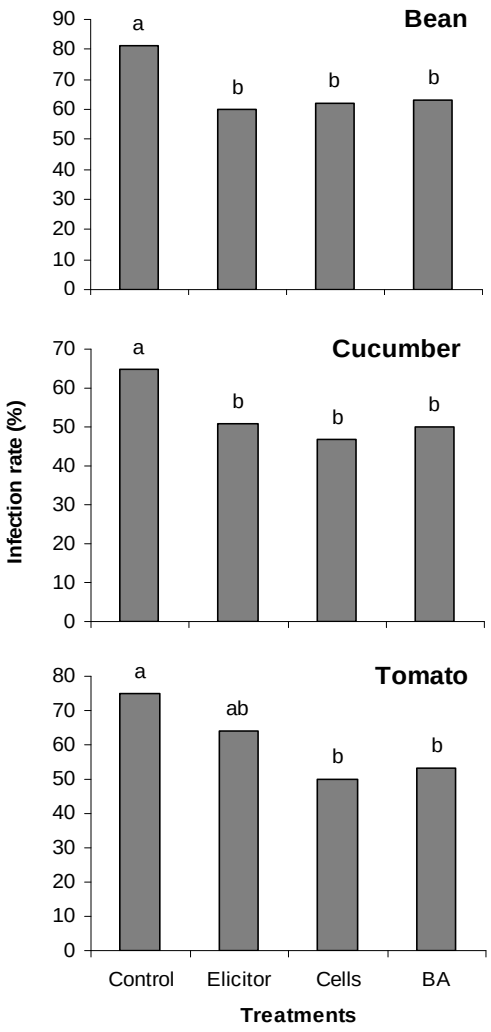



**Figure 2**. Disease reduction observed in plants treated either with P. putida BTP1 (cells), with the elicitor (NABD) or with benzylamine (BA). The last two compounds were introduced at the root level by drenching soil corresponding to each plant with 20 mL of the appropriate solutions. Control plants were treated with the same volume of 1% methanol. Bean and tomato plants were inoculated with the pathogen B. cinerea on leaves and cucumber plants were challenged with C. lagenarium on cotyledons. Data are means of three independent experiments using at least 20 plants per treatment. Values with different letters are statistically different.

These results confirm the potential of strain BTP1 at stimulating some systemic defensive response in cucumber plants to reduce leaf infection, as it was demonstrated against root rot caused by *Pythium aphanidermatum* (Ongena *et al.*, 2000). However, when tested on 5-week-old tomato plants challenged with *B. cinerea*, disease reduction provided by the purified elicitor was not significant by contrast with BTP1 cells. As it represents an important part of the elicitor structure, pure benzylamine (BA) was also tested in these ISR assays (Figure 2). Interestingly, the compound induced a significant disease protection for the three plant species tested. We have previously demonstrated that *P. putida* BTP1 is an efficient colonizer of bean and cucumber rhizosphere upon root application but does not migrate through the plant (Ongena *et al.*, 2002; Ongena *et al.*, 1999). In this work, bacterial population on tomato roots were estimated by using a spontaneous rifampicin-resistant derivative as already described

(Ongena *et al.*, 2002). BTP1 cells were observed at a concentration of $3.0 \pm 2.1 \times 10^6$ CFU/g at the time of challenge. It is thus obvious that the strain readily establishes and maintains on tomato roots. Furthermore, in the case of tomato, it does not migrate to the leaf tissues where fluorescent colonies were not observed with a detection limit of 1000 CFU/g. The inducing agent and the phytopathogen thus remained localized on different organs showing that disease suppression is due to induction of resistance in the host plant.

*Influence of cell growth phase and rate on elicitor production*

The ISR elicitor synthesized by *P. putida* BTP1 was purified from cell-free culture fluid obtained after growth of the strain in the iron-poor CAA medium. Cell concentration and NABD production in CAA were monitored in time and first compared during batch cultures realized in flask or in bioreactor without pH control. Data in Figure 3 show that in both culture modes, NABD is not detectable during the first 12–15 h of culture corresponding to the exponential growth phase. Elicitor production begins once cells have entered the stationary phase, and maximal concentrations observed after 96 h of culture were of $75 \pm 18$ ng/$10^8$ CFU and $62 \pm 12$ ng/$10^8$ CFU in flask and fermentor, respectively (based on three repeats). Final quantities measured in the bioreactor are not significantly lower than those observed in flask. The molecule is readily excreted by the bacterium into the surrounding medium because very low quantities could be recovered from cells harvested at various times (from 24 to 72 h) during the culture. For instance, mean values calculated from 48-h samples in two experiments were 22.5 ng/$10^8$ CFU in the supernatant and 2.2 ng/$10^8$ CFU in the cell lysate. Moreover, no traces of the molecule were detectable in 72-h supernatant samples after cell removal from the culture fluid after 20 h of growth. Cell removal or killing after 20 h of growth completely abolished subsequent NABD accumulation in the culture supernatant. This indicates that the molecule typically originates from the secondary metabolism of the bacterium rather than being spontaneously formed by some chemical reactions in the culture broth.

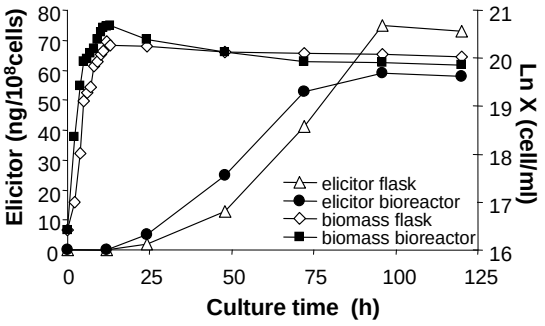



**Figure 3.** Biomass and NABD production observed during batch cultures of P. putida BTP1 in both flask and bioreactor (CAA medium). Cell concentration (X) was determined by plate-counts and is expressed as logarithmic evolution of the number of cells per ml. NABD production was estimated by HPLC. Data are from one representative experiment.

To further investigate the influence of cell growth rate ($\mu$) on NABD production, strain BTP1 was cultivated in continuous cultures (chemostat mode) at different dilution rates *D*. By contrast with batch culture where the exhaustion of a specific nutrient terminates the exponential growth phase, biomass concentration in the chemostat culture is usually controlled by the permanent limitation of a single defined nutrient (soluble iron in this case). It allows the study of metabolite synthesis upon fixed growth rate and constant cellular physiological state. Analyses of steady-state cell-free supernatants revealed 5- to 10-fold increases in NABD production upon growth at $D = \mu = 0.1$ $h^{-1}$ compared to higher growth rates. Mean values calculated from two independent experiments were 55 ± 15 ng/$10^8$ CFU at $\mu = 0.1$ $h^{-1}$, 11 ± 4 ng/$10^8$ CFU at $\mu = 0.3$ $h^{-1}$ and 5.9 ± 4 ng/$10^8$ CFU at $\mu = 0.6$ $h^{-1}$. In all cases, dissolved oxygen and nutrients never reached concentrations that could limit bacterial development. NABD production is most probably iron-regulated because it was practically undetectable in cultures supplemented with ferric chloride at a concentration inhibitory to siderophore synthesis [(Ongena *et al.*, 2002), see below]. However, similar pyoverdine (the typical iron-regulated siderophores from fluorescent *Pseudomonas*) and biomass concentrations were measured in the chemostats with different *D* values suggesting that the amounts of free iron in the various growth media were also similar (data not shown) and not inhibitory for NABD synthesis.

**Table 1.** Elicitor production yield upon growth of *P. putida* BTP1 in various culture conditions.

| Culture mode | NABD production (ng/$10^8$ CFU) |
|---|---|
| flask | 71 ± 15 |
| flask baffled | 60 ± 17 |
| flask sealed, nitrogen flushed | 5 ± 2 |
| flask sealed, semi-solid medium | 13 ± 7 |
| bioreactor 0.8 VVM, 600 rpm | 62 ± 18 |
| bioreactor 0.8 VVM, 600 rpm, pH 6 | 50 ± 20 |
| bioreactor 0.8 VVM, 600 rpm, pH 7 | 57 ± 18 |
| bioreactor 0.5 VVM, 350 rpm | 67 ± 23 |
| bioreactor 0.2 VVM, 200 rpm | 47 ± 13 |

The strain was grown either in 150-mL Erlenmeyer flask or in 2-L bioreactor in the batch mode at different oxygenation rates (VVM) and pH. Elicitor concentration was determined by HPLC after growth of the bacterium for 72 h in the CAA medium. Values are means (±standard error) calculated from three independent cultures in every condition.

*Influence of aeration and pH*

The influence of aeration rate on NABD production was first tested in batch bioreactors with various oxygen transfer rates (Kla) obtained by adapting the air flow/agitation settings. Elicitor production measured upon growth at the lower Kla (14 $h^{-1}$, 0.2 vvm/200 rpm) was reduced but

not significantly different from the one observed upon growth in the optimal oxygen transfer conditions we used (Kla = 41 $h^{-1}$, 0.8 vvm/600 rpm; Table 1). Similarly, the use of baffled Erlenmeyers, which provide a more efficient mixing of the medium and thereby improved oxygenation, did not favor significantly the production compared to usual flasks. However, more drastic oxygen limitation in sealed flasks flushed with pure nitrogen before inoculation or filled with semi-solidified CAA was clearly associated with a significant reduction of NABD production (Table 1).

As a result of ammoniac release during amino acid consumption, the exponential growth in the CAA medium is associated with a rapid increase of pH from 7.10 to 8.25 after 16 h. It remained above 8.55 until the end of the culture. To evaluate whether these alkaline conditions may influence NABD synthesis, batch fermentations were also conducted upon regulation of pH at 7.0 ± 0.2 and 6.0 ± 0.2. However no significant difference in elicitor production could be observed as compared to the unregulated cultures (Table 1). Similarly, periodic pH adjustment at a value of 6.5 in flask cultures did not markedly affected the final production level (data not shown).

**Table 2.** Influence of various carbon sources on elicitor production by *P. putida* BTP1.

| Medium | NABD production (ng/$10^8$ CFU) |
|---|---|
| CAA without added oligoelement | $68.2^{x}$ |
| CAA + Mg | $37.7^{x}$ |
| CAA + Zn | $53.9^{x}$ |
| CAA + Cu | $14.7^{z}$ |
| CAA + Cl | $49.6^{x}$ |
| CAA + Fe | $9.1^{z}$ |
| CAA + Co | $39.7^{x}$ |
| CAA + Mo | $70.5^{x}$ |
| Succinate | $13.1^{y}$ |
| Glutamate | $1.8^{y}$ |
| King's B | $2.1^{y}$ |
| Mannose | n.d. |
| Glucose | n.d. |
| Fructose | $0.5^{y}$ |
| Fructose + CAA | $34.5^{z}$ |
| Arabitol | n.d. |
| Mannitol | n.d. |
| Glycerol | $9.9^{y}$ |
| Glycerol + CAA | $48.4^{x}$ |

Substrates were added to obtain the following final concentrations in the medium: mannitol 30 mM, arabitol 35 mM, glucose 30 mM, fructose 30 mM, mannose 30 mM, glycerol 60 mM, succinate 40 mM, and glutamate 35 mM. Casamino acids were added to fructose and glycerol media at a concentration of 2 g/L. Minerals were added to the CAA medium to have a final concentration of 0.1 mM. Different superscript letters represent mean values significantly different at P=0.05 according to Fisher's least significant difference test calculated over five repeats.
n.d. Not detected.

*Influence of nutritional factors*

The effect of carbon source on elicitor synthesis was tested by growing cells in the presence of various sugars and organic acids. NABD production was compared with the one measured in CAA after 72 h of growth (Table 2). In all media tested (except those with added iron), an efficient synthesis of pyoverdines was observed with final concentrations ranging from 20 to 75 µM (data not shown). This indicates that $Fe^{3+}$ traces were not inhibitory to NABD production and thereby that elicitor biosynthesis is actually influenced by the nature of the nutrients available for growth of strain BTP1. From these results, it clearly appeared that the presence of free amino acids in the medium is necessary for efficient NABD production (Table 2). Upon addition of various oligoelements to the CAA medium, we observed that only copper and iron had a significant negative effect on NABD production (Table 2).

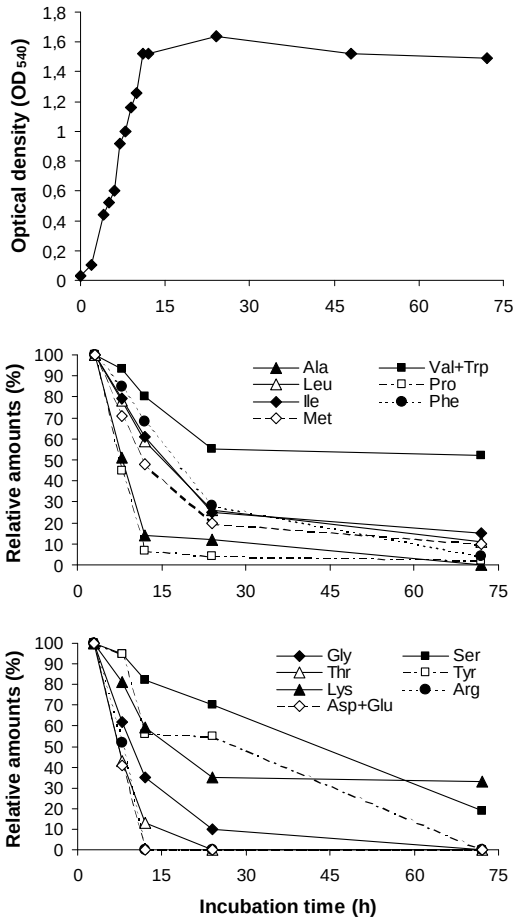



**Figure 4.** Consumption rate of individual amino acids during growth of strain BTP1 in CAA medium. Data are from one typical batch culture of the strain in a 2-L bioreactor (0.8 VVM, 600 rpm, pH not regulated). Both qualitatively and quantitatively, similar results were obtained upon growth in 3-L flask filled with 1.5 L of medium except that growth rate and thus consumption of some amino acids were slightly slower. Cell concentration was determined by optical densityat a wavelength of 540 nm = $OD_{540}$. Individual amino acids were identified and quantified as their PTH-derivatives by HPLC.

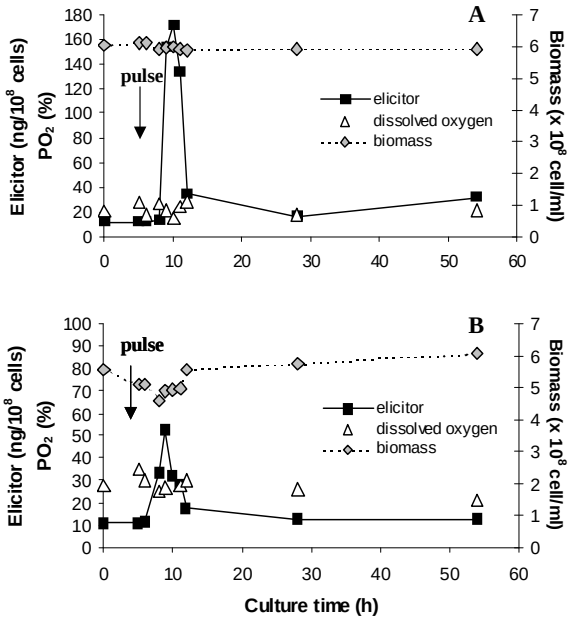



**Figure 5.** Effect of pulsed phenylalanine (a) and phenethylamine (b) on elicitor production in chemostat cultures. Cells were grown in the continuous mode in a 2-L laboratory glass bioreactor (B-Braun) with a working volume of 1.5 L of CAA medium. Cells were maintained at a constant cell growth rate of 0.15 $h^{-1}$ with aeration rate and agitation parameters that were fixed respectively at 0.5 VVM and 600 rpm. Phenylalanine was added as a pulse by injecting 5 mL of a concentrated solution to have a final concentration of 1 mM in the medium. Oxygen concentration was monitored on line with a dissolved oxygen tension electrode. Cell concentration was determined by optical density at a wavelength of 540 nm and elicitor was quantified by reverse phase HPLC. Data are from one experiment but it was repeated with similar results.

*Importance of phenylalanine*

Amino acids thus appear essential not only for BTP1 cell growth and maintenance but also for the production of NABD. We further examined the consumption rates of individual amino acids identified by HPLC as their corresponding PTH-derivatives during batch culture of BTP1 in CAA medium. Some amino acids such as Asp, Glu, Pro, Ala, and Thr are preferentially used by the bacterium during the exponential growth phase (Figure 4). Other residues such as Phe, Tyr, Trp, Lys, Ser, or Ile were still present in large amounts in the medium after 24 h, suggesting that they are preferably used for secondary metabolism rather than as nutrient for cell growth. These residues were then slowly consumed during the next 72 h, the incubation period corresponding to the NABD production phase (Figure 4). These amino acids, together with the aromatic amines phenethylamine (Pea), benzylamine, and tridecylamine, were thus tested individually for their influence on elicitor production in flask cultures. A significant effect (according to Fisher's least significant difference test at $P$ = 0,05 calculated over four repeats) on NABD production was only observed upon addition of Phe (213 ng/$10^8$ CFU) and Pea (165 ng/$10^8$ CFU) that resulted in significant 2.8–2.2-fold increases, respectively, compared to the amounts produced in control cultures (75 ng/$10^8$ CFU). These positive effects of phenylalanine and phenethylamine on NABD synthesis were also illustrated in pulsed continuous cultures at a constant cell growth rate of 0.15 $h^{-1}$. In these conditions, addition of Phe resulted in a transient ninefold increase in elicitor production without significantly

affecting the biomass level and the dissolved oxygen value (Figure 5). About 5 h after Phe addition, the NABD concentration in the medium decreased to the basal level because of the dilution rate of the medium. A lower but significant stimulation of elicitor synthesis was also observed by pulsing with phenethylamine under the same conditions (Figure 5).

**Discussion**

In light of the numerous examples of PGPR-mediated ISR reported to date, it clearly appears that the ability of a given strain to induce resistance differs in different plant species. In addition, in most inducing bacteria, more than one determinant is operative in triggering ISR in the plant. This is particularly well exemplified with the fluorescent *Pseudomonas* strain WCS358 that triggers ISR in *Arabidopsis*, tomato, and bean plants but not in radish and carnation (Meziane *et al.*, 2005; Leeman *et al.*, 1996). Three different elicitors were identified in this strain, but not all of them are recognized and active on all plant species (Meziane *et al.*, 2005). In the case of BTP1, the protective effect afforded by the pure NABD elicitor upon application on bean and cucumber roots was similar to the one provided by BTP1 living cells (Figure 2). It strongly suggests that NABD is a key factor for the resistance-inducing potential of the strain on bean and cucumber and that the molecule contains a structural motif(s) that can be recognized by root cells of different plant species. However, on tomato, the pure elicitor failed to trigger the ISR-mediated protective effect observed upon treatment with cells (Figure 2). Moreover, preliminary experiments conducted in the laboratory suggest that the addition of NABD has no effect on cultured tobacco cells by contrast with the major changes observed in the phenolic pattern upon addition of methyl jasmonate or other plant defense elicitors (Jourdan, *unpublished results*). It is thus likely that *P. putida* BTP1 produces more than one ISR determinant and that another molecule is active in triggering systemic resistance in solanacae such as tomato and tobacco. Our previous investigations led to the conclusion that metabolites identified as *Pseudomonas* determinants for ISR were not involved in resistance stimulation in bean and cucumber in the case of strain BTP1. This strain only produces traces amounts of SA, does not synthesize the pyochelin derivative, and does not produce antibiotics in significant amounts (Ongena *et al.*, 2002; Ongena *et al.*, 1999). This suggests that induction of resistance by BTP1 in tomato is not related to the production of SA or pyocyanin or diacetylphloroglucinol at the root level but may well be based on the secretion of an unknown compound. However, a role for the strain-specific pyoverdine as ISR elicitor in tomato cannot be excluded, although this molecule is not active on cucumber (Ongena *et al.*, 1999) and bean (Ongena *et al.*, 2002). Similarly, cell surface components such as lipopolysaccharides (LPS) or

flagellin may be involved in the elicitation of tomato cells by strain BTP1. NABD is clearly less active on tomato compared with the other plants tested but does retain some activity (Figure 2). The combination of NABD with another compound(s) may thus be required to fully express the eliciting potential of the molecule on this plant. Such a synergistic activity has been reported for pyochelin and pyocyanin that are both required for optimal expression of the ISR-triggering potential of *P. aeruginosa* 7NSK2 (Audenaert *et al.*, 2002).

Identification of sub-structures specifically involved in the recognition process by plant cells is essential for the understanding of early but crucial molecular talks occurring between nonpathogenic agents. In this context, the isolation of NABD from strain BTP1 further exemplify the diversity of microbial structures that can be perceived by plant cells. Results obtained by testing pure benzylamine on bean and cucumber (Figure 2) suggest that the aromatic amino part is important for the biological activity of the entire molecule. To our knowledge, this is the first report of a protective effect of benzylamine on whole plants, but this phenomenon is not surprising because previous works have already proposed that such aromatic amines may act as putative defense inducers. It is based on the rapid induction of reactive oxygen species and $Ca^{2+}$ influx observed in tobacco cell suspension cultures upon treatment with this compound (Kawano *et al.*, 2000). Recently, thiamine was demonstrated to protect plants via systemic defense response stimulation (Ahn *et al.*, 2005). 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. Further work on NABD is currently underway to appreciate the relative importance of both the charge on the quaternary ammonium and the long alkyl chain by testing multiple chemically synthesized derivatives.

Efficient root colonization is a key event required for optimal expression of biocontrol activity of beneficial rhizobacteria acting through niche exclusion/competition for substrate or antibiosis (Bloemberg and Lugtenberg, 2001). It is also true albeit to a lower extent for plant resistance inducing microbes because their populations must reach a threshold level sufficient to trigger ISR (Dekkers *et al.*, 2000; Raaijmakers *et al.*, 1995). Root colonization by *P. putida* BTP1 is efficient on the three plants tested in this study. This suggests that the strain can utilize root exudation products from the three species for its persistence and growth. Exudate organic acids are considered as the main nutritional basis of colonization in the rhizosphere, but oligosaccharides and amino acids are other major low-molecular-weight compounds available for root-associated microorganisms (Lugtenberg *et al.*, 2002). Strain BTP1 is able to use efficiently most of the organic acids for its growth. However, as in the case of phenazine production by *P. chlororaphis* plasma cell leukemia (PCL) 1391 (Van Rij *et al.*, 2004), none of these organic acids commonly found inthe rhizosphere showed a positive effect on NABD

levels (Table 2). In that sense, root exudates such as those produced by tomato plants are not optimal for elicitor production. Glucose, fructose, or mannitol are among the sugars typically found in rhizosphere for which a stimulatory effect has been demonstrated on the production of antifungal *Pseudomonas* metabolites (Van Rij *et al.*, 2004; Duffy and Défago, 1999). However, no significant positive effect has been observed on NABD synthesis upongrowth of strain BTP1 in the presence of these and other carbohydrate substrates (Table 2).

Our results show that elicitor production is tightly related to the presence of amino acids in the medium (Table 2). When tested individually, only phenylalanine displayed a significant positive impact on elicitor production. Phenylalanine is present in tomato exudates (Simons *et al.*, 1997), and in earlier experiments, we detected it among the amino acids released by bean roots. In these earlier experiments, we also observed that the concentration of phenylalanine (like most of the other residues) was significantly reduced from 0.21 µM in control plants to undetectable levels (below 100 nM) after root colonization by BTP1 suggesting an effective consumption by the strain (*unpublished results*). Although the amino acid content in root exudates may vary both qualitatively and quantitatively in function of plant genotype, plant growth stage, microbial colonization, and other environmental conditions (Kozdroj and Van Elsas, 2000), these data suggest that phenylalanine may be used by BTP1 cells *in situ*. Whether Phe can be used as a precursor for the synthesis of NABD is questionable. Phenyl-derived amines belong to the group of biogenic amines that can be produced by bacteria after decarboxylation of free amino acids (Silla Santos, 1996). However, formation of benzylamine (that could serve as "building block" for synthesis of the elicitor) from phenylalanine in such a single step is not biochemically obvious. Additional pulsed continuous cultures experiments have also shown a positive effect of phenylethylamine (Pea) on elicitor production (Figure 5). Pea is the corresponding decarboxylation product of Phe but, downstream, they enter distinct catabolic pathways. In *P. putida* KT2440, Pea is converted through phenylacetaldehyde into phenylacetic acid via the so-called phenylacetyl-CoA catabolon, whereas the homogentisate pathway is the central route for phenylalanine mineralization (Jiménez *et al.*, 2002). Therefore, the formation of a common intermediate structurally related to benzylamine is not probable. These considerations are thus more in support of a role at the regulatory level for Phe. A similar hypothesis was also raised to explain the strong stimulatory effect of this amino acid on the synthesis of phenazine in other *Pseudomonas* species (Van Rij *et al.*, 2004).

Minerals may influence the production of secondary metabolites, as they may be catalysts of many enzymes or may act at the regulatory level on the transcription and promotion of biosynthetic genes. The bivalent cations $Zn^{2+}$, $Mo^{2+}$, $Mg^{2+}$, and $Co^{2+}$ were reported to positively influence the production of some antibiotics secreted by fluorescent pseudomonads such as DAPG, pyoluteorin, pyrrolnitrin, pyochelin (Duffy and Défago, 1999), or phenazine

(Van Rij *et al.*, 2004). However, none of these elements were active on NABD production by strain BTP1. Whereas iron stimulates production of some biocontrol secondary metabolites such as phenazines (Van Rij *et al.*, 2004) and cyanide (Keel *et al.*, 1989), it negatively affected the production of NABD by strain BTP1 (Table 2) (Ongena *et al.*, 2005). Repression by $Fe^{3+}$ suggests that NABD may be under the global control of the fur protein that regulates expression of pyoverdine-type sidérophores and other factors in *Pseudomonas* (Venturi *et al.*, 1995). On the other hand, the negative influence of copper ion (Table 2) is difficult to explain, but a possibility could be that $Cu^{2+}$ can complex $NH_3$ and probably also small primary amines.

From a physiological perspective, time-course batch experiments clearly showed that NABD synthesis is stationary-phase dependent (Figure 3), and results from continuous cultures showed that the production is far more effective at low growth rate (Table 1). Although the following model does not necessarily fit with all data from the literature, root colonization by *Pseudomonas* strains can be viewed as a three-step process (Espinosa-Urgel *et al.*, 2002). It is initiated by an attraction/adhesion phase followed by a relatively short period (2 to 3 days) of intense bacterial growth and aggregation in microcolonies. The third and longer stage is a residence phase where population development is restricted by space and/or nutriment availability and thus is coupled with plant growth and root exudation rate. Based on rootcolonization data from literature, *Pseudomonas* cell growth rate should be considerably reduced (maximal doubling times about ten times lower) as compared to those observed in artificial laboratory media (Espinosa-Urgel *et al.*, 2002; Rainey, 1999; Chin-A-Woeng *et al.*, 1997; Osburn *et al.*, 1989). It implies that the actual physiology of microbial cells evolving on roots may be mainly related to some nutrient-starved state that can impose slow growth conditions (Di Mattia *et al.*, 2002). They may thus be suitable for an efficient synthesis of the NABD elicitor by BTP1 cells. In light of these results, further work will be initiated to evaluate whether the synthesis of NABD is regulated by the $\sigma^S$ transcription factor that directs RNA polymerase to multiple genes upon conditions of cellular starvation and stress (Nystrom, 2004). The quorum-sensing phenomenon and some post-transcriptional mechanisms such as the Gac two-component system may also play crucial roles in the regulation of NABD synthesis, as it was evidenced for many other secondary metabolites with biocontrol activity produced by various fluorescent *Pseudomonas* strains [reviewed in (Haas and Keel, 2003)]. As it appears to be highly conserved within the genus *Pseudomonas* (De Souza *et al.*, 2003), forthcoming work should thus also encompass the study of the influence of these regulatory systems on the synthesis of the ISR elicitor produced by strain BTP1.

Beside the C/N status and mineral content, oxygen concentration and pH are other abiotic factors that were reported to influence the production of biocontrol metabolites by rhizosphere *Pseudomonas*. Our results show that pH has no marked influence on NABD production at least

in the range of values tested (Table 1). By contrast, growth of BTP1 cells in nearly anaerobic conditions resulted in a strong reduction of elicitor production without significantly affecting the final biomass level (Table 1). This means that not only the bacterium can easily adapt to poorly aerated environments probably through the use of nitrate as an alternative electron acceptor (Dos Santos *et al.*, 2004; Ghiglione *et al.*, 2000) but also that elicitor production is somewhere dependent to oxidative biosynthetic or regulatory processes.

Understanding which and how environmental factors may affect the synthesis of the elicitors is crucial for optimal use of ISR-inducing pseudomonads as biocontrol agents. Collectively, data from this work provide some valuable information suggesting that efficient NABD production could be favored in rhizospheres corresponding to aerated (not dense, highly compacted and saturated with water) and neutral to alkaline soils where oxygen is available (Hojberg *et al.*, 1999) and iron mostly in an insoluble form (Simeoni *et al.*, 1987). However, in the case of NABD, nothing is known about the cascade of molecular events involved in the perception of environmental stimuli or about the biosynthetic genes. Further investigations will be conducted to identify this genetic background, which is a prerequisite to get further insights into the cellular regulatory network that could influence ISR induction by strain BTP1.

**Unpublished additional results**

*Influence of quorum sensing in NABD production*

As shown in chapter V, NABD production is effective during the stationary growth phase of *Pseudomonas putida* BTP1 and therefore, could be under the regulation of the quorum sensing (QS) phenomenon or population perception. In *Pseudomonas*, this phenomenon is usually related to the production of *N*-Acyl-homoserine lactones (AHLs) as signal molecules (Waters and Bassler, 2005). AHLs are constituted by a homoserine lactone core linked to a variable length acyl chain. As a first step, we have investigated the ability of *Pseudomonas putida* BTP1 to produce AHLs on plates and in flask cultures after growth in two different media (King's B and Casamino acids).

By using three biosensors reactive to specific AHLs, we have tested BTP1 products (cross-streaking against the sensor strain in plate bioassays or as supernatant concentrate added to sensor culture) for the presence of such QS signal molecules. Assays were done in parallel with *Pseudomonas aeruginosa* PAO1 used as positive control with known AHLs. The biosensor activities were estimated on the basis of violet coloration of the colonies in the case of

*Chromobacterium violaceum* CV026 (McClean *et al.*, 1997) or by bioluminescence when the two *E. Coli* pSB401 and pSB1075 strains were used (Winson *et al.*, 1998) (Table 3).

**Table 3.** Relative activity of *Pseudomonas aeruginosa* PAO1 and *Pseudomonas putida* BTP1 supernatants on AHL biosensors.

| Biosensors | Relative autoinducer activity | | |
|---|---|---|---|
| | **PAO1-KB** | **BTP1-KB** | **BTP1-CAA** |
| CV026, $C_4$-$C_6$ | +++ | - | - |
| pSB401, $C_6$>$C_8$>$C_{10}$>$C_{12}$ | +++ | - | - |
| pSB1075, $C_{12}$>$C_{10}$>$C_8$>$C_6$ | + | + | - |

Strains were grown on King-B (KB) medium or Casamino acid medium (CAA) and their respective supernatant were confronted to the biosensors CV026 mostly sensible to short-chain AHLs $C_4$-$C_6$, pSB401 sensible to AHL with $C_6$, $C_8$, $C_{10}$ and $C_{12}$ acyl chain and pSB1075 more sensible to long-chain $C_{12}$ AHLs. +++, strong activity; ++, intermediate activity; +, weak activity; -, no activity. Results presented are means from three measures of one experiment.

The observations presented in Table 3 revealed that BTP1 only produces very small amounts of *N*-acyl-homoserine lactones autoinducers, at least compared to *P. aeruginosa* PAO1 and under the conditions used. Second, supplementing exponentially growing cells with concentrated supernatant extracts containing AHL did not resulted in an earlier and/or increased production of the elicitor (results not shown).

## Materials and methods

*Microbial strains and inocula preparation.*
*Pseudomonas putida* strain BTP1, isolated from barley roots, was originally selected for its specific features regarding pyoverdine-mediated iron transport (Ongena *et al.*, 2001). It was maintained and prepared for use in the ISR assays as previously described (Ongena *et al.*, 2002). The *Botrytis cinerea* strain used in induced resistance assays on bean and tomato was kindly provided by Dr. Monica Höfte (Ghent University). The fungus was grown to sporulation on an oat-based medium (oatmeal 25 g/L; agar 12 g/L) at room temperature and the conidial suspension used for infection was prepared as described (Ongena *et al.*, 2002). The fungal pathogen *Colletotrichum lagenarium* was maintained on PDA medium (Potato Dextrose Agar, Becton, Dickinson and Company, Le pont de Claix, France) at room temperature in the dark. For long-term storage, conidia were conserved in 40% glycerol at –80°C. The suspension used for cucumber infection was prepared by harvesting spores from 4-week-old cultures in sterile peptone water containing 0.01% Tween 80. After removing mycelial debris by filtration through several layers of cheese cloth, the suspension was centrifuged for 5 min at 5000 *g* and the spores were resuspended in 0.4% glucose solution supplemented with 0.013 M $KH_2PO_4$ to the desired final concentration of $10^7$ spores/mL.

*Assays for induced resistance by the bacterium.*
In vivo assays with bean (*Phaseolus vulgaris* cv Prelude) were performed following a procedure previously described (Ongena *et al.*, 2002). Briefly, seeds were soaked, prior to sowing, for 10 min in a BTP1 cell suspension at a concentration of approximately 4 x $10^8$ CFU/mL or in NaCl 0.85% in the case of control plants. Seven days after sowing, 20 mL of the bacterial suspension at $10^8$ CFU/mL was added as a drench to the roots of each plant except the controls (watered with NaCl 0.85%). After approximately 15 days, bean plants were leaf-infected with *B. cinerea* by depositing eight 10 µl-drops

of the pathogen spore (5 x $10^5$ spores/mL) on both primary leaves. Disease incidence was recorded after 4 to 5 days on 20 plants per treatment and expressed in terms of the percentage of *B. cinerea* lesions that clearly grew out of the inoculum drop zone to produce spreading lesions.
Tomato seeds (*Lycopersicon esculentum* L. cv Merveille des Marchés) were disinfected in 5% household sodium hypochlorite for 3 min and were then washed five times with sterile distilled water and grown in autoclaved potting soil previously mixed with bacterial inoculum to a final concentration of 3 x $10^7$ CFU/mL or with an equal volume of sterile water for untreated control plants. Tomato plants were germinated at 26 ± 2°C in the greenhouse with a 16-h photoperiod. Fifteen days after sowing, 20 mL of a bacterial suspension at $10^8$ CFU/mL was added to the roots of BTP1-treated plant. Five-week-old plants were infected with *B. cinerea* on excised third leaves by using a suspension at $10^5$ spores/mL. Forty-five leaves were used per treatment in every experiment.
Before planting, cucumber seeds (*Cucumis sativus* cv. Raider) were disinfected in ethanol 70% for 1 min and rinsed three times with sterile distilled water. Seeds were then soaked for 10 min in a bacterial suspension at a concentration of approximately 4 x $10^8$ CFU/mL in NaCl 0.85%. Cucumber seeds were sown in 10-cm pots containing sterilized potting soil (Brill Substrate GmbH, KG, Germany) previously mixed with bacterial inoculum to a final concentration of 3 x $10^7$ CFU/g or with an equal volume of sterile water for untreated control plants. Cucumbers were germinated at 25 ± 2°C in the greenhouse with a 16-h photoperiod. Six days after sowing, 20 mL of a bacterial suspension at $10^8$ CFU/mL was added to each plant except the controls (watered with 20 mL of NaCl 0.85%). After approximately 10 days, cucumber plants were transferred to a high humidity chamber (19 ± 2°C) for 24 h before leafinfection with *C. lagenarium*. Each cotyledon was inoculated with six 7-µl drops of the pathogen spore suspension. Twenty-five plants were used by treatment in every experiment. Disease incidence was scored 10 days later and was expressed in terms of the percentage of typical yellow lesions that spread out of the inoculum drop area.

*Assays for induced resistance with pure compounds*
Plants were grown and infected as described above for controls without bacterial treatment. Bean, cucumber and tomato plants were treated, respectively, 7, 5 and 10 days before pathogen challenge, by adding as a drench 20 mL of solutions containing either the semi-purified elicitor, the commercial benzyldimethyltetradecyl ammonium (Sigma-Aldrich, Bornem, Belgium) or benzylamine (Acros Organics, Geel, Belgium). All compounds were tested at a final concentration of 1 µM after a 1/100 dilution of appropriate methanolic solutions in distilled water. Control plants were treated with the same volume of 1% methanol. The two commercial products were used as delivered. The elicitor extract was prepared from crude BTP1 culture supernatant by two successive solid-phase extraction steps. The cell-free fluid was first filtered through an Amberlite XAD-4 gel (Acros Organics) that retained hydrophobic material. The elicitor was eluted with pure methanol [high-performance liquid chromatography (HPLC) grade] after extensive column washing with MilliRo quality water and 50% methanol. The methanolic solution was evaporated under vacuum, and the resulting material was further submitted to solid-phase extraction on an Isolute C-18 CE-type cartridge as described previously (Ongena *et al.*, 2002). Elicitor concentration in the final methanolic extract was determined by HPLC (see below). Benzylmethylethyltridecyl ammonium was synthesized starting from benzylamine as previously described (Ongena *et al.*, 2005). This method was adapted from the one developed by Salvatore *et al.* (Salvatore *et al.*, 2002) for the synthesis of quaternary ammonium and uses CsOH to ensure chemoselective reactions favoring *N*-alkylation of the primary amine and a last methylation step resulting from an acido-basic reaction.

*Culture Conditions for Bacterial Growth in Synthetic Media*
Cells of *P. putida* BTP1 were grown in the batch mode either in agitated Erlenmeyer flask (130 rpm, 28°C) or in a 2-L laboratory glass bioreactor with a working volume of 1.5 L and equipped with automated pH control, a dissolved oxygen tension electrode and a magnetic-coupled stirrer (BiostatB, B. Braun Biotech. International, Melsungen, Germany). One-hundred-fifty-milliliter flasks filled with 50 mL of culture medium were used for testing the effect of nutrients as described below, and 3-L flasks containing 1.5 L of medium were used for time course experiments. In bioreactor, the temperature was maintained at 30°C, and pH was adjusted to the specified value by addition of $H_3PO_4$ (3 N) when required. Aeration rate and agitation were fixed,respectively, at 0.5 VVM and 300 rpm for

batch experiments, and the various settings used for testing the influence of oxygen are detailed in Table 1. In all cases, cultures were inoculated with a 2% volume of 16-h subcultures realized in the same medium. To minimize contaminating iron traces, glassware and bioreactors were rinsed with nitric acid (0.5 N). Water of Milli-Ro quality (Millipore, Massachusetts) was used to prepare all the media. All the chemicals used were of analytical grade. For chemostat culture (0.5 VVM and 600 rpm), fresh medium was fed into the reactor using a peristaltic pump to control the dilution rate. The volume of the culture was maintained constant by the use of an overflow device. The bioreactor was switched to continuous mode after an initial batch start-up. Silicone antifoam was occasionally and manually added to control extensive foam formation. Iron concentrations in the growth medium slightly increased with the dilution rate but in all cases remained below a value limiting for growth of the strain as proved (1) by efficient production of pyoverdin, the iron-regulated siderophore, which was determined on the basis of the absorbance at 400 nm using a specific molar extinction coefficient ($\varepsilon_{400\ nm,\ pH\ 7}$) of 2.75 x $10^4$ mol/L/cm, (2) by the fact that pulse with ferric chloride broke down the steady-state and rapidly led to a significant increase of the biomass, and (3) by the fact that the two amino acids that mainly support growth (aspartic acid and glutamic acid) remained in excess at any time.

*Influence of Carbon Source, Minerals, and Amino Acids*

BTP1 cells were routinely grown in the semi-synthetic iron-restricted casamino acids medium (CAA) containing casamino acids (Becton, Dickinson and Company) 5 g/L, $MgSO_4 \cdot 7H_2O$ 0.25 g/L, and $K_2HPO_4$ 0.9 g/L (addition of 3 or 12 g/L bacto agar for media described as semi-solid and gelified respectively). Several other media were used to test the effect of carbon source on elicitor production. The King's B medium was prepared by dissolving bacto tryptone (Becton, Dickinson and Company) 10 g/L, bacto proteose peptone no. 3 (Becton, Dickinson and Company) 10 g/L, $K_2HPO_4$ 0.9 g/L, $MgSO_4 \cdot 7H_2O$ 0.25 g/L, and glycerol 10 g/L in MilliRo water. The other substrates (mannitol 30 mM, arabitol 35 mM, glucose 30 mM, fructose 30 mM, mannose 30 mM, glycerol 60 mM, succinate 40 mM, and glutamate 35 mM) were tested by adding a concentrated solution to a minimal medium composed by $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $K_2HPO_4$ 0.9 g/L, and $(NH_4)_2SO_4$ 1 g/L. In all cases, pH was adjusted to 7 ± 0.2 with NaOH or HCl 0.1 N before sterilization. To test the influence of oligoelements, the following minerals $CoCl_2$, $CuSO_4$, $ZnSO_4$, $FeSO_4$, $MgSO_4$, NaCl, and $(NH_4)_6Mo_7O_{24}$ were added to the CAA medium to have a final concentration of 0.1 mM. The effect of individual amino acids was first tested in 150-mL culture flasks by adding 100 µl of a concentrated solution of either phenylalanine, tyrosine, lysine, isoleucine, or tryptophane to obtain a final concentration of 0.8 mM in the medium. For testing the effect of phenylalanine pulse in chemostat, 5 mL of a concentrated solution of the amino acid was sterilely injected (filtration through 0.2 µm membrane) into the bioreactor to have a final concentration of 1 mM in medium.

*Determination of Elicitor Production Yield*

Bacterial cell densities obtained at the end of the cultures were determined by measuring the optical density (OD) at 540 nm knowing that a value of one corresponds to 4.5 x $10^8$ CFU/mL. Cell concentration in time-course experiments was established by plate-counting on gelified CAA medium. Elicitor production in the different media was determined from 10-mL samples of culture supernatant collected after growth for 72 h as described (Ongena *et al.*, 2005). In all the media and conditions tested, time-course measurement of $OD_{540\ nm}$ over the entire culture showed that at this sampling time, cells were in the stationary phase of growth. Briefly, samples were pre-purified on C18 solid-phase extraction cartridges and analyzed by reverse phase HPLC on a Lichrospher 100 RP C-18 column (250 x 4.6 mm, 5 µm packing, Merck, Darmstadt, Germany) using the same solvent system as described above and at a constant flow rate of 1 mL/min. The molecule of interest was eluted with a gradient of acetonitrile as follows: (time in min/% acetonitrile) 0–2.5/20, 2.5–20/20–47; 20–60/47, 60–61/47–95; and 61–65/95. Amounts were calculated on the basis of the corresponding peak area at 280 nm.

*Amino Acid Analysis*

After cell elimination by centrifugation at 13,000 rpm for 15 min at 4°C, 100-µL supernatant samples (corresponding to approximately 1 µmol amino acids) were used for amino acid concentration determination in the CAA medium at every time point. Samples were first evaporated in a Speedvac concentrator and redissolved in 100 µL of a solution prepared by adding 2 mL of acetic acid 2 M and

1.2 mL pure triethylamine to 46.8 mL of 50% acetone (pH 10.1). Samples were derivatized by the addition of 100 µL 2-mM DABITC (4-(dimethylamino)azobenzene-4′-isothiocyanate, Sigma-Aldrich) dissolved in acetone and incubation for 1 h at 50°C. The resulting solution was dried, the residue was re-solubilized in 200 µL water, and the volume was adjusted to 600 µL by adding acetic acid. This solution was incubated for an additional 50-min period at 50°C and then evaporated in the Speedvac. The dried material was finally solubilized in 400 µL ethanol, centrifuged for 10 min at 10,000 rpm, and submitted to HPLC analysis (Hewlett-Packard 1100 series apparatus) by injecting a 40-µL volume onto a Supelcosil LC-18-DB column (150 x 4.6 mm, 3 µm, Sigma-Aldrich). Compounds were eluted by using a gradient of acetonitrile in sodium acetate 35 mM at a flow-rate of 0.4 mL/min and were detected at 436 nm. The following gradient was used: (time in min/% acetonitrile) 0–1/35; 1–2/35–44; 2–5/44; 5–10/44–54; 10–24/54; 24–25/54–85; and 25–31/85. An amino acid standard solution (Sigma-Aldrich) was used for determination of elution volumes of individual amino acids and for quantification based on integration of appropriate peaks. In some cases, peak identification required co-injection of known individual standards derivatized in the same way. Under the chromatographic conditions used, most of the amino acids could be identified, but Val/Trp and Asp/Glu could not be optimally resolved and were considered as a single peak for integration in HPLC profiles.

*Mass spectrometry*
Mass spectrometry investigations of the elicitor were performed with a Finnigan-MAT 900ST instrument equipped with an electrospray ion source (ESI-MS). Collision activation of mass-selected ions was performed either in the octapole region or in the ion trap. In the octapole, consecutive fragmentation processes are possible, whereas in the ion trap, all fragments must stem from the selected precursor ion. Samples were dissolved in $CH_3OD$.

*Statistical treatment of data for disease reduction in ISR assays*
The homogeneity of variances was tested with analysis of variance (ANOVA), and data from at least three independent ISR experiments with the same set-up were pooled for analysis when interaction between experiment and treatment was not significant at $P = 0.05$. Means from the different treatments were compared by the method of Newman and Keuls (least significant difference at $\alpha = 0.05$, Statistica software).

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# *Chapitre VI. Chemical synthesis and biological activities of NABD structural derivatives*

**Résumé**

Sur les trois pathosystèmes testés dans le chapitre V, des réductions significatives des symptômes des maladies ont été observés suite aux traitements des racines par la benzylamine, suggérant un rôle important du noyau aromatique dans l'élicitation des mécanismes de défense chez trois plantes de familles différentes. Nos travaux se sont donc logiquement orientés vers la recherche de sous-structures potentiellement impliquées dans la reconnaissance du NABD par les cellules de la plante. Pour cela, nous avons mis au point deux schémas réactionnels, basé dans le premier cas sur une *N*-alkylation permettant l'ajout des chaînes alkylées sur une amine aromatique ; et dans le second cas sur l'apport de la fonction amine portée par les chaînes alkylées sur le noyau aromatique grâce à une réaction d'addition-fragmentation. Ces deux méthodes ont permis de générer toute une série de dérivés synthétiques du NABD, variant aussi bien dans la nature du noyau aromatique que dans la longueur des chaînes fixées sur l'azote de l'éliciteur. Dans ce chapitre, nous décrivons tout d'abord les différentes étapes de cette synthèse chimique et présentons l'éventail des dérivés qui ont pu être produits. Nous exposons également des résultats préliminaires vérifiant l'importance de la nature du noyau aromatique ainsi qu'un rôle suggéré de la charge de l'azote, conférée par la quarternisation de la molécule. Faute de temps, ces premières observations n'ont pas pu être approfondies, ni l'ensemble de ces molécules testées. Les méthodes de synthèse décrites ici pourraient néanmoins aussi se révéler des outils intéressants et être adaptées dans l'optique d'étudier le mode de reconnaissance des éliciteurs par les cellules des plantes.

**Introduction**

As illustrated in chapter III, the chemical nature of ISR elicitors harboured or produced by rhizobacteria may greatly vary from macromolecules such as flagellins or lipopolysaccharides to very small volatile compounds such as butanediol. In addition to the structural identification of these elicitors, another important interrogation is how the plant can recognize them and more particularly which part of the molecules is responsible for its biological activity. Researches conducted with purified flagellin from a pathogenic *Pseudomonas aeruginosa* strain revealed that a 22 N-terminal amino acid sequence of the protein (flg22) is the minimal pattern recognized by *Arabidopsis* cells to activate defence responses (Felix *et al.*, 1999). As this sequence is relatively conserved between strains from different species, some authors suggest that it could also be involved in the interaction between plants and non pathogenic rhizobacteria (Meziane *et al.*, 2005). In the case of LPS, constituted by an oligosaccharide core

linking a lipid group (lipid A) to an O-polysaccharide part (O antigen), it seems that the active group conferring the elicitor activity to the molecule is the O antigen (Meziane *et al.*, 2005; Leeman *et al.*, 1995) (see chapter III). By contrast with these two cell surface components, very little is known about the way the other elicitors from beneficial bacteria are perceived by plant cells.

In the previous chapters, we have reported the isolation of NABD from *Pseudomonas putida* BTP1. NABD (*N*,*N*-dimethyl-*N*-tetradecyl-*N*-benzylammonium, $C_6H_5–CH_2–N^+(CH_3)_2–C_{14}H_{29}$) consist of a quaternary ammonium formed by a benzylamine core *N*-alkylated by three carbonyl groups. In this chapter, our aim was to generate and test various structural derivatives of the NABD molecule in order to identify the sub-structures responsible for the ISR eliciting activity. We describe organic chemistry methods developed to synthesize molecules varying in the alkyl chain length and/or aromatic core nature and we present preliminary results obtained by testing some of them for their ISR activity on bean.

## Results and discussion

### *Organic chemistry synthesis*

To generate NABD structural derivatives, two methods were used. The first one is based on *N*-alkylation of an amine with alkyl bromide (Salvatore et al., 2002) and the second method starts with an addition-fragmentation of an amine chain on an acid chloride core followed by a reductive amination. This second method was used when low production rates were obtained with the first one due to the different reactivities of the phenolic cores. In fact, the *N*-alkylation reaction (Figure 1A.) consists in sequential addition of alkyl chains first on the primary amine core (a) in order to obtain a secondary amine (b) and, finally, a tertiary amine (c). However, during the first step of *N*-alkylation, in addition to the monoalkylated compound (secondary amine, see b in Figure 1A), an undesired dialkylated compound (tertiairy amine, see b’ in Figure 1A) could also be generated. The rates of dialkylated compound formation were respectively approx. 5% and 40% when phenethylamine and benzylamine were used as precursor.

Such high di-N-alkylation rates obtained with benzylamine were avoided by using the second method adapted from reactions previously described (Meshram et al., 1998; Boldrini et al., 1974). Briefly, the mechanism of the addition-fragmentation reaction first consists in addition of the nucleophile amine on the acid halogenure carbon to form a tetrahedral intermediate. Then, this intermediate is fragmented and deprotoned to form an amide. The

amide is reduced by aluminium lithium hydride leading to secondary amine formation. During the reaction of reductive amination, the secondary amine is transformed in a tertiary form. The second alkyl chain is brought by an aldehyde compound via imine formation with the secondary amine then reduced in tertiary amine with $Na(AcO_3)BH$. The entire reaction is detailed in Figure 1B.

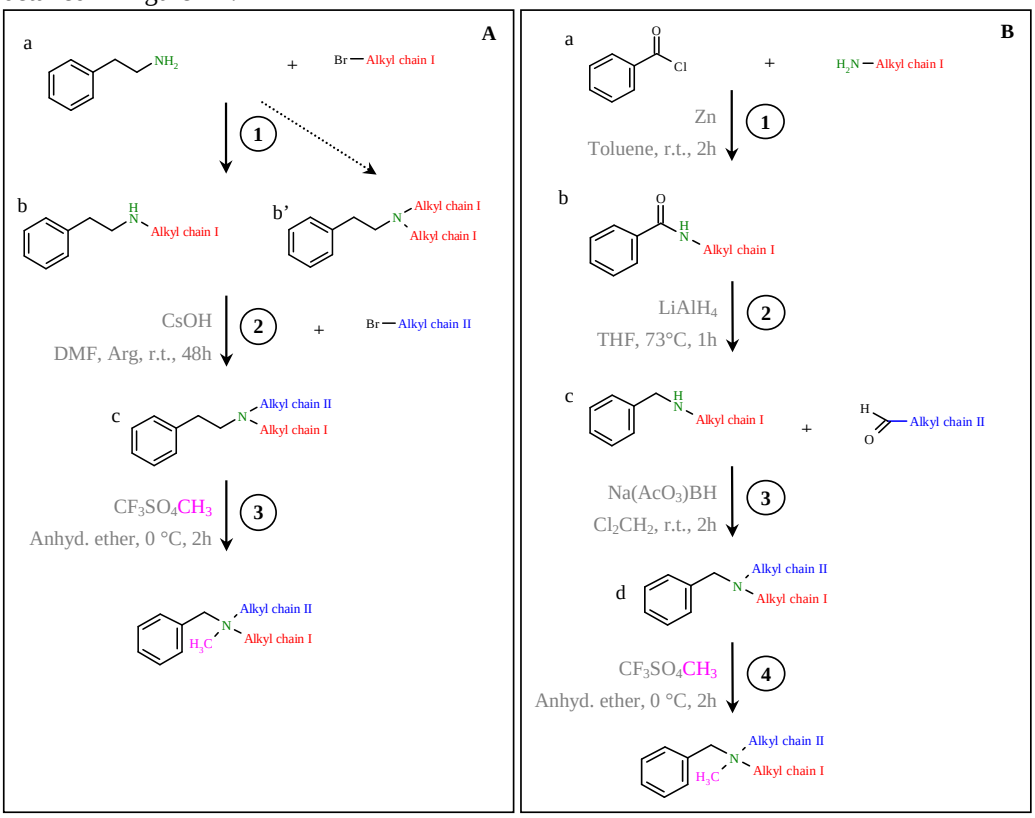


**Figure 1.** Chemical synthesis of NABD derivatives.
**A.** N-alkylation reaction. Reaction 1 allows fixation of the first alkyl chain (Alkyl chain I) on aromatic amine (a) to obtain a "monoalkylated" or secondary amine (b). During reaction 1, a "dialkylated" amine could be formed by a second reaction with the alkyl chain I (b'). A same reaction (2) is used to add the second alkyl chain (alkyl chain II) to obtain a tertiary amine (c). Respective rates (ρ) are 46% and 51% for reactions 1 and 2.
**B.** Addition-fragmentation reaction followed by a reductive amination step. The amine (alkyl chain I) reacts with acyl halogenure (a) to form an amide (1) (Fragmentation step). (ρ = 83%). This product will undergo a reduction by aluminium lithium hydride ($LiAlH_4$) (2) and lead to secondary amine formation (c) (ρ > 95%). During the reductive amination reaction (3), the secondary amine (c) will be transformed in tertiary amine (d). The second alkyl chain (Alkyl chain II) is added on secondary amine by aldehyde compound via imine formation. (ρ > 79%). Both reactions are followed by an acido-basic reaction with $CF_3SO_4CH_3$ to quaternarize the amide (ρ = 70%). DMF, *N*,*N*-Dimethylformamide; CsOH, Cesium hydroxide; $CF_3SO_4CH_3$, methyl trifluorosulfonate; $LiAlH_4$, aluminium lithium hydride; THF, tetrahydrofuran; $Na(AcO_3)BH$, sodium tri(acetoxy)borohydride; $Cl_2CH_2$, dichloromethane; Arg, under argon atmosphere; r.t., room temperature; 0°C, reaction realized at 0°C max.

Independently of the method used to obtain the tertiary amine, an acido-basic reaction with trifluoromethanesulfonate methyl is used to generate the final quaternary ammonium compound. Identity of all the synthesized compounds was verified by mass spectrometry. Using these two different approaches, various NABD derivatives listed in Table 1 were synthesized.

**Table 1.** Structural derivatives of NABD synthesized chemically in this work using either *N*-alkylation or addition-fragmentation and reductive amination both followed by acido-basic reaction reactions.

| Aromatic core | Alkyl chain R1 | Alkyl chain R2 | Alkyl chain R3 |
|---|---|---|---|
| $C_6H_5$-$CH_2$-$CH_2$-N(R1)(R2)(R3) | H | H | |
| | $CH_3$ | H | |
| | $C_4H_9$ | $CH_3$ | |
| | $C_{10}H_{21}$ | H | |
| | $C_{10}H_{21}$ | $C_4H_9$ | |
| | $C_{10}H_{21}$ | $C_4H_9$ | $CH_3$ |
| $C_6H_5$-$CH_2$-N(R1)(R2)(R3) | H | H | |
| | $CH_3$ | H | |
| | $C_3H_7$ | H | |
| | $C_3H_7$ | $C_2H_5$ | |
| | $C_3H_7$ | $C_2H_5$ | $CH_3$ |
| | $C_{12}H_{25}$ | H | |
| | $C_{12}H_{25}$ | $C_2H_5$ | |
| | $C_{12}H_{25}$ | $C_2H_5$ | $CH_3$ |
| | $C_{12}H_{25}$ | $C_3H_7$ | |
| | $C_{12}H_{25}$ | $C_3H_7$ | $CH_3$ |
| | $C_{14}H_{29}$ | H | |
| | $C_{14}H_{29}$ | $CH_3$ | |

*Biological activity of NABD derivatives*

Some of these chemically synthesized derivatives were tested for their potential to induce resistance in bean plants in comparison with the protective effect provided by the original NABD molecule and by the BTP1 strain. These preliminary experiments were performed in hydroponic conditions such as described in chapter IV. Results obtained in one assay are exposed in Figure 2.

In all cases, alkylated compounds are seemingly more active than non alkylated amines. Indeed, treatment of bean roots with benzylamine only reduced disease symptom to about 16% while tri-alkylated forms (NABD or C12-C3-C-B) provided a higher protection rate above 25%. A same tendency is observed with phenethylamine and its quaternary derivative C10-C4-C-Ph, suggesting the importance of the positive charge on the amine group. Moreover, the aromatic core, and more particularly the number of carbons between the cycle and the amine may be important since benzylamine derived molecules seem to induce a greater response in the plant than phenethylamine based compounds.

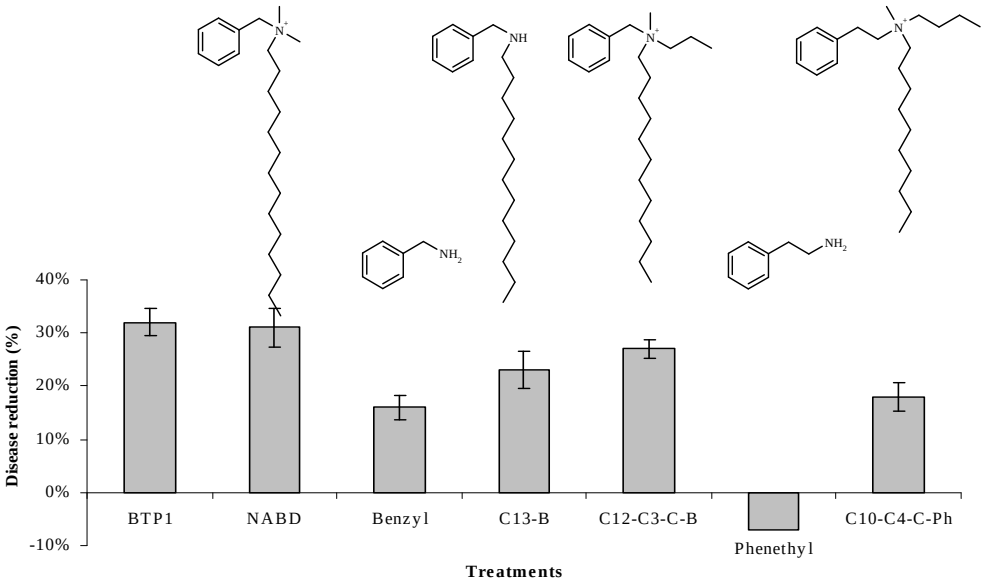


**Figure 2.** Reduction of *Botrytis* disease symptoms observed on hydroponically grown bean plants treated either with BTP1 extract, pure elicitor (NABD), benzylamine (Benzyl), *N*-tridecyllbenzylamonium (C13-B), N-dodecyl N-propyl N-methylbenzylamonium (C12-C3-C-B), Phenethylamine (Phenethyl) or N-decyl-butyl-methyl-phénéthylamonium (C10-C4-C-Ph). Compounds were added to obtain a final concentration in the nutrient solution of 1µM. Data presented are from one experiment.

In summary, this work allowed us to generate multiple synthetic compounds deriving from the *Pseudomonas putida* BTP1 elicitor. As stated above, the results presented here with some of these derivatives are preliminary and additional assays are necessary to verify these first conclusions.

## Materials and methods

*N-alkylation reaction*
Reaction was adapted from Salvatore *et al.* (2002). Anhydrous DMF was conditioned under inert atmosphere for 15 min with activated powdered molecular sieves before amine (1 eq.) addition. After 10 min, cesium hydroxide monohydrate (CsOH) (2 eq.) was added upon stirring for 30 min. Reaction was started by addition of alkylbromide (1.2 eq.) and performed during 48 h at room temperature. Alkylated amine was precipitated after incubation of the mixture at 0°C in ethyl acetate overnight.

*Addition-fragmentation reaction*
Reaction was adapted from Meshram *et al.* (1998). Zinc (powder grade) was activated 10 min with HCL 10% and washed with two volumes of $H_2O$ then one volume of acetone. Acyl chloride (1 eq.) and activated zinc (1 eq.) were mixed in anhydrous toluene (2,5 eq.) for 10 min at room temperature. After slowly amine addition (1 eq. in 2,5 eq. anhydrous toluene), the mixture was stirred during 1 h. It was then filtrated and the solid was washed with ether. Filtrate was washed with 10% $NaHCO_3$, and finally dried over $Na_2SO_4$. Solvents were evaporated under vacuum using a rotavapor apparatus (Buschi).

*Reduction of amide*
Amide (1 eq.) was dissolved in anhydrous tetrahydrofuran (THF). The mixture was incubated under inert atmosphere at -8°C and aluminium lithium hydride ($LiAlH_4$) (3,258 eq.) was added very slowly. Flask was adapted to a condenser and mixture was boiled at 67°C for 1 h. After cooling, the excess of $LiAlH_4$ was neutralized at -8°C with $H_20$ first, and then with NaOH 1N. The mixture was then stirred for 10 min before purification. Amine was purified using liquid-liquid extraction with ethyl acetate. The organic phase was dried over $Na_2SO_4$ before solvent evaporation.

*Reductive amination*
Reaction was adapted from Boldrini *et al.* (1974). Amine (1 eq.) and aldehyde (1,3 eq.) were dissolved in anhydrous dichloromethane before Sodium tri(acetoxy)borohydride ($Na(AcO_3)BH$, 2 eq.) addition. Mixture was stirred for 1h30 at room temperature. Reaction was stopped with 5% $NaHCO_3$ addition. Organic phase was washed and dried over $Na_2SO_4$ before solvent evaporation.

*Acido-basic reaction*
Tertiary amine (1 eq.) was dissolved in anhydrous diethyl ether under inert atmosphere. After methyl-trifluoromethanesulfonate (1.75 eq.) addition, resulting mixture was stirred at 0°C until a white precipitate formation (2 h). The final compound was purified from filtrate after two successive washes with cold dichloromethane and the same volume of cold diethyl ether. At least, solvents were evaporated using rotavapor and the quaternary amonium product was dried under vacuum overnight.

Purity and structure of synthetic compounds were confirmed by ESI-MS analyses. The MS instrument was a Thermoquest-Finnigan running in positive ion mode and controlled by the Xcalibur software. The capillary voltage used was 4.5 kV.

Induced systemic resistance experiments were realized as described in chapter V.

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## *Chapitre VII.* **Bacillus subtilis *M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression***

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**Résumé**

Ce premier volet de l'étude consacrée à *Bacillus subtilis* S499 (ou M4) décrit l'habilité de la souche à réduire l'impact de la maladie dans deux pathosystèmes via l'induction de résistance. Dans les deux cas, les expériences ont en effet été conçues afin de garantir une séparation spatiale de l'agent bénéfique et du pathogène.

Sur le concombre infecté au niveau des cotylédons par *Colletotrichum lagenarium*, le traitement des plants au niveau racinaire par la souche S499 confère une protection de 27-29% par rapport aux témoins. Cet effet protecteur est aussi remarqué lors du traitement des racines par des spores de la bactérie, conférant à la souche un fort potentiel d'un point de vue industriel. En effet, l'utilisation de spores plutôt que des cellules végétatives de la bactérie est un atout primordial pour une conservation à long terme d'un éventuel produit.

L'effet inducteur de résistance de la souche et de ses spores a ensuite été testé sur la tomate. Dans ce cas, une mise en contact préalable des graines avec des cellules de *Bacillus subtilis* pendant 24h permet une nette augmentation du taux de germination des graines semées ensuite dans un terreau infecté par *Pythium aphanidermatum* (de 130% à 40% selon la pression de maladie appliquée).

Par ailleurs, les lipopeptides (LPs) sont des molécules bioactives possédant des nombreuses propriétés, dont certaines, comme leurs activités antibiotiques, pouvant être impliquées dans le biocontrol grâce à des mécanismes d'antagonisme directe. Dans ce chapitre, nous avons voulu tester l'activité élicitrice de l'ISR de ces lipopeptides sur le concombre. Cependant, dans les conditions testées, l'ajout d'un mélange de plusieurs familles de LPs (surfactine, iturine et fengycine) au niveau des racines de la plante n'a pas montré de potentiel inducteur de résistance suggérant que ces derniers ne soient pas actifs sur ce végétal.

**Abstract**

Results presented in this paper describe the ability of *Bacillus subtilis* strain M4 to reduce disease incidence caused by *Colletotrichum lagenarium* and *Pythium aphanidermatum* on cucumber and tomato, respectively. Disease protection in both pathosystems was most probably due to induction of resistance in the host plant since experiments were designed in order to avoid any direct contact between the biocontrol agent and the pathogen. Pre-inoculation with strain M4 thus sensitised both plants to react more efficiently to subsequent pathogen infection. In cucumber, the use of endospores provided a disease control level similar to that obtained with vegetative cells. In contrast, a mixture of lipopeptides from the surfactin, iturin and fengycin families showed no resistance-inducing potential.

**Introduction**

Among the biological control alternatives to the extensiveuse of chemical pesticides, the application of non-pathogenicsoil bacteria living in association with plant roots is promising. Treatment with these plant growth-promoting rhizobacteria (PGPR) was in many cases associated with reduced plant diseases in greenhouse and field experiments. These PGPR strains are capable of directly antagonising fungal pathogens by competing for niche and essential nutrients, or by producing fungitoxic compounds (Whipps, 2001; Handelsman and Stabb, 1996). However, the isolation of some PGPR strains lacking the ability to exert any antagonistic activity toward pathogens has shed new light on the diversity of their modes of action, and has suggested that such strains may activate host defence systems. As bacterial treatment at the root level confers protection against foliar or systemic pathogens, such PGPR-mediated resistance has been termed induced systemic resistance (ISR) (Kloepper *et al.*, 1992). ISR is effective against a broad range of diseases (Van Loon *et al.*, 1998), and disease control strategies based on this mechanism have been successfully applied under field conditions (Zehnder *et al.*, 2001; Wei *et al.*, 1996).

ISR is phenotypically similar to the well-studied systemic acquired resistance (SAR) activated after a first infection by an incompatible necrotising pathogen or upon treatment with some chemicals such as benzothiadiazole (BTH) (Oostendorp *et al.*, 2001; Hammerschmidt, 1999). However, the signal transduction pathway and the molecular basis underlying rhizobacteria-mediated ISR differ in many aspects from pathogen-induced SAR. Typically, the expression of SAR is associated with the accumulation of salicylic acid (SA) (Métraux, 2001) and of pathogenesis-related (PR) proteins (Van Loon and Van Strien 1999). In contrast, rhizobacteria-induced ISR depends on the perception of jasmonate and ethylene rather than an early increase in endogenous SA (Pieterse *et al.*, 2001) and is currently not associated with PR protein accumulation (Van Loon *et al.*, 1998). Most of the ISR-inducing microbes identified so far are Gram-negative bacteria and are mainly species belonging to the *Pseudomonas* and *Serratia* groups (Van Loon *et al.*, 1998). To our knowledge, evidence for ISR triggered by Gram-positive bacteria has been reported for only a very limited number of strains of *Bacillus pumilus* and *Bacillus amyloliquefaciens* (Zehnder *et al.*, 2000; Benhamou *et al.*, 1996). Nevertheless, many isolates of the *Bacillus* genus, and especially strains of *Bacillus subtilis*, were reported to be effective for the biocontrol of multiple plant diseases caused by soilborne pathogens (Chen and Wu, 1999; Harris and Adkins, 1999; Raupach and Kloepper, 1998; Asaka and Shoda, 1996). Some of them are used in commercially available biocontrol products (Backman *et al.*,1997; Brannen and Kenney, 1997). These microorganisms produce a variety of powerful antifungal metabolites, including lipopeptides of the surfactin, iturin and fengycin

families. These amphiphilic cyclic peptides are composed of seven or ten α-amino acids linked to a C-13 to C-18 fatty acid chain,and display a strong antifungal activity (Akpa *et al.*, 2001; Jacques *et al.*, 1999). It was therefore suggested that antibiotic production by *B. subtilis* played a major role in disease suppression (Yu *et al.*, 2002; Yoshida *et al.*, 2001; Asaka and Shoda 1996; Leifert *et al.*, 1995; Ferreira *et al.*, 1991). However, most studies have focussed primarily on the degree of disease reduction and mechanisms of suppression in soil have not been as extensively investigated.

In this work, experiments were conducted in two different pathosystems to test the ability of one particular *B. subtilis* strain, M4, to trigger some resistance in plants. We also wanted to further characterise the enhanced resistance observed with regards to PR protein accumulation and to ISR-related gene transcripts differentially expressed in plants exposed to these bacteria.

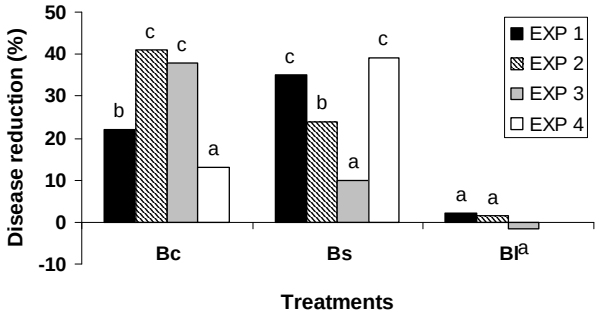


**Figure 1.** Reduction of disease caused by *Colletotrichum lagenarium* on cucumber plants following treatment at the root level with either vegetative cells of *Bacillus subtilis* M4 (Bc), spores (Bs) or semi-purified lipopeptides (Bl) from the same strain. For each independent experiment, 20 plants were used for each treatment and each individual plant was challenged with 12 7 µL-drops of the pathogen conidia suspension, thus representing a total of 240 infection sites per treatment. Disease reduction percentage was calculated by the formula (%) = 100(1−x/y) in which x and y are the total number of lesions on the leaves of treated and untreated control plants, respectively. Data from each independent experiment were subjected to analysis of variance (ANOVA). Means from the different treatments were compared by the method of Newman and Keuls (least significant difference at α = 0.05). Bars marked with different letters represent mean values that are statistically different but those marked with a do not differ significantly from the control

## Results

*Reduction of cucumber anthracnose caused by* Colletotrichum lagenarium

The protective effect of *B. subtilis* M4 was evaluated on 2-week-old cucumber plants after inoculation of the strain at the root level. Pathogen infection was assessed on the basis of lesion formation typically induced by *Colletotrichum lagenarium* on cotyledons, and disease level was rated in terms of the percentage of lesions that clearly developed and grew out of the inoculum drop zone. Under our conditions, disease incidence in control plants varied from

approximately 55% to 65% in the different assays. Results from three out of four independent experiments revealed a statistically significant disease reduction in plants treated with either vegetative cells or endospores, while semi-purified lipopeptides failed to provide any protection (Figure 1). Mean protection levels calculated from pooled results of all experiments were of 29% and 27% following treatment with vegetative cells and spores, respectively. In order to evaluate both local and systemic colonisation of plants by PGPR strains, root and leaf samples were randomly collected at the end of two independent experiments. Cell populations of *B. subtilis* M4 were 3.6 ± 1.1 x $10^6$ cfu/g and 3.0 ± 0.35 x $10^6$ cfu/g on roots from plants treated with either vegetative cells or endospores, respectively. M4 colonies were not detected in leaf samples, with a detection limit of about 100 cfu/g leaf tissue, suggesting that bacteria did not migrate through the plants.

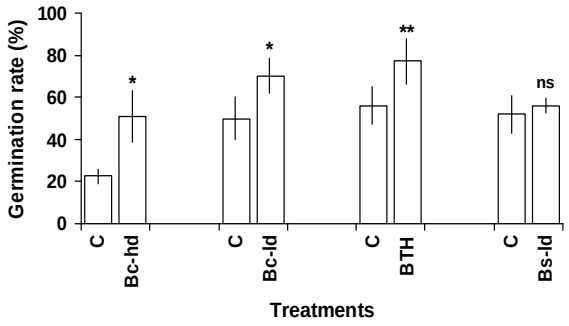


**Figure 2.** Protection against *Pythium aphanidermatum* damping-off of tomato offered by pre-treatment of seeds with either *B. subtilis* M4 or benzothiadiazole (BTH). Different treatments (see Materials and methods): C Untreated seeds, Bc-hd seeds treated with vegetative cells of *B. subtilis* M4 and sown under high disease pressure (*P. aphanidermatum* introduced in the soil at $10^5$ cfu/g soil dry weight), Bc-ld seeds treated with vegetative cells of *B. subtilis* M4 but tested for germination under low disease pressure (pathogen at 5 x $10^3$ cfu/g soil dry weight), BTH seeds pre-treated with a solution of 1 mM BTH and sown under low disease pressure, Bs-ld seeds treated with endospores of *B. subtilis* M4 and tested for germination under low disease pressure. In all cases, seedling emergence rates were determined by using 200 ± 3 seeds. Data presented are mean values and standard deviations calculated from five independent experiments with each treatment, except BTH treatment for which four experiments were performed. Means were also statistically compared using the Fisher's least significant difference (LSD) test. * and ** indicate significant differences (respectively, at $\alpha$ = 0.001 and 0.01) inseedling emergence between M4-treated or BTH-treated seeds and the corresponding untreated seeds used as controls

*Protection against* P. aphanidermatum *damping-off of tomato seedlings*

Pre-treatment of tomato seeds with vegetative cells of strainM4 significantly reduced damping-off caused by *P. aphanidermatum* (Figure 2). Interestingly, this beneficial effect was more marked upon high disease pressure conditions (less than 25% plantlet survival rate) compared to lower disease incidence levels. When compared to control seeds, germination rate increased by about 130% and 40% under high and low disease pressure, respectively. The germinability

of seeds under assay conditions, in the absence of pathogen and without any *Bacillus* treatment, ranged from 93% to 98% depending on the experiment. Bacterial treatment was effected by incubating tomato seeds for 24 h in the presence of strain M4. At the end of this period, seeds were extensively washed with water and chloramphenicol to eliminate the bacteria. The absence of M4 on the seeds obtained after this washing process was checked by plate count before sowing and no colony with morphology typical of M4 could be observed (detection limit about 100 cfu/g seeds). The protection level offered by seed treatment with vegetative cells at low disease pressure was also similar to that obtained with the SAR-inducing chemical BTH used at a concentration of 1 mM (Figure 2). However, incubation of tomato seeds in the presence of *Bacillus* endospores failed to yield any significant reduction of damping-off caused by *P. aphanidermatum*.

**Discussion**

This study shows that the non-pathogenic isolate *B. subtilis* M4 can stimulate a systemic defence response in cucumber and tomato leading to protection against *C. lagenarium* and *P. aphanidermatum*, respectively. In the case of cucumber, experiments were performed with plants pre-inoculated at the root level with bacteria before challenge with the pathogen on cotyledons. Disease suppression was, thus, undoubtedly due to induction of resistance in the host plant since colonisation studies confirmed that PGPR and pathogen remained localised on different plant organs and that bacterial populations established on roots were above the threshold necessary to trigger ISR (Raaijmakers *et al.*, 1995). In the other pathosystem studied, we also demonstrated that the beneficial strain and the pathogen *P. aphanidermatum* were never in direct contact and, thus, disease suppression could also be attributed to induction of resistance in tomato plants. Root or seed pre-inoculation with strain M4 most likely sensitised both cucumber and tomato plants to react more efficiently to subsequent pathogen infection.

The protective effect of *B. subtilis M4* was obtained in both pathosystems by inoculating seeds with vegetative cells, but biocontrol assays on cucumber also revealed that root treatment with endospores was as effective at reducing disease incidence as compared to cells. Moreover, similar disease control levels induced by treatment with spores and living cells from strain M4 were recently observed in our laboratory in challenge experiments performed on bean plants infected with the leaf pathogen *Botrytis cinerea* (*unpublished results*). From a technological point of view, the efficacy of endospores is interesting since they are more stable than vegetative cells and maintain viability for years under appropriate product storage conditions. Endospores are also tolerant to extreme pH values, far more resistant to drying processes for

powder formulation, and relatively easy to produce with industrial fermentation technology (Brannen and Kenney, 1997).

As lipopeptides of the iturin and fengycin families represent important bioactive metabolites produced in large quantities by strain M4 (Jacques *et al.*, 1999), these molecules were also tested for their ISR-inducing activity. However, semi-purified lipopeptide extracts failed to confer any protection to cucumber plants, showing that they are not involved in the resistance triggering process. The bacterial determinant responsible for ISR induction in the case of M4 thus remains to be isolated and identified among the widevariety of metabolites that are excreted by the bacterium oramong their cell envelope components. Furthermore, observations suggest that no heat-stable envelope component(s) play a major role in the ISR induction process since *B. subtilis* endospores were unable to induce a resistance response after selective heat-treatment.

## Materials and methods

*Microbial strains and inoculum preparation*

*B. subtilis* strain M4 (commercial name; formerly named S499) was isolated from soil and originally selected for the production of lipopeptides (Akpa *et al.*, 2001; Jacques *et al.*, 1999). The bacterium was maintained on plate count agar (PCA) medium (Difco, Detroit, Mich.) at 4°C before experimental use. For long-term storage, it was conserved at −80°C in cryotubes according to the manufacturer's recommendations (Microbank, Prolab Diagnostic, Richmond Hill, Canada). For the preparation of bacterial inocula used in biocontrol assays with vegetative cells, the *Bacillus* strain was grown at 30°C for 24 h in the liquid medium defined by Jacques *et al.* (1999). Cells were harvested by centrifugation at 15,000 g for 20 min and the cell pellet was washed twice with sterile saline water (0.85% NaCl). Vegetative cell suspensions were then diluted in order to obtain the desired bacterial concentration for plant treatment. Bacterial spore suspensions were prepared from 72-h-old cultures of *B. subtilis* M4 in the same way as vegetative cell suspensions. Spores were selectively recovered after incubation at 80°C for 12 min and centrifugation. They were resuspended in sterile distilled water to obtain the final desired concentration. Spore concentration of the prepared propagules was measured by plate count on PCA medium of an aliquot of the heat-treated culture broth.

The fungal pathogen *Colletotrichum lagenarium* used for cucumber infection was maintained on potato dextrose agar (PDA) medium (Difco) at room temperature in the dark. For long-term storage, conidia were conserved in 40% glycerol at −80°C. The suspension used for plant infection was prepared by harvesting spores from 4-week-old cultures in sterile peptone water containing 0.01% Tween 80. After removing mycelial debris by filtration through several layers of cheese cloth, the suspension was centrifuged for 5 min at 5,000 g and the spores were resuspended in 0.4% glucose solution supplemented with 0.013 M $KH_2PO_4$ to a final concentration of 2 x $10^7$ spores/mL. The origin of *Pythium aphanidermatum*, its maintenance and the preparation of suspensions used for challenge of tomato plants have been described previously (Ongena *et al.*, 2000).

*Biocontrol assays with cucumber*

All ISR assays were performed with cucumber (*Cucumis sativus*) cultivar Raider. Prior to planting, cucumber seeds were disinfected in 70% ethanol for 1 min and rinsed three times with sterile distilled water. Seeds were then soaked for 10 min in a bacterial suspension at a concentration of approximately 4 x $10^8$ cfu/mL in 0.85% NaCl. This concentration was used for treatment with both spores and vegetative cells. Control seeds and seeds treated with *Bacillus* lipopeptides were soaked in 0.85% NaCl

or in a 1 µM solution of semi-purified lipopeptides, respectively, for the same period of time. The lipopeptides were purified from the culture supernatant by strictly following the procedure described by Razafindralambo *et al.* (1993). Cucumber seeds were sown in 10-cm pots containing sterilised potting soil (Brill Substrate, Georgsdorf, Germany) previously mixed with bacterial inoculum to a final concentration of 3 x $10^7$ cfu/g or with an equal volume of sterile water for untreated control plants. Cucumbers were germinated at 25 ± 2°C in the greenhouse with a 16-h photoperiod. At 6 and 12 days after sowing, 20 mL bacterial suspension at $10^8$ cfu/mL was added as a drench to the roots of each plant except the controls (watered with 20 mL 0.85% NaCl). After approximately 10 days, cucumber plants were transferred to a high humidity chamber (19 ± 2°C) for 24 h before leaf-infection with *Colletotrichum lagenarium*. Each cotyledon was inoculated with six 7 µL-drops of the pathogen spore suspension. Disease incidence was scored 10 days later and was expressed in terms of the percentage of *Colletotrichum lagenarium* lesions that clearly grew out of the inoculum drop area to produce spreading lesions. Experiments were carried out four times and contained 20 plants per treatment.

*Biocontrol assays with tomato*

Tomato seeds were treated with *B. subtilis* before sowing as follows. Seeds (*Lycopersicon esculentum* L. cv. Merveille des Marchés) were washed three times (for 5 h each) with sterile distilled water and placed on sterile filters in 9-cm Petri dishes. The different treatments were realised by adding 3 mL of either sterile distilled water (controls), bacterial cell suspension at 5 x $10^8$ cfu/mL (prepared as described above), spore suspension at the same concentration, a 1 µM solution of semi-purified lipopeptides, or 1 mM benzo [1,2,3]thiadiazole-7-carbothioic acid-S-methyl ester (BTH, commercialised under the trade-name BION; Syngenta, Münchwilen, Switzerland). The plates were sealed with Parafilm and incubated for 24 h at 25°C. Seeds were then washed once with sterile distilled water, dipped for 5 min in 0.25% chloramphenicol under constant agitation and again washed three times with sterile water. They were then dried for at least 2 h in a laminar air-flow cabinet at room temperature. The absence of *B. subtilis cells* on treated seeds to be sown was checked as follows. About 0.2 g seeds was vigorously shaken in glass tubes containing 10 mL sterile peptone water [1 g/L bacto-peptone; 9 g/L NaCl; 0.02% (v/v) Tween 80] and 1 g glass beads (0.18 mm diameter). Serial dilutions were plated on PCA medium. *Bacillus* colonies were counted after incubation for 36 h at 30°C on the basis of their typical morphology.

In every experiment, 0.50 ± 0.01 g seeds from each treatment (representing 200 ± 3 individuals) were sown in large plastic trays containing the same soil as used for cucumber but previously infected with *P. aphanidermatum* by mixing with a suspension of mycelial fragments. Final concentrations of the pathogen in the substrate for plant growth were 5 x $10^3$ cfu/g and $10^5$ cfu/g soil dry weight for assays with low and high disease pressure, respectively. The trays were incubated in a growth cabinet set to maintain the temperatureat 28°C, a 95%-relative humidity and a photoperiod of 16 h. Seedling emergence was recorded after 10 days. Experiments with each treatment were repeated five times except for BTH treatment for which four experiments were performed.

## Acknowledgements

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# *Chapitre VIII. Surfactin and fengycin lipopeptides of* **Bacillus subtilis** *as elicitors of induced systemic resistance in plants*

*Environmental Microbiology* **9**(4): 1084-1090.

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**Résumé**

Dans le chapitre précédent, nous avons démontré que *Bacillus subtilis* pouvait stimuler l'ISR chez la tomate et le concombre. Toutefois, les essais réalisés avec un mélange de lipopeptides de S499 suggéraient qu'ils n'étaient pas impliqués dans le phénomène chez le concombre. Par contre, lors de recherches précédentes menées sur des tubercules de pomme de terre, une accumulation de certains composés phénoliques impliqués dans les réactions de défense chez les plantes a été mise en évidence après le traitement par des fengycines (Ongena *et al.*, 2005b). Cette observation laissait entrevoir un possible effet inducteur d'une réponse défensive chez d'autres plantes pour ces LPs. L'objectif des recherches présentées dans ce chapitre était donc de tester l'activité élicitrice de l'ISR des lipopeptides, et en particulier de la surfactine et de la fengycine, chez le haricot et la tomate.

Grâce au traitement des racines avec les lipopeptides de S499 purifiés et à une concentration de 5 µM, nous avons montré le potentiel de la surfactine, et dans une moindre mesure celui de la fengycine, à protéger ces deux plantes contre *Botrytis cinerea* avec une efficacité similaire à celle de la souche productrice. L'hypothèse d'une implication des LPs dans la stimulation de l'ISR a par la suite été vérifiée via l'utilisation de dérivés surproducteurs de la souche *Bacillus subtilis* 168, qui naturellement ne produit que très peu de LPs. La surexpression des gènes de biosynthèse de l'un ou l'autre lipopeptide dans la souche 168 est associée avec une réduction significative de la maladie chez le haricot et la tomate.

Toujours à l'appui de l'activité élicitrice de l'ISR des LPs, la protection macroscopique conférée par le mutant surproducteur de surfactine et de fengycine a été associée, au niveau métabolique, avec une stimulation de certaines enzymes connues pour intervenir dans les réponses au stress provoquées par l'attaque de pathogènes ou par des blessures. Ainsi, même si aucune différence n'est remarquée avant infection, une nette augmentation des activités lipoxygénase (LOX) et lipide hydroperoxydase (HPL) par le mutant surproducteur est observée deux et quatre jours après l'infection par le pathogène, suggérant une stimulation du métabolisme des oxilipines par la surfactine et/ou la fengycine.

Globalement, nous démontrons dans ce chapitre pour la première fois l'implication des LPs comme agents stimulateurs de l'ISR, permettant ainsi de leur attribuer un nouveau rôle dans le biocontrôle.

**Abstract**

Multiple strains of *Bacillus* spp. were demonstrated to stimulate plant defence responses. However, very little is known about the nature of molecular determinants secreted by these Gram-positive bacteria that are responsible for the elicitation of the induced systemic resistance (ISR) phenomenon. This study shows that the lipopeptides surfactins and fengycins may be involved in this elicitation process. In bean, pure fengycins and surfactins provided a significant ISR-mediated protective effect on bean plants, similar to the one induced by living cells of the producing strain S499. Moreover, experiments conducted on bean and tomato plants showed that overexpression of both surfactin and fengycin biosynthetic genes in the naturally poor producer *Bacillus subtilis* strain 168 was associated with a significant increase in the potential of the derivatives to induce resistance. In tomato cells, key enzymes of the lipoxygenase pathway appeared to be activated in resistant plants following induction by lipopeptide overproducers. To our knowledge, such lipopeptides constitute a novel class of compounds from non-pathogenic bacteria that can be perceived by plant cells as signals to initiate defence mechanisms.

**Introduction**

Members of the *Bacillus* genus are among the beneficial bacteria mostly exploited as biopesticides to control plantdiseases (Fravel, 2005). Their protective effect may rely on different mechanisms to directly antagonize pathogengrowth (Haas and Défago, 2005; Zahir *et al.*, 2004). In this context, *Bacillus subtilis* produces a variety of bioactive metabolites that could be involved in antibiosis among which lipopeptides from the surfactin, iturin and fengycin families (Bonmatin *et al.*, 2003; Peypoux *et al.*, 1999). These amphiphilic cyclic peptides are composed of seven (surfactins and iturins) or 10 α-amino acids (fengycins) linked to one unique β-amino (iturins) or β-hydroxy (surfactins and fengycins) fatty acid. The length of this fatty acid chain may vary from $C_{13}$ to $C_{16}$ for surfactins, from $C_{14}$ to $C_{17}$ for iturins and from $C_{14}$ to $C_{18}$ in the case of fengycins. Different homologous compounds for each lipopeptide family are thus usually co-produced (Akpa *et al.*, 2001). Biosynthesis of these molecules involves non-ribosomal peptide synthetases (NRPS), as well as fatty acid (FAS) and polyketide synthases (PKS) in a thiotemplate mechanism (Finking and Marahiel, 2004).

Beside direct antagonism, some beneficial bacteria can protect plants indirectly through the stimulation of inducible defence mechanisms that render the host more resistant to further pathogen ingress. Such induction of enhanced defensive capacity can be systemic as root

treatment with bacteria was shown to trigger protective effects on above-ground plant parts. This phenomenon isreferred to as “induced systemic resistance” (ISR), and is effective against a broad range of diseases (Van Loon *et al.*, 1998). Induced systemic resistance is phenotypically similar to the well-studied systemic acquired resistance (SAR) activated after a first infection by an incompatible necrotizing pathogen (Durrant and Dong, 2004). Many of the ISR-inducing microbes identified so far are Gram-negative bacteria and more particularly species belonging to the *Pseudomonas* and *Serratia* groups. However, the list of *Bacillus* strains reported as ISR inducers has grown rapidly over the last decade and includes members of the *Bacillus pumilus*, *B. mycoides*, *B. subtilis*, *B. amyloliquefaciens*, *B. pasteurii*, *B. thuringiensis* or *B. cereus* species (Kloepper *et al.*, 2004). Some plant defence responses were associated with the beneficial effect provided by these strains but much remains to be discovered about the molecular aspects of the *Bacillus*-mediated ISR. Until recently, volatile organic compounds and more particularly 2,3-butendiol were the sole determinants for elicitation identified from *Bacillus* spp. (Ryu *et al.*, 2004).

In this work, we provide strong evidence for plant resistance-eliciting activity of fengycin- and surfactin-type lipopeptides. It is demonstrated by combining two different approaches and by showing that the macroscopic disease reduction is related to metabolic changes associated with plant defence responses. Iturins were not considered in this study because they are probably not active as demonstrated by the inability of an overproducing derivative of *Bacillus subtilis* strain 6633 (Leclère *et al.*, 2004) to provide any consistent ISR effect (Ongena, *unpublished* results).

## Results and discussion

### *Protective effect of pure lipopeptides*

In two recent studies, we have demonstrated the potential of *B. subtilis* strain S499, an efficient lipopeptide producer, at inducing an elevated state of resistance in various plants (Ongena *et al.*, 2005a,b). Here, by using *Phaseolus vulgaris* (bean) as host plant and *Botrytis cinerea* as challenging phytopathogen, we first wanted to test the role of lipopeptides by comparing the protective activity afforded by purified compounds with the one provided by the producing S499 strain. Induced systemic resistance assays were performed following the method described by Ongena and colleagues (2002). Briefly, in experiments with living cells, sterilized seeds were dipped for 10 min in a bacterial suspension prior to sowing. Bean plants were then grown in sterilized potting soil previously mixed with cells [$10^7$ colony-forming units

(CFU)/g]. Additionally, 20 mL of a bacterial suspension was added to the roots of each plant 7 days after sowing. In experiments with lipopeptides, seeds were germinated in the sterilized soil and plants were treated after 5 days and 11 days by adding as a drench 20 mL of a 5 mM aqueous solution of surfactins or fengycins.

Lipopeptides were obtained from AIBI (Agricultural Universityof Gembloux, Belgium) as a mixture of homologues with a purity of 95%. The relative proportions of the different homologues in the two families were established by the combined use of matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry and reversed-phase high-performance liquid chromatography (HPLC) as described in Table 1 (surfactins: A $C_{13}$, 22%; B $C_{13}$, 17%; A $C^{14}$, 28%; A $C_{15}$, 33%; and fengycins: A $C_{14}$, 4%; A $C_{15}$, 13%; A $C_{16}$, 32%; A $C_{17}$, 30%; $AC_{18}$, 7%; B $C_{17}$, 14%). Mean values of molecular weights of the different homologues present in mixtures were used to prepare the desired concentrations: 1460 amu in the case of fengycins and 1050 amu in the case of surfactins. After 14 days, plants were infected with *B. cinerea* as described in Table 1. Experiments contained 15 plants per treatment and were repeated three times.

Results revealed statistically significant disease reduction following the treatment with pure surfactins (28%) but not with fengycins (14%) as compared with control plants. The level of disease control provided by surfactins is similar to the one observed by treating roots with cells of the *B. subtilis*-producing strain S499 (33%, see legend of Figure 1 for statistical treatment of data). Colonization of roots by S499 and possible migration through the plant were estimated by using root or leaf samples as previously described (Ongena *et al.*, 2005b). S499 colonies were counted on the basis of their typical morphology at a concentration of $2.5 \pm 1.8 \times 10^5$ cfu per gram of rhizosphere. Some of these colonies were randomly selected and tested positively for lipopeptide production on the basis of haemolytic activity on a blood sheep-containing medium. In contrast, *Bacillus* isolates were not detected in leaf samples with a detection limit of about 100 cfu per gram of leaf tissue, suggesting that the bacteria did not migrate through the plants. Disease suppression is actually due to induction of resistance in the host plant as the beneficial strain and the pathogen remained localized on different plant organs and as bacterial populations established on roots are in the range of the threshold necessary to trigger ISR (Raaijmakers *et al.*, 1995). At least in the case of surfactin, direct antagonism of pathogen growth at the sites of penetration can be ruled out as these molecules are not self-antifungal (Maget-Dana *et al.*, 1992). In contrast, fengycins are clearly fungitoxic (Vanittanakom *et al.*, 1986) but HPLC analysis of methanolic extracts from leaf tissues showed that these compounds or the producing strains do not migrate through the plant from inoculated roots to the infected leaves.

Despite several attempts, we did not succeeded in transforming the S499 strain and the selection of derivatives impaired in the synthesis of lipopeptides was therefore not possible. We thus used an alternative approach to provide further evidence for the involvement of surfactin and fengycin in ISR elicitation by testing various lipopeptide-overproducing derivatives generated from the wild-type *B. subtilis* 168 (Bs168), a naturally poor producer.

**Table 1.** Lipopeptides amounts produced by derivatives of *Bacillus subtilis* 168 (Bs168) after growth for 72 h in the synthetic optimized medium described by Jacques and colleagues (1999).

| **Strain**[a] | **Genotype** | **Surfactins mg/L**[b] | **Fengycins mg/L**[b] |
|---|---|---|---|
| Bs168 | *trpC2 sfp°* [d] | t[c] | t |
| Bs2500 | *trpC2 sfp*$^{+}$ [e] | 750 | t |
| Bs2508 | *trpC2 sfp*$^{+}$ *pamyQ-ppsa* [f] | 697 | 434 |
| Bs2504 | *trpC2 sfp*$^{+}$ *pamyQ-ppsa ΔsrfABΔsrfACΔsrfAD* [g] | t | 452 |

**a.** The three mutants of strain Bs168 were obtained following the method developed by Brans and colleagues (2004) allowing the eviction of the selectable marker used for screening of recombinant isolates. Briefly, the Bs2500 derivative was generated by inserting a functional *sfp* gene from *B. subtilis* S499 at the *amyE* locus into the chromosome of 168. The Bs2508 derivative was obtained by replacement of the weak fengycin promoter (*pfen*) by the strong *amyQ* promoter. Bs2504, a surfactin non-producer derivative of Bs2508, was obtained by interrupting the *srfA* operon (Appendix S1 in *Supplementary material*).

**b.** Supernatant samples were loaded on C18 solid-phase extraction cartridges (900 mg, Alltech) and lipopeptides were desorbed with 100% ACN. The resulting samples were analysed by reverse-phase HPLC on a LiChrospher 100 RP C-18 column using the isochratic mode (respectively 62% and 80% acetonitrile in water acidified with 0.1% trifluoroacetic acid for fengycins and surfactins) at a constant flow rate of 1 mL/min. Compounds were identified on the basis of their retention times compared with purified standards and the amounts were calculated on the basis of the corresponding peak area at 214 nm. The identity of each homologue was confirmed by using matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry. Aliquots (10 µL) of the lipopeptide extracts used for HPLC were mixed in 90 µL of a saturated solution of α-cyano-4-hydroxycinnamic acid before laser bombardment. Data are mean values from two experiments.

**c.** t means that the corresponding lipopeptide was only detected in trace amounts not sufficient for reliable quantification.

**d.** *sfp°* means that the *sfp* gene of *B. subtilis* 168 lead to a truncated form of the 4′ phosphopantetheinyl transferase involved in the thiotemplate biosynthesis.

**e.** *sfp+* means that the *sfp* gene from *B. subtilis* S499 was integrated into the *amyE* gene of the recipient strain.

**f.** *pamyQ-ppsa* means that the fengycin operon was placed under the control of the amylase promoter from *Bacillus amyloliquefaciens*.

**g.** *ΔsrfABΔsrfACΔsrfAD* means that the surfactin operon was inactivated by elimination of these genes.

*Lipopeptide overproduction by derivatives of Bs168*

The sequencing and annotation of Bs168 genome has revealed that the bacterium retains mostly functional *srfA* and fen genes as responsible for biosynthesis of surfactins and fengycins respectively (Kobayashi *et al.*, 2003; Kunst *et al.*, 1997). However, the strain is impaired in lipopeptide production due to its mutation in the 4′phosphopantetheinyl transferase gene *sfp* (Tsuge *et al.*, 1996). In this study we used three mutants of strain Bs168 that were constructed to produce specific amounts of each lipopeptide type (Table 1). As revealed by HPLC analysis of culture supernatant extracts, the Bs2500 derivative produces high levels of surfactin but still

very low amounts of fengycins upon growth in an optimized medium (Table 1). Replacement of the fengycin promoter in Bs2500 by a stronger promoter sequence yielded Bs2508 that efficiently produces both surfactins and fengycins in final concentrations higher than 400 mg/L. The third derivative used in this study, Bs2504, also secretes fengycins in large amounts at a level similar to Bs2508 but is impaired in surfactin production (Table 1). Beside the peaks corresponding to lipopeptides, no significant changes were observed in HPLC profiles of the different isolates and wild type upon analysis of concentrated culture supernatant with photodiode array detection. Final biomass concentrations and growth kinetics were also similar for both isolates (data not shown).

*Resistance induced by overproducing mutants*

The wild-type strain Bs168 was shown ineffective at conferring any protection but significant disease reductions as 22% and 27% were observed in bean plants treated, respectively, with the surfactin overproducer Bs2500 and with Bs2508 which overexpress both surfactins and fengycins (Figure 1). The derivative Bs2504 producing only fengycins also induced some resistance in bean plants but to a lower and not statistically significant extent (14%). Higher levels of protection were also induced by Bs2500 and Bs2508 in tomato plants against *Botrytis* disease (Figure 1). Average disease reductions of, respectively, 36% and 43% were observed from pooled results of three independent experiments. A significant albeit lower protective effect (25%) was also provided by treatment with the Bs2504 derivative on tomato.

The colonization rate of Bs2508 was estimated by the use of a spectinomycin-resistant derivative. This derivative was obtained by using the plasmid pDG1731 with homologous recombination at the *thrC* locus (Guérout-Fleury *et al.*, 1996) and we followed the methoddescribed by Anagnostopoulos and Spizizen (1961) for transformation. On the basis of both typical morphology and antibiotic resistance, populations of $5.3 \pm 2.35 \times 10^5$ cfu/g and $3.2 \pm 1.1 \times 10^5$ cfu/g were counted on bean roots, respectively, 8 days after sowing and at the time of pathogen challenge. Bs2508 cells were observedat a concentration of $3.0 \pm 2.1 \times 10^5$ cfu/g in the tomato soil at the time of challenge. It is thus obvious that the Bs2508 derivative readily establishes and maintains on plant roots but does not migrate to the leaf tissues where it was not detected. In addition, rhizosphere samples were collected at the end of the experiments and MALDI-TOF analyses of methanolic extracts showed the presence of $C_{15}$ surfactin. This suggests that *Bacillus* cells growing on roots are able to efficiently synthesize lipopeptides although it remains to be established whether the amounts produced in situ are sufficient or not to activate the plant.

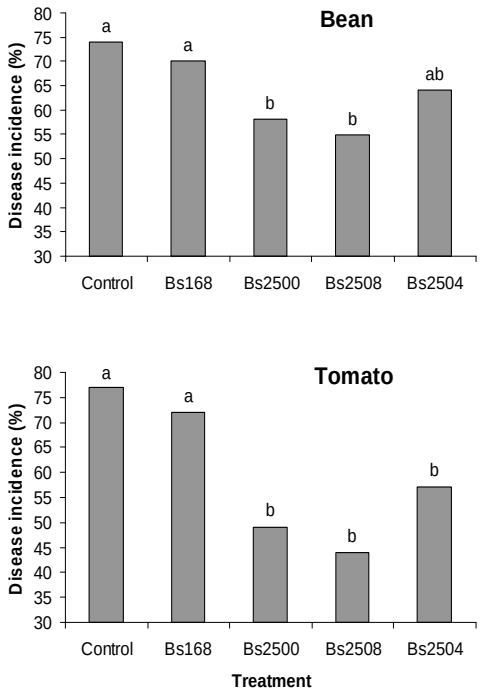


**Figure 1.** Reduction of disease caused by *Botrytis cinerea* on leaves of either bean and tomato following treatment at the root level with derivatives of the wild-type *Bacillus subtilis* strain 168 (Bs168). The effect of strains must be compared with untreated plants designated as Control. Bs2500, Bs2504 and Bs2508 are mutants of Bs168 that overproduce surfactin, fengycin and both respectively. Bacterial inoculum and pathogen spore suspension were obtained as described in Ongena and colleagues (2005a). In the case of bean, 15-day-old plants were challenged on first true leaves with 10 mL drops of a pathogen spore suspension at $10^6$ spores/mL. Five-week-old tomato plants were pathogen-challenged on third leaves with six 5 mL drops of a suspension containing $10^5$ spores mL. Data are means of three independent experiments using 20 plants (bean) or 40 plants (tomato) per treatment. Disease incidence represents the sum of spreading lesions developing on each individual plant within the same treatment reported to the total number of infection sites. Data (infection score for each individual plants) from every independent experiment were analysed for homogeneity of variances by ANOVA and data from experiments with the same set-up were pooled for analysis as interactions between experiment and treatment were not significant at $P = 0.05$. Means from the different treatments were compared by the method of Newman and Keuls (least significant difference at $\alpha = 0.05$) and bars marked with 'a' are not statistically different from the controls.

Despite similar global structures, surfactins and fengycins differ in some aspects regarding their biologicalactivities. As mentioned above, fengycins display a strong antifungal activity and may thus play a dual role in plant disease reduction. These compounds can act by enhancing host defensive capacity but also through direct microbial antagonism as we previously demonstrated (Ongena *et al.*, 2005b). Surfactins are not fungitoxic but has much stronger haemolytic, antibacterial, antiviral and antitumour properties (Peypoux *et al.*, 1999). Most of their biological activity may be related to their effect on the lipid part of biological membranes. Because of their detergent-like structure, these molecules can readily associate and tightly anchor into lipid layers. This process is facilitated by their amphiphilic nature featuring two charged sidechains protruding into the aqueous phase and apolar moieties reaching into the hydrophobic core of the membrane (Deleu *et al.*, 2003; Heerklotz and Seelig, 2001). However, the presence of surfactin at a 5 mM concentration was seemingly not detrimental for the integrity of bean root cells as no toxic effect on plant health was observed in our ISR assays. Moreover, recent experiments conducted in the laboratory showed that tobacco cells in suspension were also not affected in their viability (as revealed by Evans blue staining) and growth potential (similar increase in biomass fresh weight over a 36 h period as compared with

untreated cells) upon addition of these lipopeptides to a 10 mM concentration (Jourdan, *unpublished results*). However, surfactin could insert into the phospholipid bilayer thereby creating some disturbance or channelling in the plasma membrane that can in turn activate a cascade of molecular events leading to defensive responses. Fengycins also readily interact with lipid layers and likewise may somewhat alter cell membrane structure and permeability (Deleu *et al.*, 2005). However, this activity is reduced compared with surfactins and it can explain the lower protective effect of the fengycin overproducing derivative.

*Stimulation of lipoxygenase and lipid hydroperoxidase activities in induced tomato plants*

Lipoxygenase (LOX) introduces molecular oxygen to unsaturated linolenic and linoleic acids to yield either 9- or 13-hydroperoxides which can in turn be used by various enzymes to generate a wide array of biologically active secondary metabolites (Blée, 2002). We already demonstrated that stimulation of this so-called oxylipin pathway was related to ISR induced in bean by *Pseudomonas putida* BTP1 (Ongena *et al.*, 2004). In this study we wanted to evaluate whether the two key enzyme activities of this metabolic route could also be stimulated in tomato in response to treatment with the lipopeptide-overproducing *Bacillus* isolates. Analyses revealed significant increases in LOX activity in infected leaves of plants previously bacterized at the root level with Bs2508 compared with control plants and with those treated with the wild-type Bs168. Threefold increases in LOX activity were measured in the first 48 h after *Botrytis* inoculation (Figure 2A). This was observed concomitantly with an increase in the global activity of all hydroperoxide-degrading enzymes expressed as lipid hydroperoxidase (LHP) activity. This response was less obvious than in the case of LOX but significant 1.6-fold higher LHP activities were observed in leaves of plants pre-treated with Bs2508 (Figure 2B). These enhanced activities were maintained in elicited plants for at least 96 h.

Moreover, a clear accumulation of non-polar antifungal compounds also occurred in the infected leaves of plants bacterized with lipopeptide overproducers (data not shown). Establishing whether these accumulating compounds are related to the oxylipin metabolic route or not is beyond the scope of this work. However, all these changes at the molecular level are in support to the plant defence-inducing activity of *Bacillus* lipopeptides. We also recently observed major changes in the phenolic pattern of tobacco upon treatment with these molecules. The addition of surfactin (10 µM) to tobacco cell suspension cultures led to a increase in the concentrations of cinnamic acid (threefold as compared with controls) and benzoic acid (sixfold) after 6–10 h. It was concomitant with a strong accumulation of free salicylic acid, a marker of induced systemic resistance, which was not detected in the culture of untreated cells at any time (Jourdan, *unpublished results*). Other unidentified compounds

specifically appeared in the culture supernatant of surfactin-treated cells. These results strongly suggest induction of the phenylpropanoid metabolism, which is stimulated concomitantly with the activation of plant defence reactions against pathogen infection or after other physical ingresses such as wounding (Dixon *et al.*, 2002).

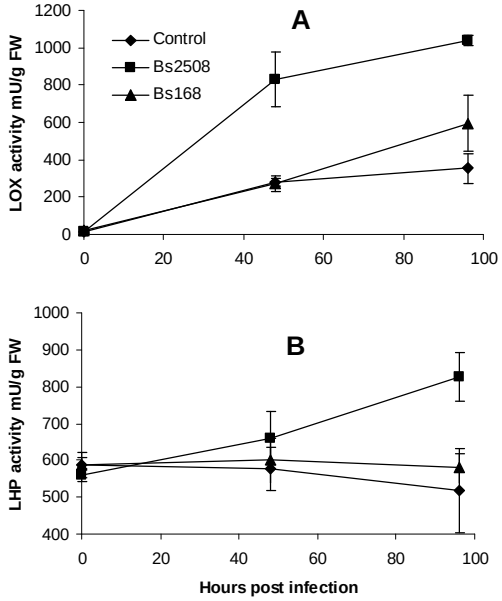


**Figure 2.** Time-course evolution of lipoxygenase (LOX, A) and lipid hydroperoxidase (LHP, B) activities in tomato plants previously treated with the wild-type *B. subtilis* Bs168 and its Bs2508 derivative overproducing surfactin and fengycin lipopeptides compared with control plants. Extracts were prepared from third leaves collected just before inoculation of the pathogen (0 h) and 2 days (48 h) and 4 days (96 h) after challenge. Lipoxygenase activity was determined spectrophotometrically on the basis of linolenic acid consumption. Lipid hydroperoxidase activity represents the activity of all hydroperoxide-degrading enzymes monitored by decrease of absorbance at 234 nm (Ongena *et al.*, 2004). Data are means and standard deviations calculated from three measurements performed at every sampling time in three independent experiments. FW, fresh weight.

## Conclusion

According to our knowledge, *Bacillus* lipopeptides constitute a novel class of compounds that can be perceived by plant cells as signals to activate defence mechanisms. Whether some amino acid sequences may represent epitopes perceived by specific receptors in the membrane of root cells or not is questionable. None of the pathogen-associated molecular patterns identified in the interaction of pathogenic microorganisms with eukaryotic cells resembles the structure of surfactins or fengycins (Nürnberger *et al.*, 2004). Moreover, there is no partial sequence shared by the peptide part of these two lipopeptides nor is there with other peptide products retaining ISR-eliciting activity (Meziane *et al.*, 2005; Gómez-Gómez, 2004). Syringopeptins and syringomycins are lipodepsipeptides produced by the phytopathogenic bacterium *Pseudomonas syringae* that are involved in plant–microbe interactions. They share a similar global structure with *Bacillus* lipopeptides. However, these lipodepsipeptides are considered as virulence factors and display a strong phytotoxic activity centred on an ability to form pores in plant plasma membranes, thereby inducing cell death (Dalla Serra *et al.*, 1999).

In contrast, surfactins and fengycins are not toxic for plant cells at the concentrations used and thus should act by a different way to activate the inducible defence responses. By testing multiple naturally co-produced or chemically synthesized homologues, additional experiments will be conducted to appreciate the relative importance for ISR elicitation of specific structural traits of *Bacillus* lipopeptides such as the length of the fatty acid chain or the nature of amino acid residues in the peptide sequence.

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# *Chapitre IX. Surfactin-type lipopeptides as MAMP for non-pathogenic* **Bacillus** *perception and defence responses elicitation in plant cells*

*Molecular Plant-Microbe Interactions* (Submitted)

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**Résumé**

Dans les chapitres précédents, nous avons démontré que la surfactine et dans une moindre mesure la fengycine, toutes deux produites par *Bacillus subtilis* S499 étaient impliquées dans l'établissement de l'ISR contre *Botrytis cinerea*. De plus, les analyses des réponses induites dans la plante avaient montré une stimulation de la lipoxygénase dans les feuilles des plants de tomate traités. Toujours dans l'optique de mieux comprendre les interactions entre les cellules des plantes et les éliciteurs bactériens, nous avons logiquement entrepris lors des travaux présentés dans ce chapitre, d'entamer une première caractérisation des événements précoces déclenchés par leur reconnaissance et de tenter d'identifier les groupements structuraux des LPs nécessaires à cette reconnaissance. Pour se faire, nous avons utilisé le modèle des cellules de tabac en culture *in vitro*.

L'addition de surfactine dans les suspensions de cellules déclenche une rapide alcalinisation du milieu extracellulaire suivie par une production d'espèces oxydantes, sans toutefois provoquer de mort cellulaire. Nous avons aussi mis en évidence une concentration seuil en surfactine, de 4 µM, pour visualiser une réponse optimale des cellules de tabac. L'ajout d'EDTA ou de $LaCl_3$, respectivement un chélateur du calcium et un inhibiteur de canaux calcique, avant le traitement par la surfactine inhibe fortement l'alcalinisation du pH, suggérant l'intervention de l'ion $Ca^{2+}$ dans la mise en place d'une réponse par les cellules.

D'autres part, la surfactine est aussi en mesure de stimuler l'activité d'enzymes comme la LOX ou la PAL quelques heures après son ajout dans les cultures pour atteindre des valeurs maximales entre 9 et 12h après le traitement. Des analyses en biologie moléculaire ont également révélé une accumulation claire d'ARNm de PAL au même moment que les pics d'activité, illustrant une transcription effective des gènes codant pour l'enzyme causée par la perception de la surfactine. Cette voie de défense a été par la suite approfondie par la mise en évidence de modification dans le profil des phénylpropanoïdes en HPLC et HPLC-MS. Une apparition d'acide salicylique et l'augmentation des taux d'acide cinnamique et hydroxy-benzoïque, ainsi qu'une diminution de ceux des acides ferulique et coumarique ont été observés, et suggèrent alors une réorientation de la voie de phénylpropanoïdes.

Des réponses différentes ont cependant été obtenu avec la fengycine et sont présentées dans les résultats additionnels. Lors de son addition dans les cultures à des concentrations similaires à la surfactine, aucune variation du pH ni de production d'espèces oxydantes n'a été observée dans les délais analysés. Mais une réponse positive a aussi été mise en évidence au niveau enzymatique, puisque son ajout est accompagné par une augmentation de l'activité PAL ainsi qu'une accumulation des transcrits correspondant à cette enzyme.

Enfin, le mode de reconnaissance et les sous-structures de la surfactine impliquées dans son activité élicitrice ont été investigués grâce à des homologues possédants des longueurs variables de chaînes lipidiques et à des lipopeptides structurellement modifiés par linéarisation ou méthylation au niveau de la partie peptidique. Les surfactines méthylées et linéaires sont moins actives, suggérant respectivement un rôle des charges présentes sur la chaîne peptidique et une importance de la structure cyclique de la molécule. De plus, une activité supérieure a été observée pour les homologues ayant des chaînes lipidiques constituées de 14 ou 15 carbones, en comparaison à ceux possédant des chaînes plus courtes. L'ensemble de ses données suggère que l'action élicitrice de la surfactine sur les cellules de tabac puisse être dépendante de ses propriétés amphiphiles et est discutée dans ce chapitre.

**Abstract**

Recognition by plant cells of some molecular patterns harboured by microbial pathogens can trigger the activation of defence pathways and eventually lead to a systemic increase of resistance to subsequent attack. In the context of systemic resistance induced by non-pathogenic rhizobacteria, we have recently demonstrated that surfactins and fengycins lipopeptides from *Bacillus subtilis* S499, were involved in elicitation of the phenomenon in bean and tomato. Here, we further investigated molecular events underlying this ISR-related interaction between such lipopeptides and plants. Addition of surfactin in the micromolar range to tobacco cell suspensions clearly induced some defence-related early events such as $Ca^{2+}$-dependent pH alkalinization and reactive oxygen species production but only limited cell death. Surfactin also stimulated defence enzymes such as phenylalanine amonia lyase and lipoxygenase, and modified the pattern of phenolics produced by the elicited cells. Reduced activity of some homologues also indicates that surfactin perception is dictated by structural clues both in the acyl moiety and in the cyclic peptide part. The present study sheds new light not only on defence-related events induced following recognition of amphiphilic lipopeptides from *Bacillus* but also more globally on the way elicitors from beneficial bacteria can be perceived by host plant cells.

**Introduction**

During evolution, plants have adapted themselves to environmental conditions. Beyond abiotic stress such as to low or to high temperatures, dryness, high salinity or wounding, plants have to

protect themselves against diseases caused by a wide range of microorganisms. To develop diseases, these pathogens have to penetrate the plant tissue, either by penetrating leave or root epidermis, or by entering through natural opening such as stomata (Chisholm *et al.*, 2006). To prevent these invasions, plants have evolved several defence strategies. Preformed physical barriers such as cell wall, or constitutively produced antimicrobial compounds can slow down or inhibit pathogen colonization. In a more active way, plants can also recognize some pathogen-associated molecular patterns (PAMPs). These general elicitors of either oligosaccharide, peptide, or lipid nature are non-specific compounds constitutively produced by the pathogen (Gómez-Gómez, 2004). They are involved in indispensable functions for the microorganism such as mobility (flagellin), enzymatic activities (Pep13) or cell surface composition (lipopolysaccharides (LPS), oligosaccharides) (Montesano *et al.*, 2003). PAMPs are recognized by associated- cell surface receptors (Jones and Dangl, 2006). In another way, plant can also recognize cellular changes caused by Avr proteins, some specific pathogen effectors which block signal transduction pathway triggered by general elicitors (Chisholm *et al.*, 2006; Van Loon *et al.*, 2006). Recognition of Avr proteins by plant resistance proteins (R proteins) activates a variety of defence response including the hypersensitive response (HR) (Heath, 2000). HR is characterized by a rapid production of oxidative species, a programmed cell death around the infection site and the synthesis of pathogenesis related proteins (PR proteins), involved in defence against pathogen (Greenberg and Yao, 2004). HR will restrict pathogen growth and confer a kind of resistance in local tissues (Kombrink and Schmelzer, 2001). By contrast to these local responses, plants can develop a systemic form of resistance that extends to all organs following localized interaction. In this context, the systemic acquired resistance (SAR) is the best characterized phenomenon. SAR is activated after a first infection by an incompatible necrotizing pathogen and renders the host plant more resistant to a subsequent attack by a range of virulent pathogens on the same or another organ (Durrant and Dong, 2004; Sticher, 1997). A systemic immunization decreasing disease impact can be triggered by specific strains of plant growth promoting rhizobacteria. This phenomenon has been termed PGPR-induced systemic resistance (ISR) and is also effective against a broad range of fungal, bacterial, and viral diseases, as well as against some insect and nematode pests (Ongena and Thonart, 2006; Van Loon *et al.*, 1998). Several beneficial rhizobacteria have been isolated for their ISR-inducing activities and many of them belong to the *Pseudomonas* and *Bacillus* genera (Bakker *et al.*, 2007; Kloepper *et al.*, 2004).

ISR can be globally viewed as a three-step process: bacterial elicitor perception, systemic signal transduction and defence gene expression leading to enhanced responsive capacity of the host plant. ISR-associated signal transduction and defence mechanisms are being well documented even if comparatively less well understood than in the case of SAR (Van Loon

and Bakker, 2005; Pieterse *et al.*, 2001). By contrast, very little is known about the molecular events governing the early interaction between the bacteria and the plant cell. In some cases, molecules responsible for the ISR-eliciting activity of these PGPR strains have been characterized. These elicitors may be i) cell surface components such as outer-membrane lipopolysaccharides (Reitz *et al.*, 2002; Coventry and Dubery, 2001; Duijff *et al.*, 1997) and flagellin from *Pseudomonas* species (Meziane *et al.*, 2005), ii) iron regulated metabolites such as pyoverdins, pyochelin, salicylic acid or a *N*-alkylated benzylamine derivative (Ran *et al.*, 2005; Audenaert *et al.*, 2002; De Meyer *et al.*, 1999; Leeman *et al.*, 1996), iii) the antibiotic compounds DAPG, pyocyanin or massetolide A (De Vleesschauwer *et al.*, 2006; Raaijmakers *et al.*, 2006; Iavicoli *et al.*, 2003; Siddiqui and Shaukat, 2003). In addition, volatiles (2,3-butanediol) and quorum sensing signals (*N*-homoserinelactone) were recently described as new active compounds capable to induce ISR (Schuhegger *et al.*, 2006; Ryu *et al.*, 2004).

Recent advances have highlighted the important role played by cyclic lipopeptides (LPs) of the iturin, fengycin and surfactin families in plant disease control by various *Bacillus* strains. *Bacillus* lipopeptides (LPs) were at first mostly studied for their antagonistic activity against a wide range of potential phytopathogens including viruses, bacteria, fungi and oomycetes (Haas and Défago, 2005; Leclère *et al.*, 2005; Ongena *et al.*, 2005a; Zahir *et al.*, 2004). It is now clear that such compounds may act not only as antagonists but also by facilitating root colonization (Bais *et al.*, 2004; Hofemeister *et al.*, 2004) and by reinforcing the host resistance potential (Ongena *et al.*, 2005b). In a recent work, we have indeed demonstrated that surfactins and fengycins but not iturins produced by *Bacillus subtilis* S499 are involved in ISR elicitation on bean and tomato (Ongena *et al.*, 2007).

Fengycins are cyclic lipodecapeptides with a ß-hydroxy fatty acid chain saturated or not with a length of $C_{14}$ to $C_{18}$ (Schneider *et al.*, 1999; Vanittanakom *et al.*, 1986). The surfactin family encompasses structural variants isolated from various *Bacillus* species but all members are heptapeptides interlinked with a ß-hydroxy fatty acid to form a cyclic lactone ring structure (Peypoux *et al.*, 1999). They are among the most powerful biosurfactant known with exceptional emulsifying and foaming properties. Because of their amphiphilic nature, surfactins can also readily associate and tightly anchor into lipid layers (Heerklotz and Seelig, 2007; Deleu *et al.*, 2003; Sheppard *et al.*, 1991).

By contrast to the numerous investigations conducted with some PAMPs used as models for the study of early defence-related events (Garcia-Brugger *et al.*, 2006; Zhao *et al.*, 2005; Gómez-Gómez, 2004), very few informations are available about the perception mechanisms of ISR-specific elicitors by plant cells. The general objective of the work presented here was to provide a first picture of the metabolic changes that can be induced in plant cells upon recognition of elicitors from non-pathogenic bacteria. More specifically, we have investigated

the responses to surfactins in cultivated tobacco cells and have identified some early events as well as later biochemical changes that could ultimately lead to an enhanced state of resistance.

## Results

### *Surfactin as main inducer of the alkalinization response of tobacco cells*

As a member of the *Bacillus* genus, the rhizobacterium *B. subtilis* has the potential to produce a vast array of biologically active molecules among which structurally diversified anti-microbial compounds (Stein, 2005). The first objective of this work was thus to evaluate whether other molecules than LPs may also be perceived by plant cells. Medium alkalinization takes part in the plant defence-associated early responses to various biotic elicitors (Felix *et al.*, 1999; Boller, 1995) and we used this readily measurable phenomenon on suspension-cultured tobacco cells to test the activity of products secreted by *B. subtilis* S499. To this end, supernatant samples were collected at various time-points during growth in the optimized medium. Crude supernatant samples were pre-purified on C-18 cartridge to yield the MeOH40 extract (fractions eluted with 40% methanol) containing molecules with intermediate hydrophobicity, and the MeOH100 extract (fractions eluted with 100% methanol), retaining more apolar compounds among which are found the LPs of the three families. The effect on tobacco cells of C-18 extracts prepared from samples collected every hour all over the culture time were first estimated with pH indicator paper. For clarity purpose, only some of them are represented in Figure 1B.

MeOH40 extract did not induce any change of the pH, suggesting that no metabolite with intermediate hydrophobicity produced by the strain S499 upon such culture conditions are active in triggering an alkalinization response by tobacco cells. The same applies for MEOH100 fractions obtained from samples collected before surfactin production, indicating that apolar metabolic products that could accumulate early in the exponential growth phase are not active on pH. By contrast, pH increases could be clearly visualized by using MeOH100 extracts prepared from culture samples collected then after. A weak but significant pH alkalinization could be observed by treating tobacco cells with extract from 16 h old culture, corresponding with the apparition of surfactin in bacterial supernatant. Stronger pH alkalinizations were observed when MeOH100 extracts from samples containing higher surfactin concentrations were added to the cells. These results were confirmed and more precisely measured with a pH meter (Figure 1C). These first results strongly suggest that surfactin lipopeptides are tightly involved in the induction of this alkalinization process since it

exactly correlates with the appearance and accumulation trend of surfactin in the medium. However, a minor contribution of other metabolites with similar production kinetic or of fengycins whose production is only 3-4 hours-delayed compared to surfactins (data not shown) could not be ruled out at this stage. By contrast, iturins that accumulate later in the culture are seemingly not involved since iturin-rich samples collected after 72 h of growth did not showed enhanced activity compared to those collected at 54 h with reduced iturin content (data not shown).

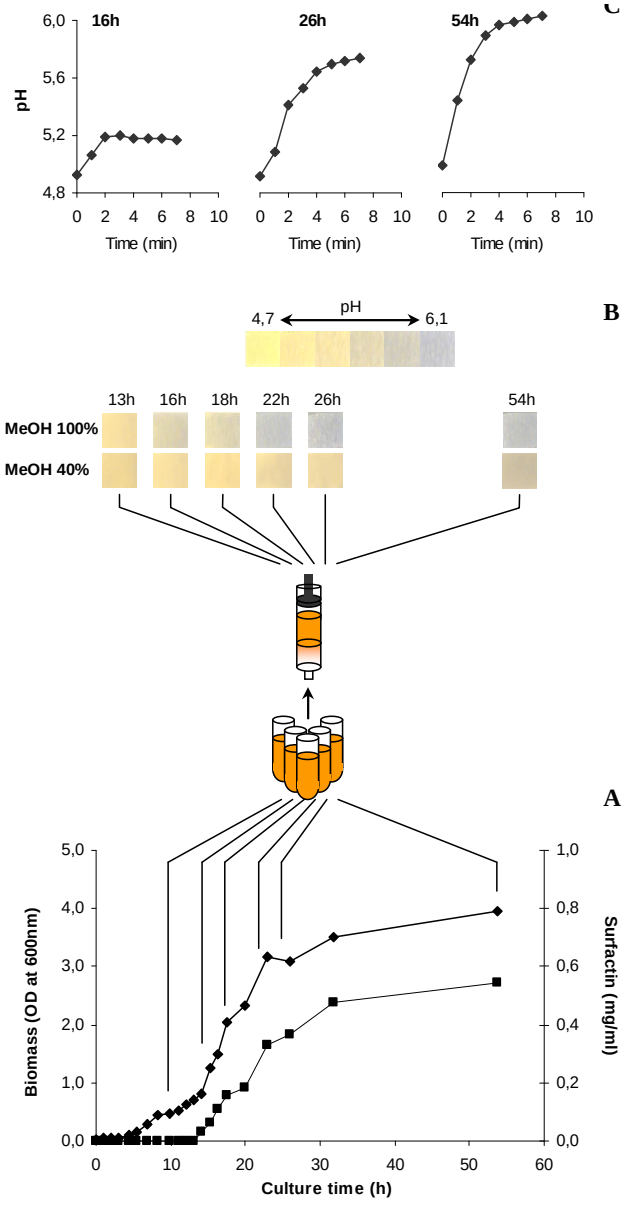


**Figure 1.** Alkalinization response of tobacco cells induced by *Bacillus subtilis* S499 culture supernatant extracts.
**A.** Bacterial growth and surfactin lipopeptide production kinetics. Biomass was estimated by optical measurements at 600 nm. Surfactin concentration was determined by HPLC.
**B.** pH changes, as revealed with indicator paper, in tobacco cell culture following treatment with semi-purified extracts of S499 supernatants collected periodically starting from 13 h to 54 h of incubation.
**C.** Extracellular pH modification (measured with glass electrode) of tobacco cell culture induced by treatment with S499 supernatant extracts respectively purified after 16 h, 26 h and 54 h of culture.

In order to get more conclusive information about the relative alkalinization-inducing activities of the three lipopeptide families, we tested MeOH100 extracts from various derivatives of the *Bacillus amyloliquefaciens* FZB42 strain affected in the biosynthesis of different lipopeptides. The AK3 mutant efficiently produces surfactin but fengycin and iturin biosyntheses are suppressed. The CH1 and CH2 derivatives retain fengycin and iturin synthesis respectively but are impaired in the production of the two other lipopeptide families. LPs production by these strains was qualitatively checked and quantified by HPLC. MeOH extracts were diluted in order to obtain a final LP concentration of 2 µM after addition into the tobacco cell culture medium. A strong alkalinization response of 0.9 pH unit was observed following treatment of tobacco cells with the AK3 extract (Figure 2). Neither CH1 nor CH2 extract induced a significant pH increase compared with cells treated with methanol and used as control. These results confirm that iturins and fengycins are poorly active in triggering an alkalinization response of tobacco cells and that, among *Bacillus* products, this activity is probably due to surfactins.

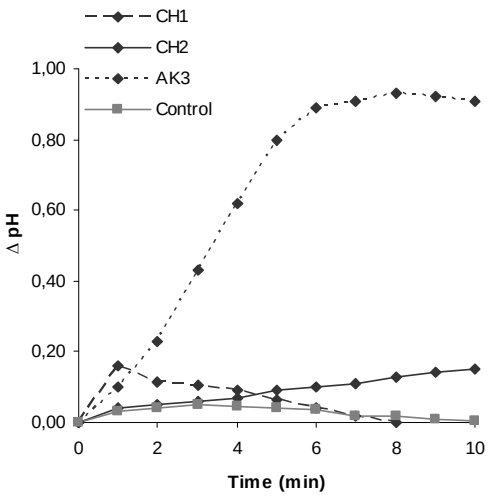


**Figure 2.** Variation in extracellular pH of culture medium of tobacco cells in response to culture extracts from *Bacillus amyloliquefaciens* FZB42 mutants. The three mutants of strain FZB42 that are affected in surfactin (surf), fengycin (feng) and/or iturin (itu) production are CH2 ($surf^-/feng^-/itu^+$), CH1 ($surf^-/feng^+/itu^-$) and AK3 ($surf^+$, $feng^-$, $itu^-$). Lipopeptides were extracted from culture broths of *Bacillus amyloliquefaciens* FZB42 mutants by using C-18 solid phase extraction cartridges. Tobacco cells suspension cultures were treated with 21 µL of culture extracts dissolved in methanol 100% to obtain a final lipopeptide concentration of 2 µM. Control consisted of cells treated with same volume of methanol 100%.

*Alkalinization as a dose/structure and calcium dependent response to surfactin*

On the basis of these informations, we focused on the effect of surfactins and we first used a 99%-pure mixture of homologues produced by the S499 strain to further investigate tobacco cell responses to this elicitor. Surfactin ability to induce a pH shift in tobacco cell medium was first evaluated using increasing amounts. Surfactins added at final concentration of 0.1 µM did not provide any effect on cultured cells but treatment with 2 µM final concentrations triggered

a similar low but significant pH shift (Figure 3). This alkalinization effect dropped off markedly in the presence of a surfactin concentration of 4 µM but further increases to 10 µM. A concentration of 20 µM was less effective than 10 µM suggesting a saturation of the phenomenon as already observed in other studies (Figure 3) (Felix *et al.*, 1999; Bourque *et al.*, 1998). Taken together, these results suggest than even if a low surfactin concentration in the medium can provoke alkalinization, a threshold concentration is seemingly required to induce a maximal cell response.

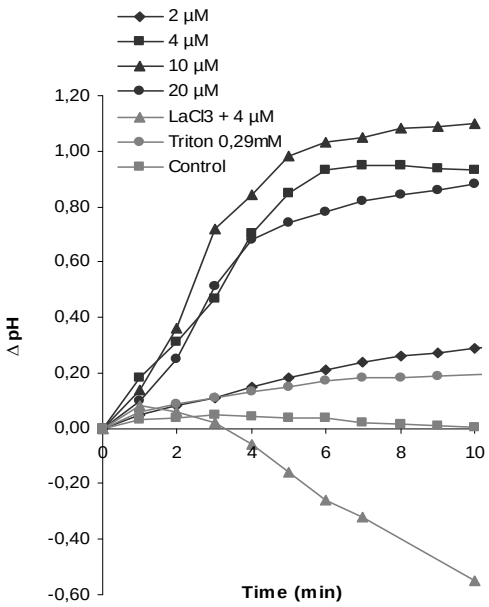


**Figure 3.** Dose-response curve for pH alkalinization induced by surfactins on tobacco cell suspensions. Cultures were treated with purified surfactin from *Bacillus subtilis* S499 (black curves) to obtain the final concentrations mentioned. In addition to treatment with surfactins (4 µM), tobacco cells were also pre-treated with $LaCl_3$, a calcium channel inhibitor before lipopeptide addition. As purified surfactins were dissolved in methanol 100%, the control consisted in cells treated with same volume of methanol. Representative data from three replicates are presented.

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 with $C_{12}$ to $C_{15}$ linear saturated acyl chains and two for iso-$C_{14}$ and iso-$C_{15}$ forms. These homologues were purified by semi-preparative HPLC and were tested individually on tobacco cells except for the two $C_{13}$ and the two $C_{15}$ forms that 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 ($C_{12}$ and $C_{13}$) failed to induce any significant pH shift (Figure 4). By contrast, $C_{14}$ and $C_{15}$ surfactins 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 low but significant difference was observed between cells treated with linear (C14n) or ramified (C14i) surfactin homologues (Figure 4B). The synthesis of linear and methylated

forms of the $C_{14}$ surfactin was performed to appreciate the importance of some traits of the peptide part of the molecule. As shown in Figure 4B, both modifications considered individually led to a significant decrease of the eliciting activity on tobacco cells and the linearized and methylated form only retained about 25% of the activity.

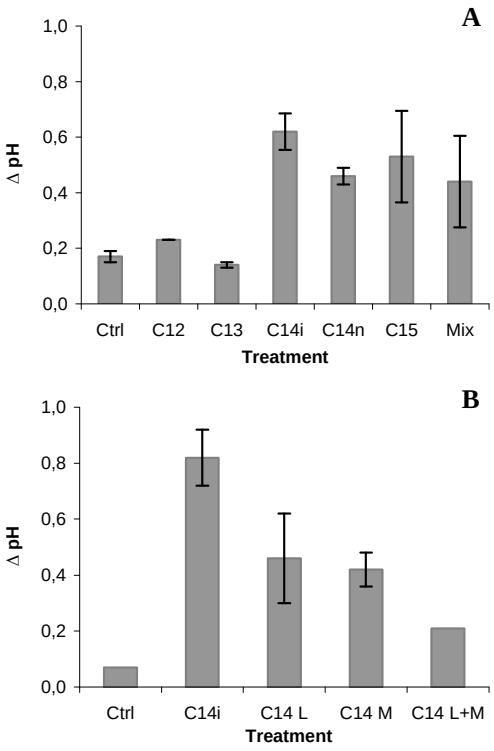


**Figure 4.** Structure-activity relationship of surfactin in eliciting pH alkalinization of tobacco cells medium.
**A.** Medium alkalinization response to treatment of tobacco cells with surfactin homologues with variable lipid chain from 12 carbons (C12) to 15 carbons (C15). For surfactin with 14 carbons in the lipid chain, a linear chain (C14n) and a ramified chain (C14i) were tested. Cells were treated with purified surfactin dissolved in methanol 100%. Treatments were added to obtain a final surfactin concentration in cell suspension of 2 µM. The mixture of the different surfactin homologues was used as a positive control (Mix). Data are means and standard variations calculated from three independent experiments.
**B.** Influence of lipid acid chain conformation and peptide core charge of C14i surfactin on cell response. This iso form of the C14 homogue was either methylated on the carboxy groups of the two aspirate residues (C14 M) or synthesised in a linearised form (C14 L) or both (C14 L+M). The natural C14i homologue and the modified surfactins were added to obtain a final concentration of 4 µM in the cell suspension. Control consisted of cells treated with same volume of methanol 100% (Ctrl). Data are means and standard variations calculated from three independent experiments.

In plant-pathogen interactions, calcium is known to have an important role in the early events after elicitor perception (Lecourieux *et al.*, 2006). In order to determine if $Ca^{2+}$ is also involved in LP-induced alkalinization, the effect of the calcium channel inhibitor $LaCl_3$ was tested in surfactin-treated tobacco cell suspensions. As shown in Figure 3, $LaCl_3$ addition before treatment with surfactin (4 µM) totally inhibited the alkalinization phenomenon suggesting a lipopeptide-induced $Ca^{2+}$ influx that can in turn inhibit the $H^+$-ATPase pump involved in extracellular pH increase. On another hand, tobacco cells were also treated with Triton X-100 used as positive control miming the effect of an anionic detergent (such as surfactin) that could provoke 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 surfactins, Triton X-100 only induced a constant, slow and limited response over the time of measurement.

*Limited cell death triggered by surfactin*

Because of their amphiphilic nature, surfactins can readily associate and tightly anchor into lipid layers. It can thus interfere with biological membrane integrity and provoke cell leakage. Viability of tobacco cells was determined in the presence of increasing concentrations of surfactin by using Evans blue coloration (Baker and Mock, 1994). Both microscopic observation and spectrophotometry quantification did not reveal any significant cell mortality in the first hours after surfactin addition at the concentrations used compared to the methanol-treated control (data not shown). Data obtained for mortality assessment 24 h after LP addition are presented in Figure 5 and showed a very limited adverse effect of surfactins on tobacco cell viability in comparison with benzylamine, reported as powerful cell death-inducer agent (Kawano *et al.*, 2000), or Triton X-100 used for calibration (data not shown). No significant cell death was observed following treatment with 2 µM surfactin and mortality rates of about 25% were induced by surfactin added at a concentration of 10-20 µM (Figure 5B). In addition, the integrity of cells was evaluated by measuring intracellular protein release and only minor leaks if any (4% to 7% increase in extracellular proteins), was induced during the first five hours after treatment with 2 µM to 20 µM surfactin (data not shown).

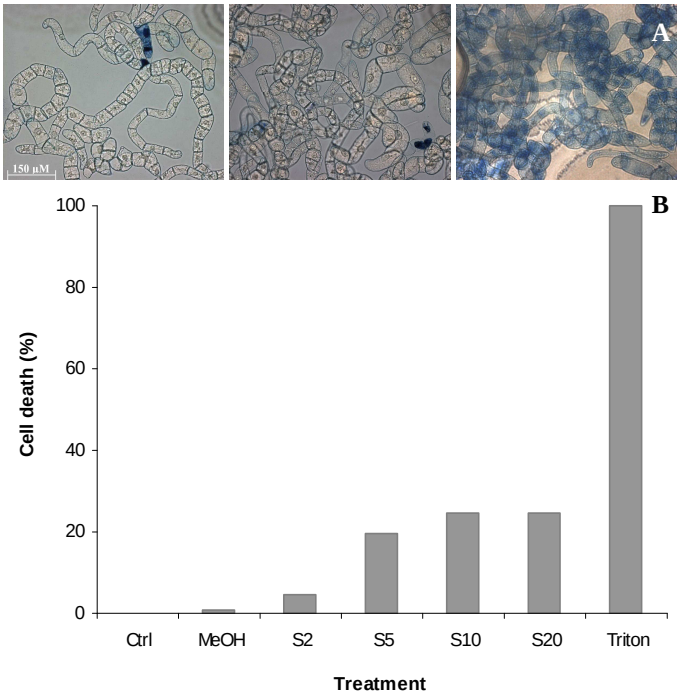


**Figure 5.** Evaluation of tobacco cell mortality upon treatment with surfactin.
**A.** From left to right, tobacco cells respectively treated with methanol 100%, surfactin 2 µM and triton X-100. Pictures were taken 24 h after treatment of cells. Dead cells are blue dyed.
**B.** Cell death caused by increasing surfactin concentration on tobacco cell suspension after 24 h of incubation. Cultures were treated with purified surfactins from *Bacillus subtilis* S499 to obtain final concentrations in the medium of 2 µM (S2), 5 µM (S5), 10 µM (S10) or 20 µM (S20). Purified surfactins were dissolved in methanol 100%. Control (Ctrl) treatment represents untreated cells and MeOH are cells treated with the same volume of methanol 100%. Tobacco cell suspensions were treated with Triton X-100 (0.1 mM) as a positive control inducing strong cell mortality. Data were obtained using optical density of sonicated washed cells coloured with Evans blue.

*Induction of an oxidative burst*

The rapid generation of reactive oxygen species has also been demonstrated to be typically involved in early events associated with the plant defence response following pathogen perception. Hydrogen peroxide release by surfactin-treated tobacco cells was investigated using a luminol-based chemiluminescence assay. Hydrogen peroxide accumulation in extracellular medium was clearly stimulated, after addition of surfactin, especially when the lipopeptide was present at concentrations of 10 µM or higher (Figure 6). In the first ten minutes, a significant accumulation of hydrogen peroxide to concentrations of 3.3 and 4 µM occurred in cell suspensions treated with 10 µM and 20 µM of surfactin respectively. At lower doses (2-5 µM), reduced but significant $H_2O_2$ accumulations were also measured. On another hand, addition of EDTA prior to surfactin treatment at 10 µM completely abolished the oxidative burst-inducing effect of the lipopeptide (Figure 6) suggesting that hydrogen peroxide production may also be governed by the calcium influx.

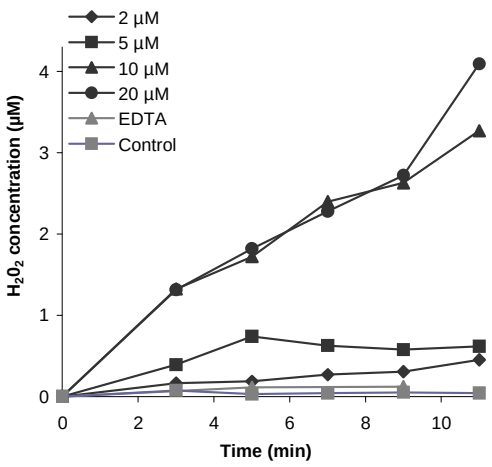


**Figure 6.** Surfactin-induced oxidative burst in tobacco cell suspensions. Cells were treated at time 0 with methanol 100% (control) or with surfactin solutions to obtain final concentrations of 2 µM, 5 µM, 10 µM or 20 µM. EDTA represents treatment with 10 µM surfactin added in tobacco cells previously treated with the chelator. The generation of $H_2O_2$ was quantified using a luminol-dependant chemiluminescence assay. Data are from one experiment but similar results were obtained in two independent assays.

*Stimulation of defence-related metabolic pathways*

Phenylalanine ammonia lyase (PAL) catalyzing the deamination of phenylalanine to yield cinnamic acid is a key enzyme involved in plant defence as it represents the entry of the phenylpropanoid pathway. Stimulation of this metabolic route may lead to the synthesis of antifungal compounds or lignin precursors and thereby contributes to the host cell response decreasing pathogen progression and pathogen-induced damages (Zhao *et al.*, 2005; Dixon *et al.*, 2002). PAL activity in tobacco cells was first tested spectrophotometrically on extracts

prepared from cells collected at various time points after surfactin addition. Results are compared in Figure 7A with those obtained for untreated cells (same volume of methanol) and for cells treated with methyl jasmonate as positive control overexpressing PAL (Sharan *et al.*, 1998). A very slow increase in PAL activity was observed in methanol-treated control cells but a strong induction of PAL activity was obtained within 6 h after surfactin addition to reach a maximal value after 9 h. Compared to control, PAL activity was 4.1 time and 8.6 time higher in the presence of 10 µM and 20 µM surfactin respectively. In addition, hybridization on Northern blots also revealed a strong accumulation of PAL transcripts in tobacco cells treated with surfactin (Figure 7B) suggesting that a *de novo* enzyme synthesis is induced by this lipopeptide.

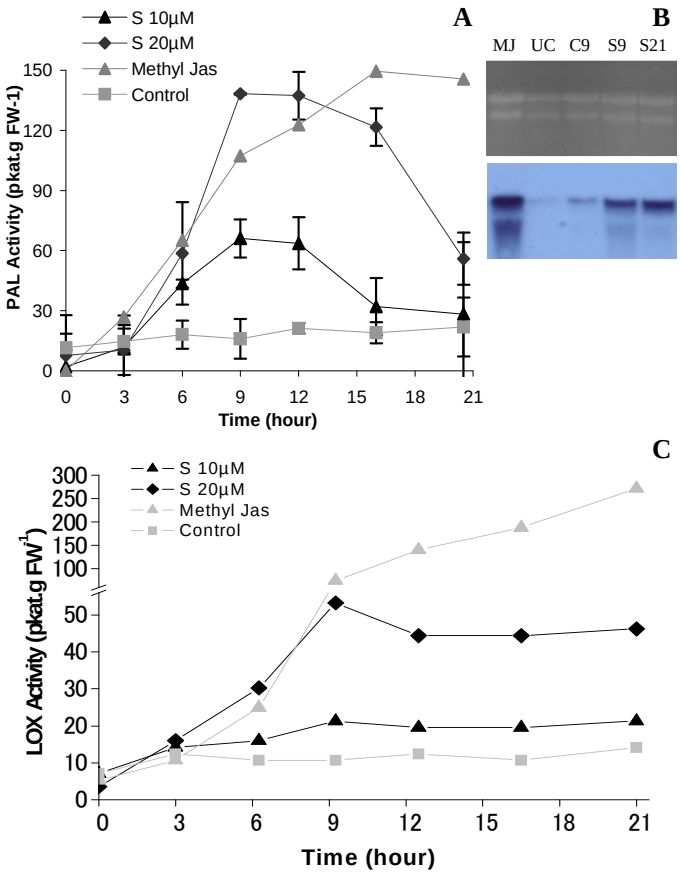


**Figure 7.** Stimulation of defence-related enzymes in tobacco cells treated with surfactin.
**A.** Time course of phenylalanine ammonia-lyase (PAL) activity following treatment with two different surfactin concentrations and with methyl jasmonate. Control consisted of cells treated with the same volume of methanol 100%. Data are means and standard deviations from two independent experiments except for treatment with methyl jasmonate which are from of one experiment.
**B.** Expression pattern of PAL in tobacco cells. Total mRNA were extracted from untreated tobacco cells (UC), cells that have been treated with methyl jasmonate as positive control (MJ) or respectively 9 and 21 h after treatment with 20 µM of *Bacillus* surfactin (S9 and S21). A negative control (C9), corresponding to ARN extraction of tobacco cells 9 h after addition of methanol, is also shown.
**C.** Time course of tobacco cells lipoxygenase (LOX) activity induced by treatment with surfactin. Data are from one representative experiment.

In a third approach, stimulation of the phenylpropanoid pathway was also investigated by analyzing the differential accumulation of PAL-derived phenolics secreted by tobacco cells.

Different samples from tobacco cell supernatants culture were purified by liquid-liquid extraction and analyzed by HPLC. Reversed-phase DAD-HPLC analyses of pre-purified supernatant extracts collected 10 h after treatment of tobacco cells with 10 µM surfactin revealed significant accumulation or decrease of several compounds (Figure 8). Cinnamic, salicylic, hydroxy-benzoic and benzoic acids could be identified among accumulating compounds on the basis of their retention time and ion fragmentation profile in LC-ESI/MS. Apparition of salicylic acid and 3-fold increase in cinnamic acid were detected 5 h after treatment and were maintained until 24 h after surfactin addition (data not shown). On another hand, ferulic and coumaric acids were poorly resolved under the HPLC conditions used but significant 1.5-fold and 2.4-fold decreases in their concentrations after surfactin addition were observed by using quantitative LC-MS. Other surfactin-induced phenolics were detected on the basis of their UV-visible spectral properties but could not be reliably identified so far.

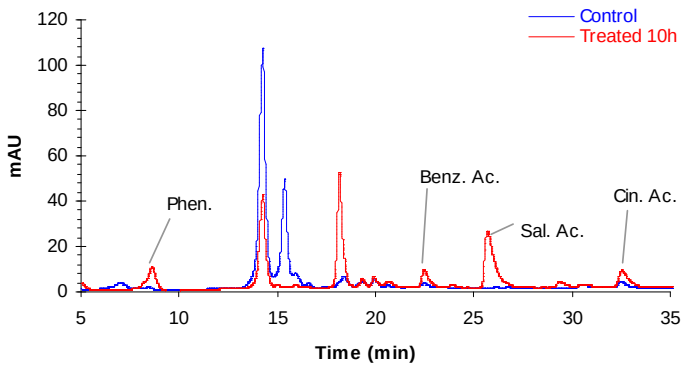


**Figure 8.** HPLC profile obtained for the analysis of phenolics in tobacco cell culture supernatants. Phenolic compounds were extracted with ethyl acetate from supernatant of cells treated with methanol (Control) or of cells 10 h after treatment with surfactin (Treated 10 h). Compounds eluting in some peaks were identified by comparison of their retention time with those of authentic standards (Phen, phenylalanine; Benz. Ac., benzoic acid; Sal. Ac., salicylic acid; Cin. Ac., cinamic acid) and on the basis of the molecular weight and fragmentation in LC-MS.

Plant lipoxygenase (LOX) is involved in the defence response to various pathogen-induced or wound-induced stresses (Shah, 2005) but previous results from our laboratory showed that this enzyme can also be stimulated following perception of non pathogenic rhizobacteria (Ongena *et al.*, 2004). LOX catalyzes the incorporation of molecular oxygen in polyunsaturated fatty acids to yield the corresponding fatty acid hydroperoxides. These are substrates for other enzymes that convert the highly reactive hydroperoxides into a wide array of bioactive oxylipins (Blee, 2002). Possible increase of LOX activity in surfactin-treated tobacco cells was thus also tested to provide an additional example of defence-related pathway induction by this lipopeptide. Evolution of LOX activity was monitored spectrophotometrically by measuring hydroperoxide production from the C18:3 linolenic acid. A significant

stimulation of LOX activity was specifically observed in surfactin-treated cells during the first hours to reach a 5-fold higher level after 9 h compared to control cells (Figure 7C). The higher LOX activity was maintained in elicited cells during at least 21 h. As in the case of PAL, the addition of methyl jasmonate also induced a strong LOX response proving adequate tobacco cells reactivity.

**Discussion**

In the so-called ISR phenomenon, selected strains of non pathogenic rhizobacteria can promote a state of enhanced resistance rendering the host plant less susceptible to subsequent attack by a phytopathogen. Systemic signal transduction in the plant during ISR and defence-associated changes in the metabolism or in gene expression are being much studied but molecular events underlying the perception of bacterial elicitors and early plant cell responses are considerably less well understood (Van Loon, 2007). Based on our recent work that showed the ability of lipopeptides produced by *Bacillus subtilis* to trigger ISR in tomato and bean (Ongena *et al.*, 2007), we have further investigated some aspects of the responses induced by these LPs in cultured plant cells. By two different approaches, we have demonstrated that surfactin-type lipopeptides are the main *Bacillus* products recognized by tobacco cells. Treatment with surfactin in micromolar concentrations (that showed a protecting activity on whole plants) triggers an alkalinization of the extracellular medium and a significant production of reactive oxygen species within minutes. Both phenomena are associated with host defence reactions in the context of plant-pathogen interactions and are induced by PAMPs such as flagellin or cryptogein (Garcia-Brugger *et al.*, 2006). The use of increasing surfactin concentrations has revealed the existence of a threshold value around 4 µM to trigger a maximal alkalinization effect, even if 1-2 µM concentrations induce a lower but significant pH increase. As surfactin addition at concentrations up to 20 µM did not induce notable cell lyses or death in the first hour, it is obvious that the observed alkalinization may rely on an active biochemical process rather than passive ion diffusion through surfactin-induced pores in the membrane. Moreover, experiments realized with $Ca^{2+}$ chelator or channel blocker strongly suggest that both $H_2O_2$ generation and pH increase are dependent on the presence of $Ca^{2+}$ ions. By analogy with PAMPs, treatment with surfactin could stimulate calcium influx through specific channels and intracellular $Ca^{2+}$ could then both inhibit the $H^+$-ATPase pump leading to an acidification of cytoplasm and activate the NADPH oxidase for $H_2O_2$ production (Lecourieux *et al.*, 2006).

Intracellular pH modification, via a cascade of MAP kinase-directed phosphorylation-dephosphorylation, can lead to the activation of defence-specific pathways and activate the

synthesis of intracellular and systemic signalling compounds (Desender *et al.*, 2007). $H_2O_2$ generation also plays multiple roles in early plant defence responses. In addition to a direct protective effect, reactive oxygen species may favour cell wall cross-linking, induce cell death, activate an array of defence genes and the biosynthesis of antimicrobial proteins and phytoalexins (Fedoroff, 2006; Apel and Hirt, 2004). But reactive oxygen species also play a potential role in systemic signalling leading to the establishment of SAR (Fobert and Despres, 2005). Following perception of surfactin, the occurrence of alkalinization and oxidative burst also typically resulted in further induction of defence-related metabolic pathways: increased PAL and LOX activities were observed in the 6-9 h following treatment with the lipopeptide. In the case of PAL, the stimulation of the activity was correlated with an accumulation of mRNA. PAL is a key enzyme in phenylpropanoid metabolism leading to a large array of phenolic compounds, including precursors for cell wall reinforcement through lignification, antifungal compounds or salicylic acid which play an important signaling role in disease resistance (Dixon *et al.*, 2002). As a result of PAL stimulation, surfactin treatment leads to major changes in the phenolic pattern produced by tobacco cells. All the accumulating or appearing compounds could not be identified but clear increases in cinnamic acid, hydroxyl-benzoic acid and salicylic acid apparition were observed concomitantly with decreases in ferulic and coumaric acid amounts. On this basis, we hypothesize a reorientation of the phenylpropanoid pathway starting from cinnamate to salicylic acid via the stimulation of enzymes such as benzoic acid 2-hydroxylase to the detriment of the other branch initiated by the cinnamic acid 4-hydroxylase to other phenolic compounds. Nevertheless, a use of coumaric, ferulic and other acids as precursor for synthesis of other defence molecules such as scopoletin or lignin precursors for cell wall reinforcement cannot be rejected. Further metabolic profiling studies are being performed to confirm this hypothesis. Interestingly, treatment of potato tuber cells with purified *Bacillus* LPs also resulted in the accumulation of some plant phenolics involved in or derived from the phenylpropanoid metabolism (Ongena *et al.*, 2005a). Increased concentrations of cinnamic acid were also observed together with higher amounts of chlorogenic acid that may act directly as an antimicrobial defence compound and that derives from the benzoate branch of the pathway (Maher *et al.*, 1994). Again, p-coumaric, ferulic, sinapic and caffeic acids that constitute building blocks for the synthesis of lignin did not accumulate. Otherwise, (Audenaert *et al.*, 2002) have observed some local PAL stimulations after treatment of tomato roots with *Pseudomonas aeruginosa* 7NSK2, another ISR-inducing rhizobacteria.

Activation of LOX enzyme is a common feature of the plant defence response to pathogen, wounding and stress (Baysal and Demirdoven, 2007; Shah, 2005; La Camera *et al.*, 2004; Feussner and Wasternack, 2002). PAMP perception by plant cells was already shown to lead to

LOX stimulation (Desender *et al.*, 2006; Klarzynski *et al.*, 2003). Here, a clear stimulation of LOX activity was observed in tobacco cells within the 9-12 h following elicitation by surfactin. It indicates a possible induction of the so-called oxylipin pathway leading to a wide range of bioactive metabolites among which signalling jasmonates or various compounds with fungitoxic activity (Matsui, 2006; Prost *et al.*, 2005). We have already demonstrated that key enzymes of the LOX pathway were activated in resistant tomato plants following induction by surfactin overproducers (Ongena *et al.*, 2007). Time-course monitoring showed that significantly enhanced activities of LOX and of all hydroperoxide-degrading enzymes were induced and correlated with disease symptom reduction. Stimulation of the entire metabolic route leading to oxylipins was also evidenced in bean (Ongena *et al.*, 2004) and tomato (Adam, *unpublished results*) treated with the rhizobacterium *Pseudomonas putida* BTP1.

Major changes in defence-associated events of tobacco cells upon treatment with surfactin were thus observed in this work. In bean and tomato plants, a role for surfactins and fengycins in resistance induction was also demonstrated by the similar protective activity of purified compounds compared to the producing strain. Treatment of potato tuber cells with purified fengycins but not surfactins and iturins, resulted, as observed here in tobacco, in the accumulation of plant phenolics derived from the phenylpropanoid metabolism. By contrast strain S499 can stimulate a systemic defence response in cucumber (Ongena *et al.*, 2005b) but, semi-purified LP extracts failed to confer any protection, suggesting that they are not involved in the resistance triggering process in that plant. It thus appears that surfactins and fengycins retain a specific ability to stimulate different plant cells.

From a global point of view, this work reinforces our previous results providing evidence for a role of *Bacillus* lipopeptides as plant resistance inducers. The biological activity of such secondary metabolites of bacterial origin is also supported by the recent work of Tran and collaborators on massetolide A (Tran *et al.*, 2007). This cyclic lipopeptide produced by *Pseudomonas fluorescens* shows ISR-eliciting activity in tomato plants for the control of *Phytophthora infestans* as demonstrated by testing the pure compound and impaired mutants. LPs thus constitute a novel class of microbial-associated molecular patterns that can be specifically perceived by plant cells as signals to activate defence mechanisms. However, there is no partial common peptide sequence shared by the *Bacillus* LPs considered here with massetolide A or with other rhizobacterial peptide products showing ISR-eliciting activity such as flagellins and pyoverdins (Meziane *et al.*, 2005; Gómez-Gómez, 2004) or, even more broadly, with molecular patterns identified in the interaction of pathogenic microorganisms with plant cells . It is therefore crucial to appreciate the relative importance of specific LP sub-structures for ISR elicitation in order to understand the molecular aspects of their perception by plant cells. Based on the amplitude of the alkalinization effect, this work suggests that the

length of the fatty acid chain is important for the ISR-eliciting activity since optimal pH shifts were observed by treating tobacco cells with surfactins of the longer chains with 14 and 15 carbons. Such an important role for the length of the acyl chain has already been documented for other biological activities of LPs and is explained by the fact that it readily inserts into phospholipid bilayers (Dufour *et al.*, 2005; Youssef *et al.*, 2005; Bonmatin *et al.*, 2003). However, our results also show that a cyclic and charged peptide part is also necessary for maximal activity. It agrees with the global model of a lipid bilayer destabilisation process facilitated by the tri-dimensional form of the surfactin molecule featuring charged side chains protruding into the aqueous phase and apolar moieties inserting into the hydrophobic core of the membrane.

Because of their amphiphilic nature and remarkable surfactant activity, most of the biological activity of LPs may be intuitively related to their effect on the lipid part of biological membranes. Recent advances mainly based on physico-chemical studies using artificial phospholipids bilayers confirmed it (Heerklotz and Seelig, 2007; Carrillo *et al.*, 2003). However, as stated above, the presence of LPs was not associated with any marked phytotoxicity or adverse effect on the integrity and growth potential of plant cells. This suggests that these molecules could interact without irreversible pore formation but in a way sufficient to induce disturbance or transient channelling in the plasma membrane that can in turn activate a biochemical cascade of molecular events leading to defensive responses. Interestingly, a similar hypothesis has been recently raised to explain the anti-proliferative effect of surfactin on colon cancer LoVo cells that did not appear to be leaky or lysed but underwent significant changes in the expression of protein factors regulating cell survival (Kim *et al.*, 2007).

Nevertheless, on the basis of results presented here, one cannot exclude the presence of surfactin-specific receptors in tobacco cells that may govern the recognition process. Further clues will certainly be gained by searching for labelled surfactin-binding protein factors associated with plasma membranes, or for a specific, reversible, and saturable binding activity and by testing a broadest range of variants for their plant defence-inducing activity. These last data should then be evaluated with regards to the changes that may be induced in the three-dimensional conformation of these variants.

In another way, it thus appears that some homologues within the particular family of surfactins may be more active than others at stimulating the plant immunity potential. Unfortunately, only a few studies have been devoted to date to investigate LP production level and/or gene expression during growth of biocontrol *Bacillus* strains on the surface of plant tissues (Ongena *et al.*, 2007; Romero *et al.*, 2007; Bais *et al.*, 2004; Asaka and Shoda, 1996). Very little information is thus available about the quantities and pattern of surfactin

homologues secreted by strains growing in the rhizosphere. However, recent results from our laboratory indicates that some abiotic, nutritional and physiological factors somewhat influence the production of surfactins by *B. subtilis* strains, not only quantitatively but also qualitatively (Nihorimbere, *unpublished results*). Different patterns of surfactin variants with specific activities may thus be naturally synthesized by a given *Bacillus* strain depending on the nutritional context imposed by the host plant and the variability of physico-chemical parameters inherent to the soil such as mineral content, pH, temperature and oxygen availability. That said, such a structure/activity relationship has also been demonstrated for the antifungal activity of iturins (Bonmatin *et al.*, 2003). Hence, the variety of the LPs naturally synthesized may explain why some *Bacillus* strains are more efficient to reduce plant diseases than others via ISR or direct antagonism toward the pathogen.

The way ISR elicitors from PGPR are perceived by plant cells remains largely unknown except for some of them that were already identified as PAMPs harbored by pathogenic isolates of the same bacterial genus such as flagellin and outer membrane lipopolysaccharides (Chinchilla *et al.*, 2006; Gerber *et al.*, 2006; Piater *et al.*, 2004). The study presented here sheds new light on defence-related events induced following perception of amphiphilic surfactin lipopeptide produced by *B. subtilis* S499. This lipopeptide readily interacts with tobacco cells and triggers a series of defence-associated events that are not specific of any group of elicitors (Desender *et al.*, 2007). It suggests a convergence of the early cellular reactions with those initiated by PAMPs whatever the type and specificity of molecular interactions governing the initial recognition of ISR elicitors at the membrane level.

In plant-pathogen interactions, the general concept is that elicitors or avirulence factors must be recognized by plant receptors or R proteins localized to the plasma membrane or the cytoplasm before initiating defence reactions. Physical interaction between the eliciting molecule and a specific receptor kinase induces conformation that initiates intracellular signalling processes (Chisholm *et al.*, 2006). It is still not yet clear whether bacterial LPs are recognized by plant cells via specific receptors but the importance of the acyl chain and of the amphiphilic nature of surfactin strongly suggest that these LPs can mainly interact via a less specific mechanism based on some destabilization of the lipid bilayer structure. This perturbation could remain limited due to the specific composition in sterols of the membrane of plant cells which is known to influence the LP interaction with biological membranes.

**Additional results**

*Effect of fengycin on Phenylalanine Ammonia Lyase (PAL) activity in tobacco cells*

In this chapter, we have observed an increase in PAL activity in tobacco cell suspension culture after treatment with surfactins of *Bacillus subtilis*. Since fengycins are also active to induce ISR in tomato and bean plant (chapter VIII), these compounds were also tested for their potential to induce some (early) defence responses in cultured tobacco cells. By using the same method as for surfactin, we have thus followed PAL activity and mRNA expression pattern after treatment with fengycin. The results obtained are shown in Figure 9.

A similar response to previous results is observed upon surfactine addition at 10 or 20 µM. By contrast, the stimulation of PAL activity in tobacco cells treated with fengycin is delayed compared to surfactin and only clearly occurred 16h after treatment. Upon addition of a mixture of both LPs, a two-step on PAL activity was observed with an early increase after 3h due to surfactin, followed by a second and slower increase starting at 12h which is probably induced by fengycins. It also appeared from these results that the effects of individual LPs may be additive.

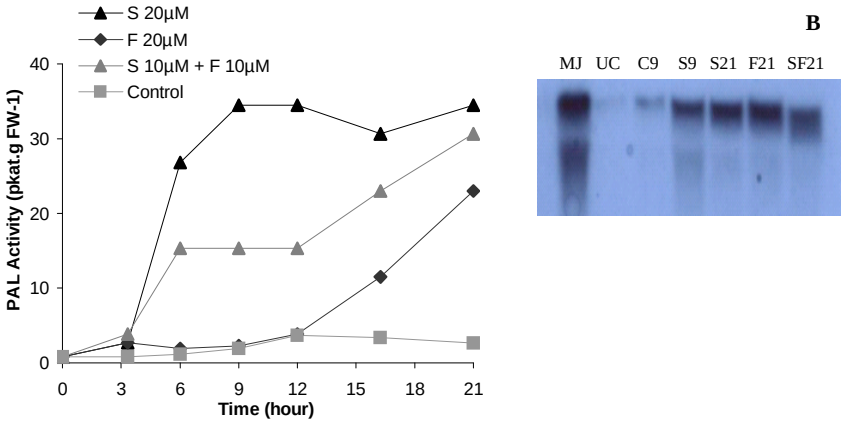


**Figure 9.** Stimulation of defence-related enzymes in tobacco cells treated with fengycin and surfactin.
**A.** Time course of phenylalanine ammonia-lyase (PAL) activity following treatment with surfactin and fengycin. Control consisted of cells treated with the same volume of methanol 100%. Data are from one experiment.
**B.** Expression pattern of PAL in tobacco cells. Total mRNA were extracted from untreated tobacco cells (UC), cells that have been treated with methyl jasmonate as positive control (MJ) or respectively 9 and 21 h after treatment with 20 µM of surfactin (S9 and S21) or fengycin (F21) or both compounds (SF21). A negative control (C9), corresponding to ARN extraction from tobacco cells 9 h after addition of methanol, is also shown.

## Materials and methods

*Bacterial and plant cell growth*

The wild-type *Bacillus subtilis* strain S499 was isolated from soil by Dr. L. Delcambe (CNPEM, Liège, Belgium) and studied in our laboratory for several years. *Bacillus amyloliquefaciens* CH1, CH2 and AK3 strains were kindly given by Professor Borriss, Center of Bacterial genetic, Biology Institute, Humboldt University of Berlin, Germany. *Bacillus amyloliquefaciens* strains and *Bacillus subtilis* S499 were cultured in aerobic condition in flask containing optimised medium with the following composition per liter: 7 g yeast extract, 30 g casein peptone, 20 g saccharose, 1.9 g $KH_2PO_4$, 0.45 g $MgSO_4$, 10 mg citric acid, 3.6 mg $MnSO_4.H_2O$, 0.014 mg $ZnSO_4.7H_2O$, 0.01 mg $H_3BO_3$, 0.005 mg $FeCl_3.6H_2O$, 0.004 mg $NaMoO_4$, 0.002 mg KI, 0.001 mg $CuSO_4$, and adjusted to pH 7 with KOH. All cultures were inoculated with a 16 h old pre-culture 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 (Spectronic® 20 GenesysTM, Spectronic Instruments, Model 4001/4).

Tobacco cells (Nicotinum tabacum L. cv. Bright Yellow-2) were cultivated in Murashige and Skoog medium (4.4 gL) at pH 5.8, completed with 30 g/L saccharose, 0.2 g/L $KH_2PO_4$, 50 mg/L myo-inositol, 2.5 mg/L thiamine and 0.2 mg/L 2,4-dichloro-phenoxyacetic acid. Tobacco cells were grown at a constant temperature (28°C) in the dark on a rotary shaker (110 rpm, Multi-Shaker PSU 20) and sub-cultured weekly by addition of 4 mL cells to 100 mL fresh liquid medium. Experiments were realized using cells in exponential growth phase after 5-6 days.

*Semi-preparative purification of surfactin homologues*

Milligrams of surfactin were obtained from AIBI (Agricultural University of Gembloux, Belgium) as a mixture of homologues with a purity of 95%. The different homologues were identified and relative proportions established by the combined use of matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry and reversed-phase high-performance liquid chromatography (HPLC) as described in the next section (surfactins: A C13, 22%; B C13, 17%; A C14, 28%; A C15, 33%). A mean value of 1050 amu for the different homologues present in the surfactin mixture was used as molecular weight to prepare the desired concentrations. The various homologues were purified from this mixture (solubilised in pure methanol) by semipreparative reverse phase HPLC (HP 1100 series system from Hewlett-Packard) on a Chromspher 5 C-18 (250 mm x 10 mm, 5 µmpacking, Chrompack) column by repeated injections of 100 µL aliquots. Surfactins were eluted isocratically with 78% acetonitrile in 0.1% trifluoroacetic (TFA) acid in milliQ water and at a flow rate of 4 mL/min. Samples were monitored spectrophotometrically at 280, 214, 254, 320 and 375 nm by means of a diode array detector (DAD). The collected fractions corresponding to each peak were pooled and evaporated to dryness (Speed-Vac® Plus SC110A, SAVANT). The residues were weighted, re-suspended in methanol to a concentration of 1 mg $ml^{-1}$ and adequate volumes were used to treat tobacco cells.

*Quantification of surfactins*

Surfactin production by the strains was determined from 10 mL samples of culture supernatant collected after growth for 72 h. Supernatant samples were loaded on Alltech Maxi-Clean SPE 900 mg C-18 column after activation and conditioning of the cartridge by flushing with ACN and water. After loading, the packing bed was washed successively with 10 mL of water and 5 mL of 40% ACN. The remaining material containing the elicitor then was desorbed with one ml of 100% methanol and evaporated to dryness. The residue was resolubilized in 80 µL of methanol and analyzed by HPLC on an Purospher® RP-18 analytical column (250 x 4.6 mm, 5 µm packing; Merck), in the isocratic mode (78% ACN + TFA 0.05% in MilliQ water). Amounts were calculated on the basis of the corresponding peak area at 280 nm.

The methylation of surfactins 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 equal volume of distilled water, methylated surfactin was purified by HPLC (same method than describe above). Linear derivatives of the $C_{14}$ surfactin homologue were kindly given by Dr Dufour S. (Laboratoire de Biologie Industrielle, University of Gembloux, Belgium) and were synthesized using method described (Dufour *et al.*, 2005).

*Extracellular pH measurements*

For pH variation estimations with indicator paper (Merck pH 4.0-7.0/0.2), 2 mL –samples of tobacco cell culture in exponential phase (approximately 0.15 g fresh cell/mL) were distributed in 12-wells microtitre plates placed at 28°C on an orbital shaker (110 rpm). Experiments were then realized with 10 mL of cells and the pH was registered every minute with a glass pH electrode (Microprocessor pH Meter 211; HANNA Instruments). Experiments of calcium channel blocker inhibition were realized by a previous addition of a solution of $LaCl_3$ 1.8% to obtain a final concentration of 20 mM and pH was adjusted before measurement to 4.8 with diluted KOH.

*Measurements of $H_2O_2$*

The production of $H_2O_2$ was monitored by chemiluminescence from the ferricyanide-catalysed oxidation of luminal using a luminometer (TD-20/20 Luminometer, TURNER DESIGNS). Briefly, tobacco cells in exponential phase were filtrated on Whatman® filter and resuspended in HEPES solution (31.8 g/L mannitol, 480 mg/L HEPES, 90 mg/L $K_2SO_4$, 73 mg/L $CaCl_2$, adjusted to pH 7) to obtain a final concentration of cell of about 0.15 g/mL. Cell suspensions were incubated in the dark at 28°C during 3 h on 110 rpm before being dispensed into 2 mL open vials. After treatment with surfactin, a 100 µL aliquot of the cell suspension was added to 100 µL of phosphate buffer (50 mM pH 7.9) and 100 µL of luminol 1.1 mM. The reaction was started by addition of 100 µL of $K_3[Fe(CN)_6]$ 14 mM freshly prepared and signal was integrated over the first 30 sec after reaction start. AOS production was compared to calibration values established with known concentrations of $H_2O_2$. In inhibition experiments, 50 µL of EDTA 2% was added 10 min before treatment with lipopeptides.

*Cell mortality determination*

A volume of 1.5 mL of treated tobacco suspension cells was filtered (Miracloth pore size 22-25 µm, Calbiochem) and resuspended in 1 mL of HEPES medium at pH 5.7. 100 µL of a 5% (w/v distilled water) Evans Blue solution was added and the mixture incubated during 10 min at 27°C on a rotary shaker (110 rpm). 800 µL were then filtered and washed with 10 mL of water to remove non-fixed dye. The fixed dye was finally solubilised by suspending washed cells in 1 mL of a 50% MeOH/1% SDS solution at 55°C during 30 min. The quantification of dead cells was carried out by measuring the absorbance of the coloured supernatant at 620 nm (Beckman Coulter AD 340). A calibration curve was realized by treating tobacco cells with rising 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).

*PAL and LOX activities*

Experiments for measurements of PAL activity were realized with cells in exponential phase cultured in 250 mL flask (50 mL of medium). After sampling and until spectrophotometric measures, samples were always manipulated at 4°C. At each samples, 5 mL of cell suspension were rapidly filtrated, washed with 10 mL of cold distilled water, and resuspended in extraction buffer (borate buffer 50 mM, 5% glycerol, 5 mM ß-mercaptoethanol, pH 8) to obtain a final concentration of 0.5 g/mL. Cells were then sonicated (Sonoplus, Bandelin electronic, Berlin) during 1 min at 70% maximal power. After 10 min incubation, cell suspensions were centrifuged during 10 min and supernatants were directly analysed. The reaction mixture consisted in 200 µL of extract added to 2.8 mL of dosage buffer (Sodium tetraborate 35.7 mM pH 8) containing L-phenylalanine 5.35 mM. PAL activity was measured by apparition of cinnamic acid at 290 nm. The reaction was performed at 40°C and absorbance was followed for 20 min on spectrophotometer UVIKON XS, BIO-TEK instruments.

For LOX measurements, cells were resuspended in phosphate buffer 100 mM pH 7 containing 0.4 g/L $Na_2S_20_5$ and 2.5 g/L Tween 20. After sonication and incubation for 1 h on ice, cells suspensions were centrifuged and supernatants were directly analysed. 50 µL aliquot was added to 2.940 mL of oxygened dosage buffer (phosphate buffer pH 7) and reaction was initiate by addition 10 µL of substrat (linolenic acid 18 mM in phosphate buffer and NaOH 0.05 M). LOX activity was measured by apparition of hydroperoxyde acid at 234 nm. Reaction was performed at 30°C and absorbance was followed for 20 min. The rates of increase were calculated from the initial linear portion of the curve and activities are expressed in pKatal.

*Northern blot analyses*
cDNA probes (565 pb) were synthesized by polymerase chain reaction (PCR) amplification of genomic DNA using forward primer (5'-TCTTGAATGCTGGAGTTTTTGG-3') for PAL1 and reverse primer (5'-TGTTCCATAATAGCAGCAGCCTC-3') for PAL2. Primers were chosen by comparison on five conserved sequences of PAL genes (X78269, D17467, AB008200, AB 008199 and M84466; Genbank, Pubmed). Probes (TabPal1 and TabPab2) were cloned with pGEM-T easy vector (Promega). For expression pattern, total RNA was extracted from tobacco cell powder (obtained by grinding fresh cells in liquid nitrogen and stocked at -80°C) by the phenol/sodium dodecyl sulphate (SDS) method, as described (Ausubel *et al.*, 1990). 15 µg of total RNA were separated on formaldehyde gels, blotted onto Hybond $N^+$ membranes (Amersham, Little Chalfont, UK) and hybridised with a mix of DNA probes TabPal1 and TabPal2 labelled by random priming in the presence of [α-32P]dATP, according to the procedure recommended by the manufacturer (Random Primers DNA Labelling System; Invitrogen, Carlsbad, Calif.). After hybridization, the blots were washed and then exposed to X-ray film (Fujifilm, Japan) for at least 24 h.

*Phenolic analyses*
Analyses of phenolic compounds were realized on tobacco cell culture supernatants extracted with ethyl acetate (1/1, vol/vol). Extracts were concentrated (Speed-Vac® Plus SC110A, SAVANT) and 10 µL were injected in HPLC on a lichrospher 100 RP C-18 column (250 x 4.6 mm, 5 µm packing; Merck, Darmstadt, Germany), using acetonitrile/water with trifluoroacetic acid (TFA) 0.1%. Compounds were eluted with a gradient of acetonitrile as follows: (time in min:% acetonitrile:% TFA 0.1%:flow in mL/min) (0:5:95:0,5; 2:5:95:0,5; 2,5:5:95:1; 5:5:95:1; 15:20:80:1; 25:25:75:1; 35:32:68:1; 36:95:5:1; 40:95:5:1; 41:5:95:1; 46:5:95:1). Identification of phenolic compounds was performed with LC-MS. The HPLC system was a Waters Alliance 2690. Elution program and column used were the same as described above. The MS instrument was a Waters Micromass Quattro Ultima Platinum triple quadrupole mass spectrometer running in positive ion mode. The MS was tuned to optimize the signal of each standard of the various phenolics by direct injection of diluted solutions. The capillary voltage used was 2.6 kV, and the source and desolvatation temperatures were set at 115 and 250°C, respectively. The cone and desolvatation gas flows were 50 and 630 L/h, respectively. The collision cell pressure was set at 2 µbars. Both instruments were controlled by the MassLynx software.

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# Discussion générale et perspectives

Que ce soit très concrètement pour son intérêt dans le développement de biopesticides ou d'un point de vue plus fondamental, l'ISR induite par les rhizobactéries est un phénomène particulièrement intéressant. Bien que les connaissances sur le sujet progressent à grand pas depuis quelques années, de multiples aspects demeurent assez méconnus. C'est notamment le cas du dialogue moléculaire qui s'établit entre l'organisme inducteur et la plante hôte pour initier le phénomène. Dans ce contexte, notre objectif principal est donc de contribuer à l'étude du langage utilisé par certaines souches de rhizobactéries pour pouvoir être perçues par les cellules végétales.

**Identification de nouveaux éliciteurs de l'ISR**

Par des approches similaires, telles que l'utilisation de mutants non ou surproducteurs et le traitement des racines par des extraits enrichis ou par les éliciteurs purifiés, la première partie des recherches menées durant cette thèse a conduit à la caractérisation des métabolites intimement liés à l'activité inductrice d'ISR de *Pseudomonas putida* BTP1 et *Bacillus subtilis* S499.

L'éliciteur de *P. putida* BTP1, l'ion *N*,*N*-dimethyl-*N*-tetradecyl benzylammonium (Figure 1A), est une molécule constituée d'un noyau benzylamine dont l'azote est quaternisé par trois chaînes carbonées, d'où sa dénomination, le NABD (*N*-alkylated benzylamine derivative). Son isolement permet tout d'abord d'élargir et de diversifier la gamme relativement restreinte des déterminants microbiens de l'ISR connus jusqu'alors (chapitre III). D'un point de vue plus général, le NABD apparaît aussi comme un nouveau type de molécule isolée chez les *Pseudomonas* car, à notre connaissance, une telle structure n'a jamais été rapportée.

Chez *Bacillus*, même si les lipopeptides sont des molécules connues pour leur activité anti-microbienne ou participant à la formation de biofilm et à la colonisation (Ongena and Jacques, 2008), leur capacité à induire une réponse défensive chez les plantes n'avait pas encore été mise en évidence, leur attribuant ainsi un nouveau rôle biologique immuno-stimulateur. Le potentiel des lipopeptides cycliques comme éliciteurs d'une résistance chez les plantes est appuyé par les récents travaux de Tran et ses collaborateurs, qui ont démontré que la massétolide A produite par *Pseudomonas fluorescens* protégeait les plants de tomate contre *Phytophthora infestans* (Tran *et al.*, 2007). La surfactine (Figure 1B) et la fengycine (Figure 1C), ainsi que la massétolide A sont de nouvelles molécules de type antibiotique impliquées dans l'induction de l'ISR à ajouter à celles précédemment identifiées, comme le DAPG ou la pyocyanin (De Vleesschauwer *et al.*, 2006; Weller *et al.*, 2004; Siddiqui and Shaukat, 2003).

En raison de leur nature chimique, ces lipopeptides et le NABD peuvent également être considérés comme une nouvelle classe d'éliciteurs représentée par des molécules amphiphiles.

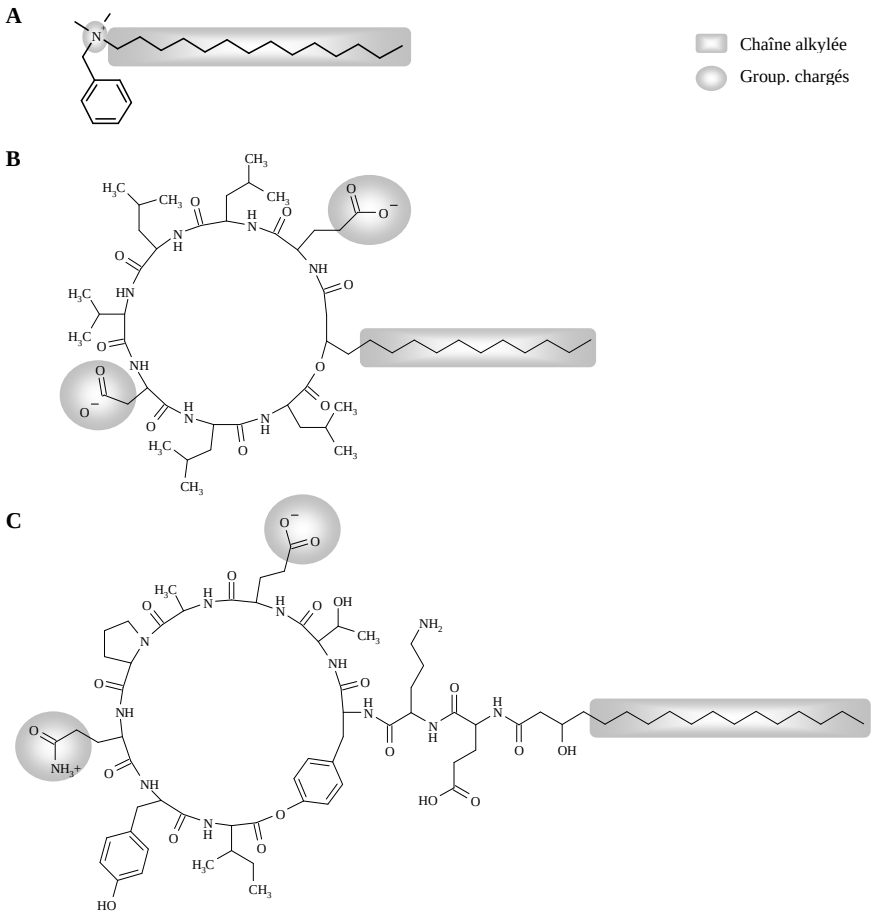


**Figure 1. A.** Structure du NABD produit par *Pseudomonas putida* BTP1 (NABD, *N*-alkylated benzylamine derivative). B et C. Exemple de lipopeptides issues de *Bacillus subtilis* S499 (surfactine, **B** et fengycine, **C**). Des structures plus détaillées sont illustrées dans une revue récente dédiée aux lipopeptides de *Bacillus* (Ongena and Jacques, 2008).

### Facteurs influençant la biosynthèse et la production *in situ* des éliciteurs

La biosynthèse des molécules élicitrices de l'ISR par le PGPR peut être modulée par de multiples facteurs physiologiques et environnementaux. Une preuve supplémentaire de leur implication passe donc par la démonstration d'une production effective lorsque les cellules se développent sur les racines. Plus généralement, l'objectif est également de mieux comprendre l'influence des paramètres de la rhizosphère sur l'expression des gènes de synthèse et/ou sur la

production de métabolites impliqués dans le contrôle biologique par les rhizobactéries bénéfiques.

Même si elles n'ont pas pu être mesurées de manière précise, des quantités significatives de NABD ont été retrouvées dans la rhizosphère de plants traités par BTP1, suggérant une production effective *in situ* par la souche. Cependant, aucune information n'est disponible sur les gènes de biosynthèse de cette molécule. Cela empêche, à ce stade, de générer dans BTP1 un système rapporteur qui nous aurait permis de visualiser la distribution des populations de BTP1 associées aux racines qui expriment les gènes de biosynthèse, mais aussi de quantifier cette expression de manière relative comme il a été rapporté pour l'étude du DAPG produit par *Pseudomonas fluorescens* CHA0 (Notz *et al.*, 2001), de la putisolvine chez *Pseudomonas putida* PCL1445 (Dubern and Bloemberg, 2006) ou, d'une manière plus générale, chez des microorganismes de la rhizosphère (Bloemberg, 2007). Toutefois, une approche *in vitro* a été développée afin d'étudier l'influence de certains paramètres physiologiques, nutritionnels et physico-chimiques sur la production du NABD par *P. putida* BTP1.

Sa cinétique de production est typique d'un métabolite secondaire. En exploitant la technologie du chémostat, nous avons montré que sa synthèse n'est effective qu'à un taux de croissance faible. Il est très probable que ce soit également le cas lors du développement de la bactérie dans la rhizosphère, le taux de croissance étant limité par la disponibilité en nutriments libérés dans les exsudats racinaires ainsi que par la compétition avec les autres populations (Di Mattia *et al.*, 2002; Espinosa-Urgel *et al.*, 2002; Rainey, 1999). Chez les bactéries, dans des conditions de stress ou pendant la phase stationnaire de croissance, la transcription de certains gènes est intimement liée au facteur $\sigma^S$ (Nystrom, 2004). Il est dès lors envisageable qu'il puisse aussi intervenir dans la régulation de la biosynthèse du NABD chez BTP1. D'autres part, celle-ci pourrait également être dépendante du système GacA/GacS, qui régule la production de certains métabolites impliqués dans le biocontrol (Bertani and Venturi, 2004; Chin-A-Woeng *et al.*, 2003; Haas and Keel, 2003). Enfin, toujours concernant les systèmes de régulation potentiellement impliqués dans la biosynthèse du NABD, des expériences préliminaires réalisées avec des biosenseurs sensibles aux N-Acyl-homosérine lactones, ne semblent pas suggérer l'implication du quorum-sensing.

D'une manière générale, les acides aminés qui sont une composante importante des exsudats racinaires peuvent jouer un rôle essentiel pour le développement et la production de métabolites secondaires par *Pseudomonas* dans la rhizosphère, notamment dans le cas de la phenazine-1-carboxamide, un métabolite antifongique produit par *Pseudomonas chlororaphis* PCL1391 (Van Rij *et al.*, 2004). Notre recherche montre que la synthèse du NABD est aussi étroitement conditionnée par la présence d'acides aminés dans le milieu, et en particulier la phénylalanine, dont l'ajout accroît significativement la production. Le rôle exact de la

phénylalanine dans la voie de biosynthèse du NABD reste encore à être élucidé. Cependant, la non détection de la benzylamine, le noyau de l'éliciteur, lors d'analyses du surnageant de culture de BTP1 (résultats non montrés) ainsi que l'absence, à notre connaissance, d'enzymes permettant sa formation à partir de la phénylalanine seraient défavorables à l'hypothèse d'une utilisation de l'acide aminé comme précurseur de biosynthèse de l'éliciteur. Une seconde hypothèse peut être formulée qui se baserait sur un rôle régulateur de la phénylalanine. En effet, chez *Escherichia coli*, la protéine TyrR agit comme répresseur ou un activateur de transcription. Lorsqu'elle est fixée à certains promoteurs, cette protéine peut interagir avec la RNA polymérase en présence de phénylalanine (Pittard *et al.*, 2005). Par ailleurs, une protéine similaire, PhhR, a été récemment décrite chez *Pseudomonas putida* KT2440. Elle activerait la synthèse d'une enzyme impliquée dans le métabolisme de la phénylalanine (Herrera and Ramos, 2007). Il est donc imaginable que ce facteur de régulation puisse intervenir dans la régulation d'une enzyme impliquée dans la biosynthèse de l'éliciteur.

La production du NABD par la rhizobactérie est aussi négativement influencée par certains oligo-éléments dont le fer. La forte inhibition de production en présence de fer suggère une régulation négative comme c'est le cas pour les sidérophores et notamment ceux de type pyoverdines (Budzikiewicz, 2004). Chez les *Pseudomonas*, la transcription des gènes de biosynthèse des pyoverdines est effective suite à la fixation au niveau du promoteur de la protéine pfrI. Son activité est cependant contrôlée par la protéine Fur, un senseur de la concentration en fer dans le milieu extérieur. En présence d'ions $Fe^{2+}$, la protéine Fur inhibe pfrI qui n'est alors pas en mesure d'exercer son rôle d'activateur de transcription (Venturi *et al.*, 1995). La synthèse du NABD pourrait donc également être sous la dépendance de la protéine régulatrice Fur.

Enfin, l'étude d'autres paramètres abiotiques montre également qu'une restriction en oxygène dissous disponible ou une acidification du milieu n'ont pas d'effet négatif drastique sur le taux de production du NABD. Globalement, tous ces résultats suggèrent que les conditions nutritionnelles et physico-chimiques dictant l'état physiologique des cellules bactériennes *in situ* peuvent être propices à une synthèse effective de l'éliciteur au niveau des racines.

Quelques études ont été réalisées avec des bactéries gram-négatives mais peu de recherches se sont focalisées sur la manière dont est influencée la synthèse des composés bioactifs chez *Bacillus* en fonction de facteurs liés à la plante hôte. Or, la présence de lipopeptides éliciteurs de l'ISR produits par *Bacillus subtilis* dans la rhizosphère de haricot a également été démontrée lors de nos expériences. Comme dans le cas du NABD, il est difficile de quantifier avec précision les lipopeptides extraits du sol. Cependant, de très récents résultats obtenus au laboratoire confirment la production de surfactine *in situ* par *Bacillus subtilis* S499.

Ces études ont notamment montré un bon taux d'expression des gènes de synthèse de ce lipopeptide dans les cellules en contact avec les racines mais aussi dans des cultures *in vitro* reconstituées à partir d'exsudats racinaires. Enfin, tout comme la production de l'éliciteur de BTP1, celle de la surfactine produite par *Bacillus* est accrue à des taux de croissance faibles et est aussi effective en milieu peu oxygéné (Nihorimbere, *unpublished results*).

**Spécificité des éliciteurs par rapport à l'hôte végétal**

D'un point de vue fondamental comme appliqué, la détermination de l'activité biologique d'un microorganisme est un élément important, puisqu'elle permet d'éventuellement comprendre le mode d'action de la souche ou de diversifier le spectre d'action d'un produit commercialisable.

Les recherches menées avec *Pseudomonas putida* BTP1 ont permis de montrer son potentiel à stimuler l'ISR chez plusieurs familles végétales, puisque la souche est en mesure de réduire de façon significatif les maladies du concombre et du haricot ou de la tomate respectivement causées par *Colletotrichum lagenarium* et *Botrytis cinerea*. De plus, des résultats encourageants ont également été obtenus chez le riz (De Vleesschauwer, *unpublished results*), suggérant une activité biologique de la souche sur les monocotylédones. Par ailleurs, la souche ne se limite pas à protéger les plants contre des pathogènes foliaires mais induit également l'ISR contre des agents infectieux des racines, comme *Pythium aphanidermatum* (Ongena *et al.*, 1999). Sur plusieurs phytosystèmes, l'activité élicitrice du NABD, utilisé à une concentration de 1µM dans des systèmes hydroponiques, est équivalente à celle conférée par les cellules vivantes de BTP1, suggérant que le composé soit l'éliciteur principal de la souche. Cependant, dans le cas de la tomate, il semble qu'un composé supplémentaire intervienne, puisque la protection conférée par le NABD est sensiblement réduite par rapport à la bactérie.

Dans le cas des plants traités par *Bacillus subtilis*, un phénomène similaire est remarqué, puisque les lipopeptides sont actifs sur des plants entiers de haricot et de tomate, ainsi que sur des cellules de tabac en culture *in vitro* ou sur tubercule de pomme de terre (Ongena *et al.*, 2005), mais ne semblent pas être impliqués dans l'induction de l'ISR contre *Colletotrichum lagenarium* chez le concombre.

D'une manière générale, une variation de l'activité des éliciteurs est mise en évidence selon le pathosystème étudié et une spécificité semble donc se dégager selon le pathogène et/ou la plante utilisés. Cependant, dans le cas du NABD, celle-ci serait plutôt axée sur la plante, puisque les protections observées varient chez le haricot et la tomate alors que le même pathogène fut utilisé dans les deux cas. Par contre, dans le cas de *Bacillus subtilis* S499, il est encore difficile d'établir une spécificité vis-à-vis du pathogène ou de l'espèce végétale, et

celle-ci ne pourra être éventuellement mise en évidence qu'après des expériences utilisant des pathogènes communs au concombre et au haricot ou à la tomate. Cette spécificité a déjà été relatée chez d'autres souches de PGPR. C'est le cas de *Pseudomonas putida* WCS358, active sur le haricot, une fabacée, et sur *Arabidopsis*, une brassicacée mais cependant inactif sur le radis (Bigirimana and Höfte, 2002; Van Wees *et al.*, 1997; Leeman *et al.*, 1995). D'autre part, une spécificité de l'éliciteur vis-à-vis du pathogène ou de la plante a aussi été observée et est très bien illustrée par les travaux de Méziane et ses collaborateurs (Meziane *et al.*, 2005). En effet, via l'utilisation de mutants ou des éliciteurs purifiés, ils ont démontré que WCS358 pouvait induire l'ISR chez *Arabidopsis* infectée par *Pseudomonas syringae* grâce à ses lipopolysaccharides (LPS), ses flagelles ou sa pseudobactine, alors que chez la tomate ou le haricot infectés par *Botrytis cinerea*, les LPS et les pseudobactines, mais à priori pas les flagelles, étaient impliqués. Enfin, un dernier exemple est celui du 2,3-butandiol, produit par *Pseudomonas chlororaphis* O6, qui participe à l'ISR chez le tabac contre *Erwinia carotovora* mais pas contre *Pseudomonas syringae* (Han *et al.*, 2006).

**Reconnaissance des éliciteurs par les cellules végétales.**

Les lipopeptides et le NABD constituent les principaux éliciteurs de l'ISR de leurs souches respectives dans les phytosystèmes étudiés. Or, *Pseudomonas putida* BTP1 et *Bacillus subilits* BC25, une souche surproductrice à la fois de surfactine et de fengycine, sont toutes les deux en mesure de stimuler la lipoxygénase (LOX) (Ongena *et al.*, 2004; Chapitre VIII), une enzyme considérée pour intervenir dans les réponses au stress provoqué par l'attaque de pathogènes ou par des blessures (Shah, 2005). Ces deux éliciteurs pourraient ainsi agir de manière similaire en stressant les cellules de l'hôte grâce à leurs propriétés amphiphiles. Durant la dernière partie de notre travail de thèse, nous avons cherché à approfondir ces investigations en utilisant des cellules de tabac en culture *in vitro*. Ces recherches ont montré que des réactions de défenses étaient fortement exprimées lors de traitement des suspensions de cellules avec de la surfactine (voir section suivante). En testant divers homologues purifiés de cette molécule, nous avons également observé une dépendance de l'activité élicitrice des réactions de défense en fonction de la longueur de la chaîne lipidique qui elle-même influence le caractère amphiphile et le pouvoir détergent de la molécule. Ces observations suggèrent que la perception de la surfactine par les cellules végétales puisse résulter principalement d'une interaction avec la bicouche lipidique de la membrane plasmique. Les propriétés élicitrices de la surfactine sont aussi fortement altérées après linéarisation de la chaîne peptidique et/ou suite à la méthylation des deux groupements carboxyliques portés par l'acide glutamique et l'acide aspartique. Or ces

deux réactions diminuent également notablement le caractère amphiphile de la molécule, confirmant l'hypothèse d'une perturbation des membranes de la cellule hôte grâce aux propriétés surfactantes du lipopeptide. D'autre part, aucun effet néfaste sur la croissance ni aucune mort cellulaire significative n'ont été observés respectivement sur plantes entières ou sur cellules de tabac traitées par la surfactine pure. Cela suggère que la surfactine puisse interagir avec la membrane plasmique sans former de pores irréversibles, mais en perturbant suffisamment celle-ci afin d'activer une cascade d'événements aboutissant à des réactions de défense. Une hypothèse similaire a d'ailleurs été récemment émise pour expliquer l'effet anti-prolifératif de la surfactine sur les cellules LoVo du cancer du colon, qui ne paraissent pas être lysées alors que des changements sont notés dans l'expression des protéines régulant la survie de la cellule (Kim *et al.*, 2007).

Toujours sur les cellules de tabac, les autres lipopeptides de *Bacillus subtilis* ont des effets différents. En effet, les iturines ne semblent pas actives pour induire l'ISR sur plantes entières (Leclère *et al.*, 2005). Selon nos résultats, elles ne provoquent pas non plus de réactions pouvant être associées à la mise en place d'une réponse défensive dans les cellules de tabac tout comme elles étaient inactives sur cellules de tubercule de pomme de terre (Ongena *et al.*, 2005) . Par ailleurs, un effet notable mais moins important qu'avec la surfactine, est observé lors du traitement des racines par des mutants surproducteurs de fengycine ou par ce lipopeptide purifié. De plus, sur les cellules de tabac, la fengycine induit globalement des réactions moins marquées et/ou plus tardives. Même si ces deux lipopeptides ont des structures assez proches, la fengycine présente des activités détergentes plus faibles que la surfactine, pouvant aussi expliquer la différence d'action sans contredire l'hypothèse de perturbation membranaire.

Pour l'éliciteur de BTP1, des essais préliminaires n'ont montré aucune activité détectable sur les cellules de tabac (données non présentées), suggérant soit que le NABD n'ait pas de propriétés suffisamment détergentes sur les membranes de tabac ou soit que son mode de reconnaissance est différent. Le test des multiples dérivés de la molécule que nous avons synthétisés n'a malheureusement pas pu être réalisé afin de confirmer cette hypothèse. Cependant, le groupement aminé aromatique de la molécule semble important puisque le traitement par la benzylamine au niveau des racines de trois types de plantes engendre un effet protecteur du même ordre que celui obtenu en appliquant la molécule complète ou en traitant par les bactéries. De plus, Kawano et al ont montré l'apparition d'un burst oxydatif et l'augmentation de la concentration intracellulaire en ions $Ca^{2+}$ dans les cellules de tabac après le traitement des cultures par la benzylamine (Kawano *et al.*, 2000). Ces résultats suggèrent ainsi que l'aspect amphiphile du NABD soit moins important que pour la surfactine pour la perception par les cellules végétales.

| | |
|---|---|
| Pyo. CHAO | Lys—Asp*-Ser—Thr—Ala—aThr-Lys—Asp—Orn* |
| Pyo. WCS358 | Ser—Lys—Gly—Orn*-Lys—Orn*-Ser |
| Pyo. WCS374 | Asp—Orn*-Lys—Thr—Ala—Ala—Orn*-Lys |
| MALP-2 | Cys—Gly—Asn—Asn—Asp—Glu—Ser—Asn—Ile—Ser—Phe—Lys—Glu—Lys |
| Flg15 | Arg—Ile—Asn—Ser—Ala—Lys—Asp—Asp—Ala—Ala—Gly—Leu—Gln—Ile—Ala |
| Surfactine | Glu—Leu—Leu—Val—Asp—Leu—Leu |
| Fengycine | Glu—Orn—Tyr—aThr-Glu—Ala—Pro—Gln—Tyr—Ile |
| Massétolide A | Leu—Glu—aThr-aIle—Leu—Ser—Leu—Ser—Ile |

**Figure 2.** Séquence d'acides aminés de différentes molécules actives. Pyo CHAO, Peptide de la pyoverdines de *Pseudomonas aeruginosa* CHAO ; Pyo WCS358, Peptide de la pyoverdines de *Pseudomonas putida* WCS358 ; Pyo WCS374, Peptide de la pyoverdines de *Pseudomonas fluorescens* WCS374 ; MALP-2, séquence d'acides aminés du lipopeptide de *Mycoplasma fermentans* reconnus par les TLR2 de certaines cellules animales ; Flg15, peptide de la flagelline de *Pseudomonas putida* et *Pseudomonas syringae* ; Surfactine, séquence d'acides aminés de la surfactine ; Fengycine, séquence d'acides aminés de la fengycine ; Massétolide A, séquence d'acides aminés de la massétolide A. *Acides aminés hydroxylés.

Cependant, sur base de nos résultats, l'implication de récepteurs dans la reconnaissance des éliciteurs de BTP1 et S499 ne peut pas être exclue. En effet, la flagelline, qui est un éliciteur de réponses défensives chez les plantes, est perçue par *Arabidopsis* via les récepteurs FLS2 reconnaissant le fragment flg15, une séquence de 15 acides aminés (Felix *et al.*, 1999). D'autre part, chez les mammifères, certains lipopeptides bactériens sont connus pour activer les macrophages, les fibroblastes ou les lymphocytes pour déclencher la production de cytokines (Okusawa *et al.*, 2004). Ceux-ci seraient reconnus grâce à des récepteurs Toll-like (TLRs), et en particulier les TLR2. Toutefois, la présence de tels récepteurs à la surface des cellules de plantes reconnaissant spécifiquement une séquence d'acides aminée n'est à ce jour pas connue. D'autre part, aucune séquence partielle n'est partagée entre la surfactine, la fengycine et les autres molécules de nature peptidique impliquées dans l'ISR, comme le massétolide, la flagelline ou les pyoverdines (Figure 2). Il est donc difficile d'imaginer que ces diverses molécules, dans leurs conformations tridimensionnelles, présentent un motif similaire reconnaissable par une même protéine membranaire. Dans l'éventualité d'une interaction avec un récepteur, la présence sur les cellules de la plante de récepteurs spécifiques aux différentes molécules est probable.

**Evénements précoces associés à la perception des éliciteurs**

Le traitement des suspensions de cellules avec de la surfactine à des concentrations de l'ordre du micromolaire déclenche une alcalinisation du milieu extracellulaire suivie par une production d'espèces oxydantes, sans toutefois provoquer de mort cellulaire. De plus, l'ajout du lipopeptides à des concentrations variables a permis la détermination d'une concentration seuil pour une stimulation optimale de ces deux paramètres. Enfin, l'utilisation de substances chélatrices du calcium ou bloquant les canaux calciques inhibe fortement le phénomène, suggérant l'implication du $Ca^{2+}$ dans la mise en place des réponses par les cellules de tabac. Les événements déclenchés dans les cellules de tabac suite à la perception de la surfactine sont repris dans la Figure 3.

A plus long terme, la perception de la surfactine induit aussi une nette stimulation d'enzymes liées aux réponses défensives des plantes, comme la phénylalanine ammonia lyase (PAL), qui est l'enzyme d'entrée dans la voie des phenylpropanoides, et la lipoxygénase (LOX), appartenant à la voie des oxilipines (Shah, 2005; Dixon *et al.*, 2002). L'activation de la LOX par la fengycine n'a pas pu être investiguée, cependant, le traitement des suspensions de cellules de tabac avec la surfactine induit une bonne stimulation de la voie des oxilipines. Celle-ci peut aboutir à la production de plusieurs métabolites antimicrobiens et de molécules impliquées dans la signalisation de la réponse au stress immunitaire, comme le jasmonate. Ce phénomène a également été observé sur des plantes entières, puisque les mutants de *Bacillus* surproducteurs de surfactine sont en mesure de déclencher une augmentation de l'activité LOX qui est corrélée avec une restriction de la maladie dans les feuilles de tomate (chapitre 8). Enfin, des résultats similaires ont aussi été observés sur la tomate (Adam, 2008) et le haricot initialement traités par *Pseudomonas putida* BTP1 (Ongena *et al.*, 2004).

Dans le cas de la PAL, l'augmentation de l'activité enzymatique est accompagnée d'une accumulation d'ARNm spécifiques, signifiant une transcription effective des gènes de biosynthèse codant pour l'enzyme. Enfin, via l'analyse par chromatographie, puis par chromatographie couplée avec un spectromètre de masse, une modification du profil des phénylpropanoides a été observée. Bien que tous les composés accumulés suite au traitement par la surfactine n'aient pas été identifiés, l'apparition significative d'acide salicylique et l'augmentation des taux d'acide cinnamique et hydroxy-benzoïque au dépend de l'acide ferulique et coumarique suggèrent une réorientation de la voie de phénylpropanoïdes. Celle-ci pourrait s'expliquer par une plus grande activité de l'acide benzoïque 2-hydroxylase, qui catalyse la formation de l'acide salicylique, et une répression de l'acide 4-hydroxylase. Toutefois, l'utilisation de l'acide coumarique ou de l'acide ferulique comme précurseur d'autres composés intervenant dans les mécanismes de défenses ne peut être exclue.

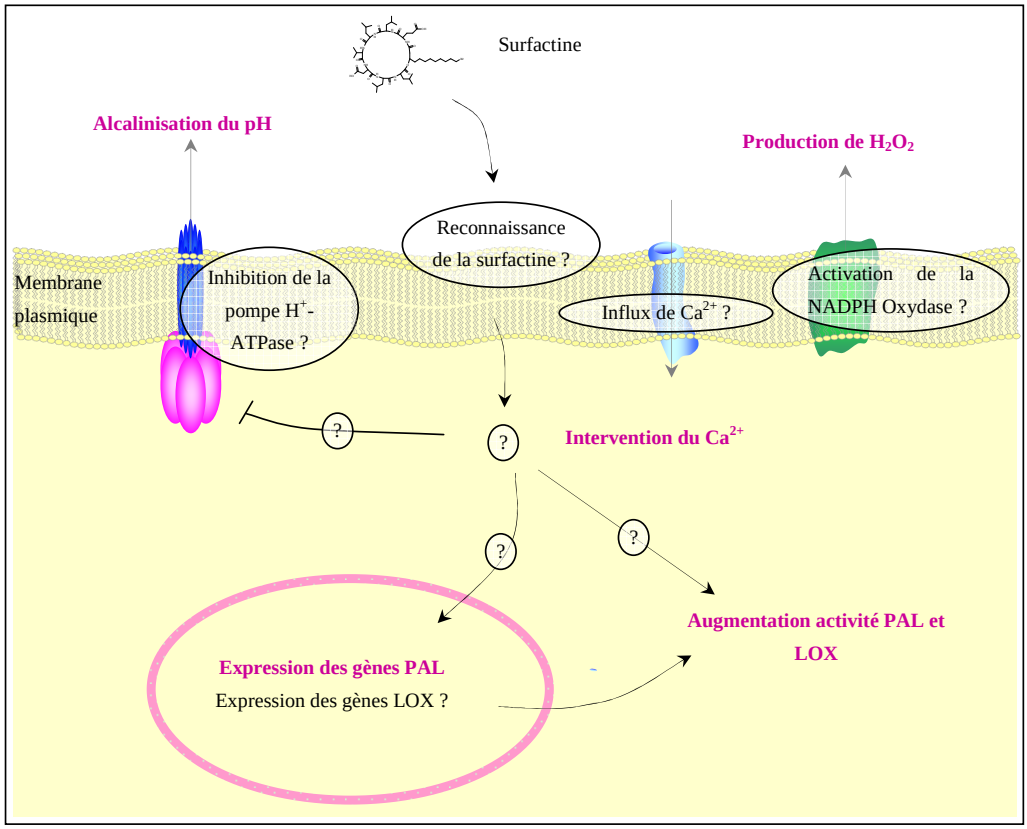


**Figure 3.** Evénements déclenchés chez les cellules de tabac après élicitation par la surfactine.

Les expérimentations avec les cellules de tabac en culture *in vitro*, qui sont les premières à être réalisées avec des éliciteurs spécifiques de l'ISR, ont abouti à la caractérisation de plusieurs événements précoces. De façon intéressante, certains de ces phénomènes sont communs à ceux induits par des éliciteurs non spécifique de l'ISR, comme la flagelline (Felix *et al.*, 1999) et les lipopolysaccharides (Gerber *et al.*, 2004), ou isolés uniquement chez des agents pathogènes, comme la cryptogéine (Garcia-Brugger *et al.*, 2006). Dans les recherches basées sur la reconnaissance de la crytogéine, certaines auteurs supposent que les premiers événements déclenchés, comme l'alcalinisation du milieu extracellulaire et la production d'espèces oxydantes soient respectivement liées à l'inhibition de la pompe $H^+$-ATPase par le $Ca^{2+}$ et à l'activation de la NADPH oxidase, responsable de la production de peroxyde d'hydrogène (Lecourieux *et al.*, 2006). Il est donc envisageable que ces deux protéines interviennent aussi dans les réponses à la surfactine.

Toujours concernant les réponses de la plante à l'élicitation, des études récentes réalisées *in situ* montrent une augmentation des niveaux de $H_2O_2$ à la surface des racines de riz, ainsi que dans les feuilles, suite au traitement par la pyocyanine produite par *Pseudomonas*

*aeruginosa* 7NSK2 (De Vleesschauwer *et al.*, 2006). Selon l'auteur, les microburst oxydatifs ainsi générés seraient responsables de la résistance conférée par la souche contre *Magnaporthe grisea* et permettrait à la plante de répondre plus fortement et rapidement après l'infection par l'agent pathogène. Un effet de mise en alerte est aussi décrit chez la même plante traitée par *Serratia plymuthica* IC1270, qui accentue la production d'espèces oxydante lors de l'infection. Transposer les mécanismes déclenchés chez les cellules végétales en culture *in vitro* à ceux induits chez une plante entière traitée par un PGPR reste difficile. Cependant, il peut être envisageable que les événements précoces observés *in vitro* soient aussi effectifs *in vivo* et soient à la base de la réponse qui conduira à une résistance de l'hôte à une attaque ultérieure par un pathogène.

Nos recherches ont également permis de mettre en évidence une variation des réponses en fonction de la plante. Sur le concombre, les lipopeptides de *Bacillus subtilis* n'interviennent pas dans l'ISR contre *Colletotrichum lagenarium*, suggérant qu'ils ne soient pas reconnus par la plante ou pas en mesure de suffisamment stimuler l'hôte. Dans les suspensions de cellules de tabac, aucune variation du pH n'a été mesurée après l'addition de fengycine. De plus, même si ce lipopeptide induit une accumulation de transcrits codant pour la PAL, ainsi qu'une stimulation de son activité, celle-ci est plus tardive comparé à la surfactine. Chez la pomme de terre, la fengycine induit l'accumulation de composés phénoliques alors que rien n'est observé avec la surfactine (Ongena *et al.*, 2005). Ainsi, chez une même famille végétale, les deux lipopeptides présentent des actions différentes, suggérant que la surfactine et la fengycine ne déclenchent pas les mêmes mécanismes. Cependant, il est aussi imaginable que les deux lipopeptides aient une activité additionnelle, puisque les expériences réalisées sur plantes entières présentent souvent des taux de protection plus élevés lors de traitements par des mutants surproducteurs de surfactine et de fengycine (ou par les deux lipopeptides purifiés) en comparaison à celles traitées par l'un des deux uniquement. Ainsi, selon le végétal utilisé, l'un ou l'autre lipopeptide n'aurait pas d'activité, cependant, un traitement combiné permettrait d'obtenir une action synergique et d'observer une meilleure réponse globale de la plante. Une hypothèse similaire a aussi été soumise par Audernaert et ses collaborateurs, qui ont observé une stimulation de la PAL au niveau des racines de la tomate, et ont suggéré que la pyochéline et la pyocyanine produites par *Pseudomonas aeruginosa* 7NSK2 agiraient en synergie pour protéger la plante contre *Botrytis cinerea* (Audenaert *et al.*, 2002).

**Perspectives**

Que ce soit au niveau fondamental de la compréhension du phénomène d'immunité induit par les rhizobactéries ou d'un point de vue plus appliqué, de nombreuses perspectives découlent de nos recherches.

Le NABD et les lipopeptides sont des molécules élicitrices de l'ISR possédant des propriétés amphiphiles. Une des premières questions d'intérêt est la façon dont elles interagissent avec les cellules de la plante. Une approche serait d'approfondir les recherches initiées sur les sous-structures nécessaires pour leur activité biologique. Celles-ci pourraient utiliser les dérivés synthétisés lors de ce travail dans le cas du NABD, ou des homologues de lipopeptides variant dans leur longueur de chaîne, leur composition en acides aminés ou dans leur conformation. Une seconde approche complémentaire pourrait se focaliser sur le mode de perception de ces molécules afin de savoir s'il résulte de la présence de récepteurs sur/dans les cellules de la plante ou d'une perturbation membranaire. Pour ce faire, une estimation de l'ampleur et de la durée de la déstabilisation de la bicouche lipidique pourrait être entreprise par des méthodes biophysiques (Isothermal Titration Calorimetry) qui permettraient en outre de déterminer la nature chimique de l'interaction (hydrophobe, électrostatique) entre éliciteurs et membrane. La recherche de récepteurs, quant à elle, pourrait s'accomplir en employant des molécules élicitrices marquées. Dans le cas des lipopeptides, une solution serait l'ajout, au milieu de culture de *Bacillus subtilis* S499, d'acides aminés constitutifs de la surfactine ou de la fengycine marqués au $^{14}C$ ou au $^{3}H$. Pour le NABD, vu que sa synthèse chimique a été mise au point, il serait aisé d'y insérer un groupement radioactif. Enfin, la synergie d'action des éliciteurs mériterait d'être explorée plus en profondeur, en particulier entre la surfactine et la fengycine.

Nos résultats suggèrent une production effective des éliciteurs *in situ*, mais une étude plus complète doit être envisagée si on veut mieux comprendre les facteurs de la rhizosphère susceptibles d'influencer leur biosynthèse. D'une manière plus globale, cette recherche contribuerait à la compréhension du fonctionnement de ces rhizobactéries dans l'environnement spécifique qu'elles colonisent. Cette aspect comprend notamment l'optimisation des méthodes d'extraction et de quantification des composés sécrétés *in situ* via des méthodes analytiques de pointe en LC-MS. Une seconde approche est basée sur la mesure de l'expression des gènes de biosynthèse par un système rapporteur. Le système de gène rapporteur pourrait alors permettre la quantification du niveau d'expression en réponse à différents facteurs physiologiques ou biotiques et abiotiques influencés par la plante

(composition des exsudats racinaires, influence de son stade de développement) sur la production des éliciteurs. La présence d'autres microorganismes dans la rhizosphère pourrait aussi être envisagée et ainsi faire intervenir la notion de compétition pour l'espace et les nutriments et donc nous rapprocher des conditions réelles. Concernant *Bacillus subtilis*, les gènes intervenant dans la biosynthèse des lipopeptides ainsi que certains facteurs de régulation sont connus (système ComA/ComP du quorum sensing, facteurs de transcription CodY, DegU et PerR) rendant possible le développement de cette perspective. Cependant, dans le cas du NABD produit par *Pseudomonas putida* BTP1, le(s) gène(s) impliqué(s) n'est(ne sont) malheureusement pas connu(s), empêchant la mise au point d'un tel système rapporteur. Il serait donc nécessaire au préalable d'identifier ce(s) gène(s) (par exemple par hybridation soustractive), ainsi qu'un éventuel système de régulation

Toujours d'un point de vue fondamental, des recherches plus axées sur la plante méritent aussi d'être approfondies, en particulier sur les événements précoces et les mécanismes de défenses systémiques. Par exemple, l'implication du calcium dans l'initiation des événements précoces doit être confirmée, ainsi qu'une éventuelle cascade faisant intervenir des MAP kinases. D'un point de vue métabolomique, il est intéressant d'identifier les métabolites secondaires induits (par LC-MS/MS) et de tester leur activité fongitoxique. L'extrapolation des premiers événements visualisés dans les cellules de tabac sur des plants entiers serait aussi une formidable occasion de démontrer l'action des éliciteurs. Visualiser les mécanismes induits au niveau des cellules racinaires des plants traités serait un bon moyen d'y parvenir. Enfin, d'un point de vue plus global, un aspect important concerne la spécificité de la réponse, à savoir si ce type de réponses est présent chez plusieurs éliciteurs de l'ISR et tracer un possible modèle développant les événements déclenchés suite à leur perception, ainsi que les divergence/similitudes avec les éliciteurs de pathogènes.

La notion de spécificité devrait aussi être abordée par rapport au microorganisme producteur. L'effet inducteur de l'ISR du NABD et des lipopeptides est-il spécifique aux deux souches étudiées dans ce travail ou au contraire, ces molécules peuvent-elles être considérées comme éliciteurs « généraux » pour d'autres souches de *Pseudomonas* ou de *Bacillus*? Le NABD est également produit *in vitro* par *Pseudomonas putida* KT2440 mais rien n'indique que cette souche induise ou non l'ISR. Il serait donc intéressant de savoir si d'autres *Pseudomonas* sont en mesure de produire le NABD et d'induire l'ISR. Un raisonnement similaire peut aussi être appliqué dans le cas des lipopeptides de *Bacillus* puisque de nombreuses souches de *B. subtilis* et d'autres espèces sont capables de produire de la surfactine. On peut donc imaginer qu'elles soient toutes potentiellement stimulatrices de

résistance chez les plantes. S499 pourrait se distinguer par son aptitude à coloniser la rhizosphère ou par les quantités et types de lipopeptides produits *in situ*.

D'un point de vue appliqué, nos résultats démontrent une capacité réelle des deux souches à conférer une protection des plantes dans plusieurs pathosystèmes mais nous ne disposons pas encore de suffisamment d'informations pour concevoir une utilisation en tant que biopesticides. C'est pourquoi, des expériences sur une plus large gamme de plantes sont nécessaires. De même, l'élargissement du spectre d'efficacité contre d'autres pathogènes (champignons, virus, bactéries ; pathogène racinaire, foliaire) serait un atout primordial pour la commercialisation d'un produit. Des tests en conditions plus proches de la réalité (serres industrielles, champs) et à plus grande échelle pourraient alors être entrepris, que ce soit avec des cellules vivantes des rhizobactéries, ou avec des spores dans le cas de *Bacillus subtilis*. En effet, toujours dans le cadre du développement d'un biopesticide, la formulation est un autre facteur important et doit prendre en considération un bon stockage du produit, tout en conservant ses propriétés biologiques. Par ailleurs, les lipopeptides sont également impliqués dans la colonisation des racines ou dans un antagonisme direct des phytopathogènes, renforçant alors l'intérêt de la souche comme biopesticide. Enfin, toujours dans le but du développement d'un produit, il serait également intéressant de tester la viabilité à long terme des souches au niveau des racines de la plante. En effet, la commercialisation d'un produit ne nécessitant pas d'applications régulières serait un avantage conséquent d'un point de vue marketing.

Enfin, des observations préliminaires lors des manipulations mais non décrites dans ce travail laissent entrevoir une meilleure résistance de la plante face à des stress abiotiques suite au traitement par BTP1. De plus, cette bactérie stimule des enzymes impliquées dans les réponses au stress en général. Il serait donc intéressant de tester l'implication de cette bactérie dans des phénomènes inductibles autres que ceux liés aux maladies biotiques, comme la résistance à des stress hydriques, salins ou des blessures. Cela permettrait le cas échéant d'élargir et de renforcer son intérêt pour une application agronomique.

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