UNIVERSITÉ DE REIMS CHAMPAGNE-ARDENNE

UNIVERSITE DE REIMS CHAMPAGNE-ARDENNE

ECOLE Doctorale N° 358 : Sciences, Technologies, Santé

Faculté des Sciences Reims

THESE

présentée à l'UFR des Sciences Exactes et Naturelles

pour obtenir le grade de

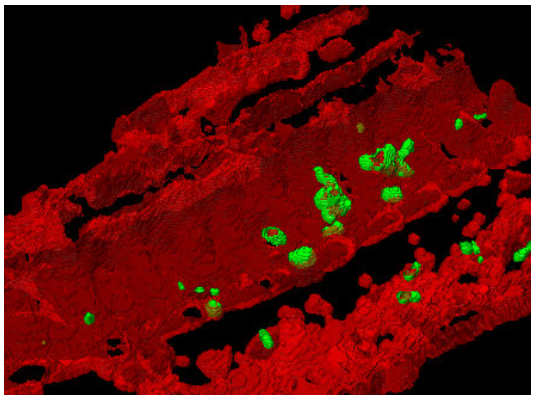
Docteur de l'Université de Reims Champagne-Ardenne

Spécialité : Biologie et Physiologie Végétales

par Stéphane Compant

Sujet :

Interaction entre la vigne, *Vitis vinifera* L., et une bactérie endophytique, *Burkholderia phytofirmans* souche PsJN : colonisation, induction de défenses et résistance systémique contre *Botrytis cinerea.*



Soutenue publiquement le 16 Novembre 2007

devant le jury composé de :

Pr. E. Benizri	INPL-ENSAIA-INRA Nancy, France	Rapporteur
Pr. M. Höfte	Université de Gent, Belgique	Rapporteur
Pr. J. Nowak	Virginia Polytechnic Institute and State University, Blacksburg, USA	Examinateur
Dr. A. Sessitsch	Austrian Research Centers GmbH, Seibersdorf, Autriche	Examinateur
Pr. P. Jeandet	Université de Reims Champagne-Ardenne	Examinateur
Dr. E. Ait Barka	Université de Reims Champagne-Ardenne	Co-directeur
Pr. C. Clément	Université de Reims Champagne-Ardenne	Co-directeur

En couverture :

Reconstruction en 3 dimensions de l'observation sous microscope confocal de *Burkholderia phytofirmans* souche PsJN marquée avec le gène *gfp* dans les vaisseaux du xylème de la vigne.

Remerciements

Je tiens tout d'abord à exprimer ma reconnaissance à Essaïd et Christophe pour m'avoir accepté au sein de leur équipe et du laboratoire SDRP ainsi qu'aux différents partenaires socio-économiques (la ville de Reims, le Conseil régional, le CIVC et Moët et Chandon pour les boutures de vigne) et Europol' Agro pour le financement de ces travaux.

Saïd, merci de m'avoir mis entre les mains ce qu'allaient être les fruits de ce travail et de m'avoir permis de me passionner sur ce sujet. Je ne sais pas si j'en serai là actuellement si j'avais été encadré par une autre personne. Merci pour m'avoir laissé faire cette thèse comme je le voulais, la confiance que tu as su m'apporter et pour notre amitié partagée depuis toutes ces années.

Je tiens également à te remercier Christophe pour m'avoir aidé surtout dans les moments difficiles. Je ne sais pas si cette thèse aurait été soutenue si on ne s'était pas justement parlé tous les deux à la fin. Je te remercie également pour les longs trajets que j'ai pu effectuer à l'étranger et pour m'avoir laissé voler de mes propres ailes avec cette thèse.

Mes remerciements vont en outre à Jerzy et Angela pour nos nombreuses discussions sur le sujet, vos soutiens scientifiques et notre amitié partagée lors de cette thèse de doctorat. J'espère juste que l'on continuera à travailler ensemble. Et quelque chose me dit que ceci n'est que le commencement. On verra bien ce que nous dit l'avenir... Merci également pour votre participation au Jury de cette thèse.

Merci également aux autres membres du Jury : monsieur le Professeur Emile Bénizri ainsi que madame le Professeur Monica Höfte et monsieur le Professeur Philippe Jeandet pour l'intérêt que vous portez à ces travaux.

Mes remerciements vont en outre aux personnes qui m'ont aidé dans la réalisation de certaines expériences : Hervé Kaplan, Birgit Reiter, Sophie, Sandra, Fabienne et Fanja. Merci à vous pour m'avoir consacré un peu de votre temps libre.

En outre, je tiens à remercier les chercheurs qui ont participé à la rédaction de certains articles ou ont eu un aspect critique avant qu'ils soient soumis. Un grand merci aux Docteurs Tom Coenye, Jacques Balandreau, Euan K. James, Geoffrey N. Elliot, Guy Abell et Richard Veilleux.

Je tiens également à remercier les stagiaires qui m'ont aidé au cours de cette thèse et en particulier David (M'sieur « *Frankia* sp. »). C'est juste dommage que tu n'ai pas continué dans ce domaine d'étude avec un nom comme ça...

Merci également à Alexandra, Philippe et Hervé dont nos travaux sur le chimiotactisme bactérien et le resvératrol, non aboutis, ne seront pas présentés lors de cette thèse. Mes remerciements vont en outre à messieurs les Docteurs Yves Dessaux et Denis Faure pour une étude de quorum sensing sur la bactérie PsJN dont les résultats n'ont pas fait non plus l'objet de publication. Tout ne peut pas marcher... malheureusement.

Merci à vous également, Steph et Sylvain, pour la correction du manuscrit. Ca m'a bien aidé. Merci de m'avoir accordé un peu de votre temps.

Un grand merci aussi à Andreas ("Trailos", "agafi mou", "moussi moussi moussaka"…), Cédric ("le Piot", je ne serais plus là pour les pauses Piot...fallait bien que je parte), aux Pépettes (inséparables), Gaël, Bibiz, Chantal, "PhiPhaï", JLD, Flof, Laurence, Dédé, Pat, Flo, Hélène, Sophie, Nath, Seb, Sylvain et Sandrine, "Mamezelle Jacquens", "Zhou-Zhou", "Coq", "Dude", Farid ("Alain"), Kinkin, Bénito, Hassan et les "Momos" des différents labos. Merci aussi à Ben et Gaëlle "family" avec le petit Elouan et également le petit Nico pour votre soutien et notre amitié partagée depuis ces longues années. En dernier lieu, un énorme merci au Piot et au Ben pour ces années passées au labo, ça va faire un vide quand je vais partir. Va falloir retrouver un autre Steph. Ca ne va pas être trop difficile, il suffira de regarder sous un microscope...

Un grand merci aussi aux chercheurs qui m'ont passionné par le biais de leurs travaux. Je pense qu'ils se reconnaîtront dans les stratégies de cette thèse ou dans la bibliographie.

Marie, un grand merci à toi également pour ta patience et ton soutien depuis ces longues années. Je ne saurai jamais assez te remercier pour ce que tu as fais. J'ai l'impression que cette thèse n'aurait pas été possible sans toi. Un grand merci pour ce que tu as fait pour moi. Je suis désolé pour ce qu'il s'est passé à la fin de cette thèse et je m'en excuse. J'espère juste que tu comprendras en lisant cette thèse qu'une carrière se dessine de plus en plus.

Enfin, pour terminer un grand merci à mon père (je serai toujours là pour t'aider à surmonter ce qu'il t'arrive actuellement), ma mère et mon frère ainsi que toute ma famille (oncle, tante et cousins) pour leurs soutiens au cours de ces années. Un grand hommage en dernier lieu à mes grands parents Gian-Franco, Hélène et Odette qui n'ont pu voir les fruits de ce travail. J'aurai du passer plus de temps avec vous. J'espère que ceci n'aura pas été en vain...

A mes grands parents,
à ma famille,
à Marie,
à mes amis

et aux PGPR endophytiques...

Sommaire

Résumé-Abstract

Résumé

Lors de ces travaux, l'interaction entre la vigne, *Vitis vinifera* L., et une PGPR endophytique, *Burkholderia phytofirmans* souche PsJN, a été étudiée. Ceci a permis de caractériser des phénomènes de colonisation bactérienne, des réponses de défense de la plante, ainsi qu'une résistance systémique induite (ISR) permettant une protection contre l'agent pathogène *Botrytis cinerea* Pers.

L'association entre la vigne et la souche PsJN a tout d'abord été étudiée en conditions gnotobiotiques en utilisant des plantules *in vitro*, la souche bactérienne de type sauvage ou des dérivés génétiques (PsJN::*gfp*2x et PsJN::*gusA*11). Ceci a permis de déterminer une colonisation épi- et endophytique des racines de vigne par la souche PsJN ainsi qu'une migration de la bactérie de l'intérieur des racines jusqu'aux feuilles, par le biais du flux d'évapo-transpiration de la plante *via* les vaisseaux du xylème.

La colonisation de la vigne par la souche PsJN a ensuite été étudiée en conditions non stériles en utilisant des boutures fructifères dans le but de décrire une possible colonisation des inflorescences. Les résultats obtenus ont permis de montrer une colonisation épi- et endophytique du système racinaire par la bactérie et ensuite, une migration des racines jusqu'à la rafle et les jeunes baies, en présence d'autres micro-organismes.

La mise en place de composés de défense ainsi qu'une ISR induite par la souche PsJN a été ensuite déterminée sur des boutures fructifères. Ceci a été caractérisé avant sa progression systémique et avec des conditions plus stériles. Des événements précoces tels que l'accumulation de peroxyde d'hydrogène et d'oxyde nitrique, ainsi que la synthèse de composés phénoliques ont été caractérisés au niveau racinaire. De plus, il est apparu, d'après des résultats préliminaires, que la souche PsJN induit, au niveau local ainsi qu'au niveau systémique, des gènes codants des protéines PR dont la signalisation dépend de la voie de l'acide salicylique et / ou de l'acide jasmonique. Ceci a permis de suggérer que la souche bactérienne induit des mécanismes communs des phénomènes de résistance systémique acquise (SAR) et d'ISR. Ces phénomènes permettent, par la suite, une protection de la vigne au niveau floral vis à vis de l'infection causée par *B. cinerea* Pers.

Mots Clés : *Vitis vinifera* L., *Burkholderia phytofirmans* souche PsJN, PGPR, endophyte, colonisation, défense, ISR, SAR, *Botrytis cinerea* Pers.

Abstract

The interaction between grapevine, *Vitis vinifera* L., and an endophytic PGPR, *Burkholderia phytofirmans* strain PsJN, has been studied in this work. This has allowed to characterize phenomenons of bacterial colonization, some plant defence responses as well as induced systemic resistance (ISR) confering protection against the phytopathogen *Botrytis cinerea* Pers.

Association between grapevine and strain PsJN has been studied, firstly, under gnotobotic conditions by using *in vitro* plantlets, PsJN wild-type strain or some genetically derivatives (PsJN::*gfp*2x and PsJN::*gusA*11). This has allowed to determine epi- and endophytic colonization of grapevine roots by strain PsJN as well as a migration of the bacterium from the endorhiza to the leaves, mediated by the plant transpiration stream *via* xylem vessels.

Grapevine colonization by strain PsJN has been then monitored under non-sterile conditions by using fruiting cuttings, with a special emphasis on putative inflorescence colonization. Results have demonstrated an epi- and an endophytic colonization of the root system by strain PsJN and then, its spreading from the endorhiza to grape inflorescence stalk and to young berries, even in presence of other microorganisms.

Plant defence compounds as well as a ISR induced by strain PsJN has been then determined on fruiting cuttings. This has been characterized before its systemic spreading inside plants and with some more sterile conditions. Some early events such as hydrogen peroxide and nitric oxide accumulations as well as phenolic compound synthesis have been characterized at the root level. In addition, preliminary results have demonstrated that strain PsJN induces locally and systemically some PR-encoding genes, dependent of salicylate and/or jasmonate signaling pathways. This has allowed to suggest that this bacterium induces common mechanisms of Systemic Acquired Resistance (SAR) and of ISR phenomenons. These phenomenons allow then a protection of grapevine flowers against infection caused by *B. cinerea* Pers.

Keywords: *Vitis vinifera* L., *Burkholderia phytofirmans* strain PsJN, PGPR, endophyte, colonization, defence, ISR, SAR, *Botrytis cinerea* Pers.

Liste des abréviations

ACC : Aminocyclopropane carboxylate

AOC : Appellation d'origine contrôlée

AVR : Avirulent

BABA : Acide β-aminobutyrique

Big : "*Botrytis*-induced grapevine gene" ou gène de vigne induit par *Botrytis*

BTH : Acide benzo (1, 2, 3) thiadiazole-7-carbothioïque S-méthyl-ester

Brg : "*Botrytis*-repressed grapevine gene" ou gène de vigne réprimé par *Botrytis*

CWE : Enzymes dégradants les parois cellulaires.

DAPG : 2,4-diacetylphloroglucinol

EPR : "Emergence-Promoting Rhizobacteria" ou rhizobactéries stimulant l'émergence

ET : Ethylène

FAO : Forme active de l'oxygène

GFLV : "Grapevine fanleaf virus"

GUS : β-glucuronidase

GFP : "Green Fluorescent Protein" ou protéine verte fluorescente

H_2O_2 : Peroxyde d'hydrogène

$HO_2^{\bullet}$: Radical hydroperoxyle

HR : "Hypersensitivity Response" ou réponse hypersensible

HRGP : "Hydroxyproline Rich Glycoprotein" ou glycoprotéines riches en hydroxyproline

INA : Acide 2,6-dichloroisonicotinique

ISR : "Induced Systemic Resistance" ou résistance systémique induite

JA : Acide jasmonique

LAR : " Local Acquired Resistance" ou résistance locale acquise

LOX : Lipoxygénase

LPS : Lipopolysaccharides

LTP : "Lipid Transfer Protein" ou protéine de transfert de lipides

MAMP : "Microbe-Associated Molecular Pattern" ou profil moléculaire associé aux microbes

MIMP : "Microbial-Induced Molecular Pattern" ou profil moléculaire induit par des micro-organismes

MF : Matière fraîche

NO : Oxyde nitrique

$O_2^{\bullet-}$: Anion superoxyde

OEPP : Organisation européenne et méditerranéenne pour la protection des plantes

OGM : Organismes génétiquement modifiés

$OH^{\bullet}$: Radical hydroxyle

PAL : Phénylalanine ammonia-lyase

PAMP : “Pathogen-Associated Molecular Pattern” ou profil moléculaire associé aux agents pathogènes

PGIP : “PolyGalacturonase Inhibiting Proteins” ou protéines inhibant la polygalacturonase

PGPB : “Plant Growth-Promoting Bacteria” ou bactéries stimulant la croissance des plantes

PGPF : “Plant Growth-Promoting Fungi” ou champignons stimulant la croissance des plantes

PGPR : “Plant-Growth-Promoting Rhizobacteria” ou rhizobactéries stimulant la croissance des plantes

PGPY : “Plant Growth-Promoting Yeasts” ou levures stimulant la croissance des plantes

PR : “Pathogenesis-Related” ou associé à la pathogénécité

R : Résistant

SA : Acide salicylique

SAR : “Systemic Acquired Resistance” ou résistance systémique acquise

UFC : Unité formatrice de colonies

UV : Ultra-violet

YIB : “Yield-Increased Bacteria” ou bactéries augmentant le rendement

Liste des publications

Compant S., Duffy B., Nowak J., Clément C. and Ait Barka E. (2005a) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology* 71: 4951-4959.

Compant S., Reiter B., Sessitsch A., Nowak J., Clément C. and Ait Barka E. (2005b) Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium, *Burkholderia* sp. strain PsJN. *Applied and Environmental Microbiology* 71: 1685-1693.

Compant S., Nowak J., Clément C. and Ait Barka E. (2007) Polyphenolic compound accumulation as a response of grapevine (*Vitis vinifera* L.) to colonization by *Burkholderia phytofirmans* strain PsJN. In: Macromolecules from Grape and Vine (Jeandet P., Conreux A. and Clément C., eds), Lavoisier, Paris, France, pp. 89-93.

Compant S., Kaplan H., Sessitsch A., Nowak J., Ait Barka E. and Clément C. Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: from the rhizosphere to inflorescence tissues. *FEMS Microbiology Ecology,* révision soumise.

Compant S., Nowak J., Coenye T., Clément C. and Ait Barka E. Interaction between plants and *Burkholderia* spp. *FEMS Microbiology Reviews,* publication invitée, en révision.

Compant S., Paquis S., Bordiec S., Rabenoelina F., Sessitsch A., Nowak J., Baillieul F., Clément C. and Ait Barka E. Induced defense responses in *Vitis vinifera* L. by the endophyte *Burkholderia phytofirmans* strain PsJN and its related systemic resistance towards early flower infection by *Botrytis cinerea* Pers. En preparation.

Avant-propos

Dans les écosystèmes naturels et cultivés, des interactions diverses s'établissent entre les plantes et des micro-organismes (Welbaum *et al.,* 2004). Ces relations, qu'elles soient neutres, à l'origine de maladies ou de symbioses, ont toujours suscité un très grand intérêt. Dans un premier temps, l'homme a su utiliser ces interactions dans le but d'améliorer le rendement de ses plantes cultivées. Ensuite il les a étudiées en vue d'accroître ses propres connaissances.

De nos jours, il devient courant d'apprendre que des micro-organismes ayant des propriétés bénéfiques pour un hôte végétal naturel sont utilisés chez d'autres plantes en vue d'augmenter leurs capacités. Le potentiel d'un tel micro-organisme a notamment été étudié chez la vigne et concerne la bactérie endophytique, *Burkholderia phytofirmans* souche PsJN (Sessitsch *et al.,* 2005). Cette bactérie a été utilisée dans un premier temps afin de combattre le champignon *Botrytis cinerea* responsable de la pourriture grise (Ait Barka *et al.,* 2000). *B. phytofirmans* souche PsJN inhibe la croissance *in vitro* de *B. cinerea*, permet d'augmenter la résistance de la plante vis à vis de cet agent pathogène, tout en stimulant directement la croissance de son hôte végétal (Ait Barka *et al.,* 2000 ; 2002). *B. phytofirmans* souche PsJN présente donc un potentiel intéressant pour la viticulture. Néanmoins, certains points de l'interaction entre la vigne et cette souche bactérienne avaient besoin d'être étudiés. En effet, les défenses associées au phénomène de résistance contre *B. cinerea* ainsi que l'interaction entre la vigne et la souche bactérienne n'ont été que superficiellement abordées. Elles méritaient, ainsi, d'être étudiées d'avantage. De plus, la résistance induite contre *B. cinerea* avait été montrée sur feuilles de plantules *in vitro* de vigne mais pas sur les inflorescences. Compte tenu de l'importance de ces organes chez la vigne, les inflorescences méritaient ainsi de faire l'objet d'une étude.

En outre, la bactérie a été détectée au niveau foliaire après inoculation d'explants de tige et il a été démontré qu'elle est transmise par micro-bouturage *in vitro* des plantules bactérisées sans un besoin de ré-inoculation (Ait Barka *et al.,* 2002). Néanmoins, au début de cette thèse, aucune étude de suivi de la colonisation bactérienne n'avait été réalisée sur les plantules *in vitro*. De plus, aucune donnée ne concernait sa propagation et sa localisation sur un modèle *in vivo.* L'étude de la colonisation bactérienne de la plante paraissait de ce fait intéressante. Cependant, il fallait utiliser des souches modifiées (dérivées *gfp* et *gusA*), ceci permettant de visualiser la souche PsJN, de la différencier des autres micro-organismes et de mieux caractériser le phénomène de colonisation de la vigne par cette souche bactérienne.

Ainsi lors de cette thèse, l'interaction entre la vigne et *B. phytofirmans* souche PsJN a été étudiée. Ceci a porté aussi bien sur la colonisation bactérienne et ses mécanismes associés, que sur l'induction de défense chez la plante et la résistance de ses inflorescences contre *B. cinerea.*

Chapitre 1 :

Introduction générale

1. La Vigne

1.1. La vigne : présentation botanique

La vigne, plante angiosperme dicotylédone est une liane de la famille des *Vitacae*, anciennement famille des *Ampelideae* (Planchon, 1887). Cette famille, associée aux familles des *Rhamnaceae* et des *Leeceae*, forme l'ordre des Rhamnales (Chadefaud et Emberger 1960). Les *Vitaceae* sont, pour la plupart, des plantes ligneuses ou herbacées, ainsi que des arbustes à tiges sarmenteuses (Hellman, 2003).

1.2. Le système racinaire de la vigne : généralités

Comme toutes les dicotylédones, la vigne est composée d'un système racinaire pivotant permettant son ancrage dans le sol (Öpik et Rolfe, 2005). Cependant, suite aux attaques de phylloxéra en Europe dont les premiers foyers ont été observés en France dès 1865, les vignes cultivées sont portées de nos jours par des portes-greffes d'espèces américaines (Huglin et Schneider, 2003).

1.3. Le système végétatif aérien et reproducteur : généralités

Le système aérien chez la vigne est porté par le cep de vigne et se trouve constitué d'un appareil végétatif avec la tige feuillée ainsi que d'un système reproducteur avec les inflorescences (Mullins *et al.*, 1992 ; Hellman, 2003).

En 1952, Baggiolini a décomposé le développement annuel de ces organes en 16 stades phénologiques. La tendance fut ensuite d'affiner cette description en subdivisant les stades existants. Ceci fut notamment l'objet de la description de Meyer en 2001 dans l'échelle BBCH.

Dans cette échelle BBCH, il est décrit qu'après l'étalement des premières feuilles (stades 11 à 13), les inflorescences apparaissent (stade 53), puis se séparent grâce à l'allongement des entre-nœuds (stade 55). Ensuite, les boutons floraux se séparent (stade 57) et les pédicelles des fleurs s'allongent, ce qui permet de différencier chaque fleur lors de la floraison (stades 60 à 69). L'ovaire est ensuite fécondé et devient fruit, ce qui correspond à la nouaison (stade 71). Puis, les fruits se développent et passent progressivement aux stades de plomb de chasse (stade 73), de petit pois (stade 75) avant que les grappes pendent sous le poids des baies. Les fruits grossissent ensuite et finissent par se toucher, ce qui induit la

fermeture de la grappe (stades 77 à 79). Enfin, au cours de la maturation, les baies commencent à s'éclaircir et à changer de couleur (stade 81) et à la véraison, les baies deviennent molles au toucher (stade 85) puis mûrissent jusqu'à la vendange (stade 89).

1.4. La vigne, le vignoble et le Champagne

D'un point de vue économique, la vigne (*Vitis* spp.) est considérée comme une plante très importante compte tenu de l'utilisation de ses fruits (Aigrain, 1999). Parmi le genre *Vitis*, *Vitis vinifera* L. est l'espèce la plus cultivée en raison de ces nombreuses qualités, en particulier pour la production de raisins de table ou l'élaboration des vins (Galet, 1997).

De nos jours, la France est le premier producteur de vin au niveau mondial. *V. vinifera* L. est, de ce fait, bien cultivée sur notre territoire. Elle y représente plus de 3,3 % des surfaces agricoles (Viniflhor, données 2006), faisant ainsi de la viticulture une composante majeure de notre agriculture.

En Champagne, 32400 hectares sont actuellement consacrés à la culture de la vigne (Viniflhor, données 2006). Cette vigne sert à l'élaboration d'un vin blanc de renommée, le Champagne. Ce vin effervescent à appellation d'origine contrôlée (AOC) représente actuellement un chiffre d'affaire d'environ 3,4 milliards d'euros pour 300 millions de bouteilles vendues chaque année (Viniflhor, données 2006). Il est élaboré à partir de raisins provenant majoritairement des cépages Chardonnay, Pinot noir et Pinot meunier (Figure 1 a-c) bien que d'autres cépages tels que Arbanne et Petit Meslier peuvent également être utilisés.

En vue d'obtenir le meilleur Champagne, ces vignes font actuellement l'objet de pratiques culturales diverses en vue de contrôler leur rendement. Ceci inclut notamment des méthodes permettant d'augmenter la taille et le poids des raisins, une stimulation de la croissance de la plante, ainsi qu'une lutte contre les stress abiotiques pouvant intervenir sur ces plantes (Aigrain, 1999).

1.5. La vigne et ses phytopathogènes

La vigne, comme toutes les plantes, est sensible à différents agents pathogènes. Elle peut ainsi être attaquée par des virus (GFLV pour "Grapevine fanleaf virus" responsable du court-noué, *Closterovirus* sp. responsable de l'enroulement), des phytoplasmes (flavescence dorée causée par des micro-organismes de type mycoplasmes), des insectes (tordeuse de la grappe, cochenille, …) ou des bactéries (nécrose bactérienne due à *Xylophylus ampelinus*,

Figure 1 : Principaux cépages utilisés dans la région champenoise : (a) Chardonnay, (b) Pinot noir et (c) Pinot meunier. Photographies d'après www.kobrandwine.com. Barres d'échelles : (a) 6 cm, (b) 4 cm et (c) 6 cm.

galle du collet avec *Agrobacterium vitis*, maladie de Pierce avec *Xylella fastidiosa* (Galet, 1977 ; Huglin et Schneider, 2003).

Des champignons phytopathogènes peuvent également infecter la vigne et sont à l'origine de différentes maladies. *Plasmopara viticola* est ainsi responsable du mildiou, *Erysiphe necator* de l'oïdium, *Eutypa lata* de l'eutypiose, *Botrytosphaeria* sp. du "black dead arm" et divers champignons (*Phaemonacremonium* sp., *Phaemoniella* sp., *Phellinus* sp…), de la maladie de l'esca (Galet, 1977 ; Huglin et Schneider, 2003).

1.6. La vigne et *Botrytis cinerea*

Un autre agent pathogène est le champignon nécrotrophe *Botrytis cinerea.* Ce phytopathogène mondialement répandu présente la particularité d'être polyphage et ainsi de s'attaquer à diverses plantes dès que les conditions climatiques lui sont favorables (Elad *et al.,* 2004). Chez la vigne, *B. cinerea* peut être à l'origine de la pourriture noble sur de rares terroirs, ce qui permet d'obtenir des vins plus liquoreux (Sauternes, Coteaux du Layon, Monbazillac). Dans d'autres régions en revanche, il est responsable de la pourriture grise (Pezet *et al.,* 2004). C'est notamment le cas en Champagne où cette maladie provoque des pertes de récoltes autant quantitatives que qualitatives (Long, 1979 ; Pezet *et al.,* 2004). Dans les dernières années, des pertes de rendement allant jusqu'à 40% ont été ainsi constatées (Viniflor, données 2006). De plus, il a été montré que la pourriture grise est à l'origine de conséquences graves sur la qualité des vins. Elle provoque ainsi une diminution de la moussabilité en même temps qu'une altération de leurs qualités organoleptiques (Marchal *et al.*, 2002). Il apparaît, de ce fait, important de combattre la pourriture grise. Néanmoins, avant de lutter contre cette maladie, il importe de mieux connaître, au préalable, les sites d'infection de la vigne par le champignon *B. cinerea*.

1.7. L'infection de la plante par *B. cinerea*

Chez la vigne, *B. cinerea* peut infecter tous les organes de la plante après leur pénétration *via* des enzymes de virulence (cutinases, polygalacturonases, laccases, xylanases,…). Ce champignon peut ainsi être détecté sur la tige, les feuilles (Figure 2 a et b), les bourgeons préfloraux, les fleurs et les baies (Figure 3 a-c ; Jeandet *et al.*, 1999 ; Pezet *et al*., 2004). Les conséquences de son infection sont cependant plus importantes au niveau des fleurs et des baies. L'infection des fleurs est même considérée comme une étape importante

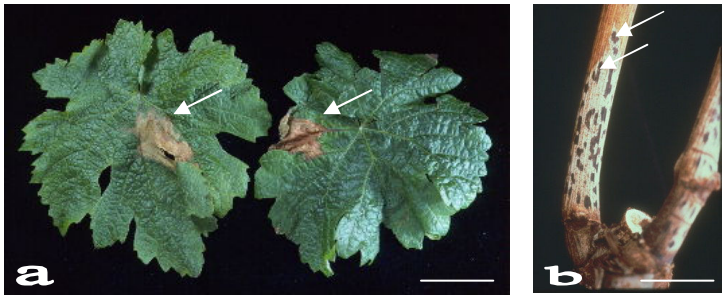


Figure 2 : Symptômes de *Botrytis cinerea* sur des organes végétatifs de la vigne : (a) au niveau foliaire et (b) au niveau caulinaire. Photographies INRA. Barres d'échelles : (a) 1cm et (b) 2 cm.

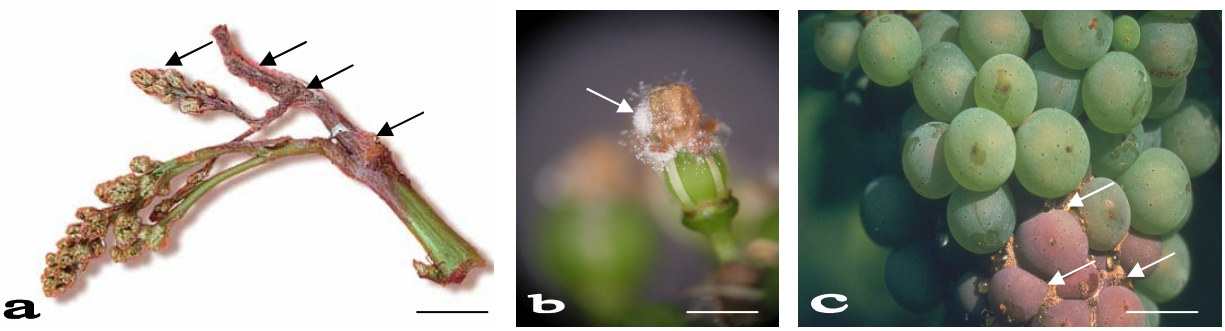


Figure 3 : Symptômes de *Botrytis cinerea* sur des organes reproducteurs de la vigne : (a) avant floraison, (b) pendant la floraison et (c) au niveau du fruit mature. Photographies (a) et (c) INRA et (b) original. Barres d'échelles : (a) 4 mm, (b) 2 mm et (c) 2 cm.

dans le développement de la pourriture grise au niveau des fruits. *B. cinerea* peut ainsi infecter divers organes floraux, puis entrer en phase de latence pour se développer ensuite au moment de la maturité des baies (Keller *et al.*, 2003). Une étude a démontré, néanmoins, que différents groupes de populations de *B. cinerea* peuvent être à l'origine de l'infection de ces organes. Le groupe I et le groupe II (*vacuma*) sont ainsi trouvés principalement au niveau des fleurs, tandis que le groupe III (*transposa*) domine au niveau des fruits (Martinez *et al.*, 2005). Cependant, l'infection des fleurs peut expliquer de nos jours jusqu'à 78% de la pourriture grise identifiée au niveau des baies (Nair *et al.*, 1995). A la floraison, les déchets floraux (étamines déhiscentes, capuchons floraux), le réceptacle floral, le stigma et l'ovaire offrent ainsi un support pour le champignon qui peut y établir un inoculum primaire (Wolf *et al.*, 1997 ; Keller *et al.*, 2003 ; Viret *et al.*, 2004). Puis, à la maturité des baies, les fruits présentent des défenses affaiblies contre l'agent pathogène (Jeandet *et al.*, 1991 ; Bais *et al.*, 2000). Si les conditions climatiques sont favorables (forte humidité, chaleur), la croissance du champignon est alors amplifiée (inoculum secondaire). La contamination s'étend ensuite par simple contact entre les baies contaminées et celles qui sont encore saines (Elad *et al.*, 2004 ; Pezet *et al.*, 2004).

Afin de protéger la vigne contre les agents pathogènes, que ce soit *B. cinerea* ou d'autres micro-organismes agresseurs, différentes méthodes existent actuellement (Figure 4). Celles ci incluent notamment l'utilisation de micro-organismes bénéfiques comme dans le cas qui nous intéresse. Néanmoins, la résistance naturelle des plantes, la lutte chimique ainsi que la transgénèse sont également utilisées. Il apparaît ainsi intéressant de les développer.

2. La résistance naturelle des plantes

Les plantes ont toujours pu se protéger contre les agents pathogènes grâce à un système de défense complexe impliquant des défenses constitutives et des défenses induites (Jones et Dangl, 2006).

Des défenses constitutives, peuvent ainsi se retrouver à différents niveaux de la plante (racines, tige, feuilles ou inflorescences) et sont constituées par des barrières physiques (écorce, épiderme, cuticule et composition des parois cellulaires) et chimiques (toxines et

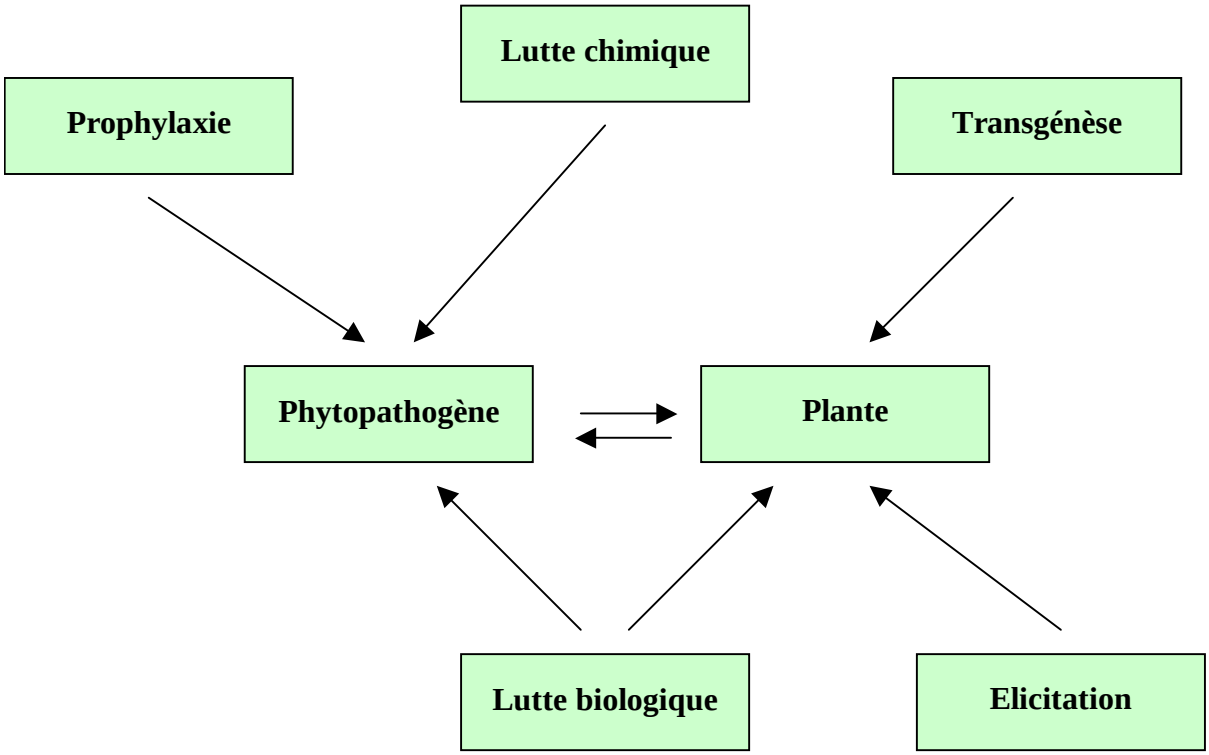


Figure 4: Illustration des différentes méthodes de lutte contre les agents pathogènes des plantes.

exsudats antimicrobiens ; Wittstock et Gershenzon, 2002). Ces défenses préformées permettent de restreindre l'infection par certains micro-organismes. Néanmoins, dans certains cas, elles peuvent être contournées par d'autres micro-organismes, tels que ceux à l'origine de maladies (Ton *et al.*, 2006).

Des défenses induites peuvent également se mettre en place lors de l'interaction entre la plante et un agent pathogène (Jones et Dangl, 2006). Elles sont les conséquences d'une reconnaissance entre l'hôte végétal et son agresseur (Figure 5).

2.1. Reconnaissance de l'agent pathogène

Lors de l'attaque d'une plante par un agent pathogène, une reconnaissance spécifique race/cultivar peut avoir lieu et impliquer un système gène pour gène comme défini par Flor dès 1942. Dans ce modèle, les gènes dominants de résistance (*R*) de la plante confèrent une résistance aux races d'agents pathogènes portant les gènes *Avr* correspondants. Cette combinaison précoce induit la mise en place de réactions de défense, ce qui permet de restreindre la progression de l'agent agresseur. Toute autre combinaison est à l'origine d'une réaction compatible, c'est à dire induisant une maladie (van der Hoorn *et al*., 2002).

Néanmoins, le système *Avr*-*R* ne fonctionne pas dans tous les cas et un modèle de garde a été établi. D'après cette hypothèse, le produit du gène *R* est une protéine de garde qui reconnaît la cible de virulence modifiée par le produit du gène *Avr* et participe ensuite à l'activation des réactions de défense (Dangl et Jones, 2001 ; Mackey *et al*., 2002 ; van der Hoorn *et al.*, 2002).

La reconnaissance des agents pathogènes par les plantes peut également provenir de molécules sécrétées ou présentées par l'agent agresseur : les PAMPs pour "Pathogen-Associated Molecular Pattern" ou MAMPs pour "Microbial-Associated Molecular Pattern" (Nürnberger et Brunner, 2002 ; Jones et Dangl, 2006). Ces éliciteurs généraux sont à l'origine de réactions de défense qui, dans ce cas, se font indépendemment du cultivar (Nürnberger et Brunner, 2002).

2.2. Evénements précoces

Après la phase de reconnaissance de l'agent pathogène, des réactions rapides de transduction du signal se mettent en place dans les cellules de la plante agressée. Ceci

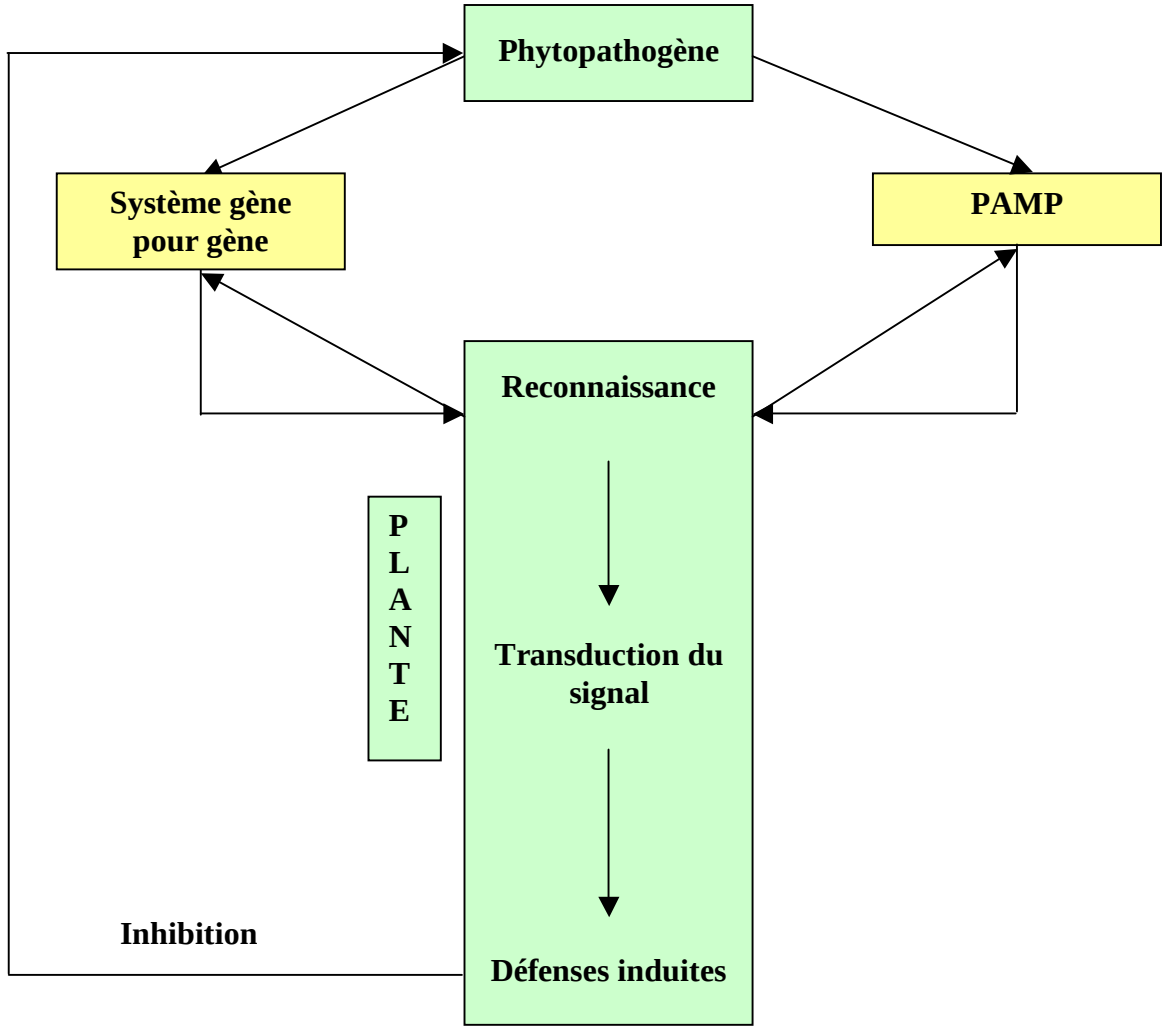


Figure 5 : Illustration du phénomène de résistance induite par les agents pathogènes. PAMP : Pathogen-Associated Molecular Pattern ou éliciteurs généraux.

intervient tout d'abord au niveau intracellulaire puis ensuite au niveau intercellulaire (Garcia-Brugger *et al.,* 2006 ; Kachroo et Kachroo, 2007).

Dans le cas de la transduction intracellulaire, des changements de flux d'ions (influx de Ca^{2+}, efflux de K^+, Cl^-), des activations de protéines kinases et de phosphatases, la production de formes actives de l'oxygène (FAO), d'oxyde nitrique ainsi qu'une oxydation lipidique prennent place suite à l'infection. Ces événements ont notamment pu être décrits lors de l'interaction entre le tabac et la cryptogéine, un PAMP sécrété par des champignons du genre *Phytophtora* spp. (pour revue Garcia-Brugger *et al.,* 2006), ou entre la vigne et l'éliciteur BcPG1 issu de *B. cinerea* (Poinssot *et al.,* 2003 ; Vandelle *et al.,* 2006).

Parmi ces événements précoces, la production de FAO est très intéressante. Elle intervient lors du développement de la plante comme dans le cas de la croissance cellulaire (Foreman *et al.,* 2003) ou de l'élongation racinaire (pour revue Torres et Dangl, 2005) mais également lors de l'interaction entre la plante et un agent agresseur (Mitller *et al.,* 2004), ou comme nous le verrons dans un autre chapitre, lors d'un contact entre la plante et des micro-organismes bénéfiques (Gerber *et al.,* 2004).

Les FAO sont essentiellement représentées par l'anion superoxyde ($O_2^{\bullet -}$), le radical hydroperoxyle ($HO_2^{\bullet -}$), le peroxyde d'hydrogène (H_2O_2, la forme la plus stable) et enfin le radical hydroxyle ($OH^{\bullet}$) (Figure 6). Lors de l'interaction entre la plante et son environnement biotique, ces composés oxygénés vont s'accumuler pour former un "burst oxydatif" (Mitller *et al.,* 2004). Ceci peut être à l'origine d'autres réactions de défense de la plante (Figure 7 ; Mellersch *et al.,* 2002). Ainsi, les FAO sont impliquées dans le renforcement des parois cellulaires (Brisson *et al.,* 1994), la mise en place de la réponse hypersensible (cf. paragraphe 2.5.1. ; Gechev et Hille, 2005) et contribuent à l'expression de gènes de défense (Grant et Loake, 2000), tout en étant potentiellement cytotoxiques vis à vis des micro-organismes (Mehdy, 1994).

Le monoxyde d'azote (NO) est également considéré comme une autre forme activée de l'oxygène (Delledonne, 2005). Ce gaz originellement décrit chez les bactéries et chez l'animal peut également être produit par les plantes (Lamotte *et al.,* 2005). Il se retrouve par exemple lors de l'élongation racinaire (Lamattina *et al.,* 2003 ; Stöhr et Stremlau, 2006) ou de la fermeture stomatique (Bright *et al.,* 2006). Néanmoins, comme décrit pour les FAO, le NO intervient également lors de l'interaction entre un micro-organisme pathogène et son hôte

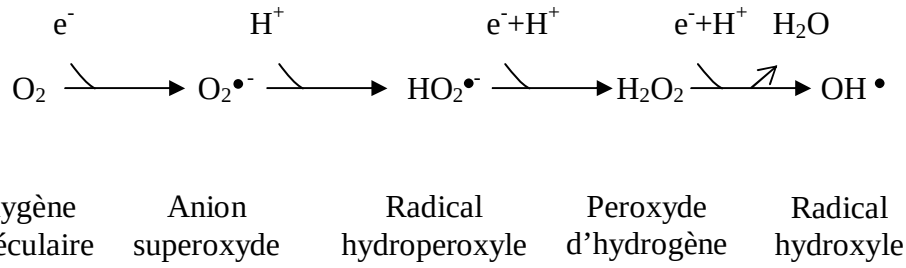


Figure 6 : Formes actives de l'oxygène *via* la réduction séquentielle de l'oxygène moléculaire.

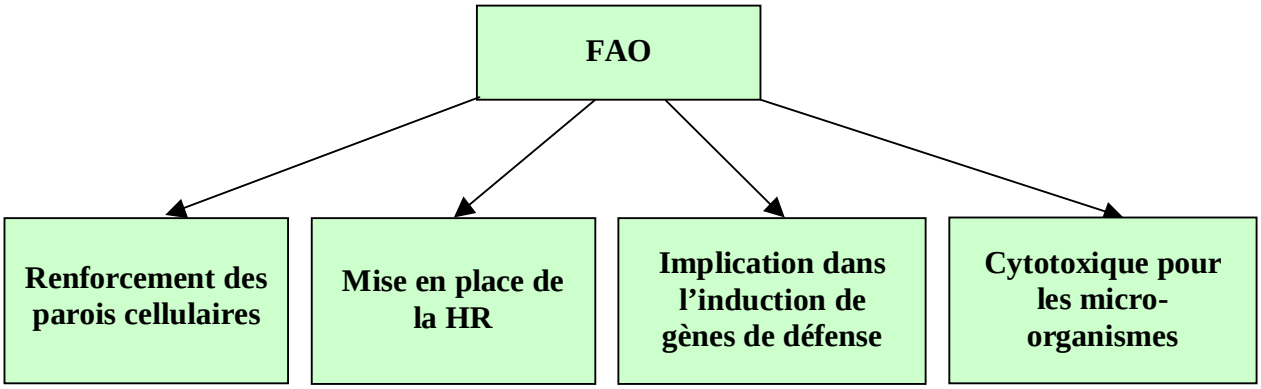


Figure 7 : Principaux rôles des FAO lors de l'interaction de la plante avec des agents pathogènes. FAO : Formes Actives de l'Oxygène.

végétal (Wendehenne *et al.,* 2004 ; Mur *et al.,* 2006), ainsi que lors d'une association avec des micro-organismes bénéfiques, comme nous pourrons le constater lors de cette introduction.

Lors de l'interaction entre une plante et un agent pathogène (ou ses PAMPs), le NO s'accumule et est à l'origine d'un "burst nitrique" (Wendehenne *et al.,* 2004 ; Delledonne, 2005 ; Mur *et al.* 2006 ; Zaninotto *et al.* 2006). Ce gaz peut présenter divers rôles dans les interactions plantes / pathogènes (Figure 8). Il peut ainsi être cytotoxique vis à vis des micro-organismes (Stamler *et al.,* 2001), être impliqué dans les cascades de signalisation (Lamotte *et al.,* 2005), la mise en place de la réponse hypersensible avec l'H_2O_2 (Zaninotto *et al.,* 2006), contribuer aux renforcements pariétaux (Prats *et al.,* 2005) voire même induire l'expression de gènes de défense (Parani *et al.,* 2004).

2.3. Signalisation intercellulaire

Suite à ces événements précoces se met en place une signalisation intercellulaire au niveau de la plante agressée (Kachroo et Kachroo, 2007).

Les phytohormones sont bien connues pour intervenir dans le développement de la plante (Davis, 2004). Elles interviennent également lors de la mise en place des réactions de défenses. Ainsi, lors de l'attaque d'un agent pathogène, une signalisation *via* ces molécules signals se met en place dans toute la plante. Ceci permet aux différentes parties de la plante d'être informées de l'agression extérieure (cf. paragraphes suivants). Cette signalisation fait intervenir principalement l'acide jasmonique (JA), l'acide salicylique (SA) et l'éthylène (ET) (Figure 9 ; Kachroo et Kachroo, 2007), bien qu'il y ait également participation de la systémine et de l'acide abscissique (Mauch-Mani et Mauch, 2005). Ces molécules signals interviennent lors de divers mécanismes de défenses et sont même caractéristiques de certains phénomènes de résistance. Nous pourrons le constater lors des paragraphes concernant les composés de défenses, ainsi que les mécanismes de résistance de la plante, induits en réponse aux agents microbiens.

2.4. Les composés de défenses

Lors de l'infection de la plante par un agent pathogène, divers composés de défenses peuvent être synthétisés (van Loon *et al.,* 2006b).

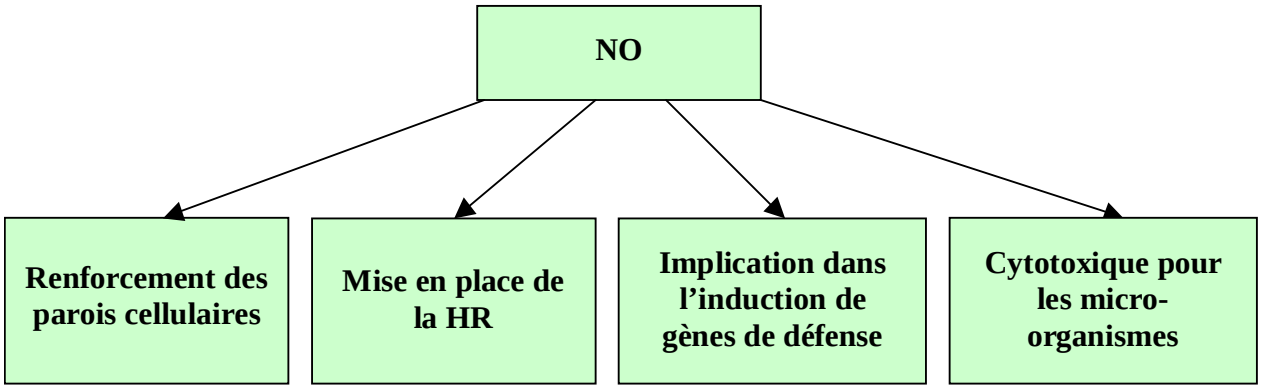
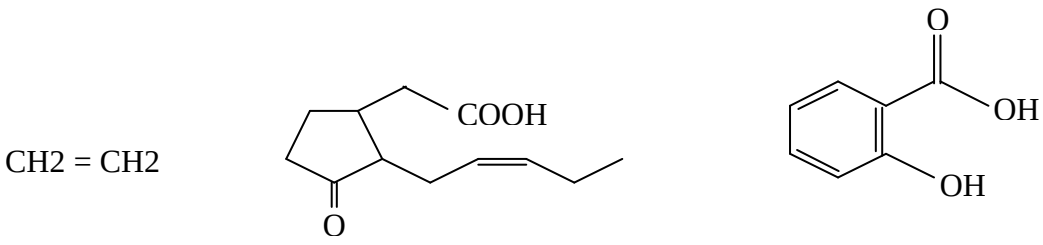


Figure 8 : Principaux rôles du NO lors de l'interaction de la plante avec des agents pathogènes. NO : Oxyde nitrique.



Ethylène Acide jasmonique Acide salicylique

Figure 9 : Formules chimiques des trois composés majeurs (éthylène, acide jasmonique et acide salicylique) impliqués dans la signalisation intercellulaire chez les plantes.

2.4.1. Les Protéines PR

Des protéines PR (pour "Pathogenesis-Related") peuvent être produites par la plante lors de l'interaction avec un agent pathogène (Tuzun et Somanchi, 2006). Les protéines PR ont été initiallement décrites chez le tabac suite à l'infection par le virus de la mosaïque du tabac (Gianinazzi *et al.*, 1970 ; van Loon et van Kammen, 1970) et depuis dix-sept familles de protéines PR ont été répertoriées (Tableau 1). Celles ci incluent des isoformes basiques ou acides qui présentent des activités différentes (van Loon *et al.*, 2006b).

Les activités de toutes les protéines PR ne sont pas encore connues, mais la majorité d'entre elles présentent des activités antimicrobiennes. Parmi ces familles, existent les PR-1 à fonction encore inconnue (Alexander *et al.*, 1993 ; Tahiri-Alaoui *et al.*, 1993 ; Niderman *et al.*, 1995), ainsi que les β-1,3-glucanases (PR-2) et les chitinases (PR-3, PR-4, PR-8 et PR-11) capables de dégrader les parois fongiques (Tuzun et Somanchi, 2006). A celles-ci s'ajoutent les PR-5, des osmotines qui perturbent la perméabilité membranaire des agents pathogènes (Vigers *et al.*, 1992 ; Hu et Reddy 1997), les PR-6, des inhibiteurs de protéases (Ryan, 1990 ; Lorito *et al.*, 1994), ainsi que les PR-7, des endoprotéases qui seraient impliquées dans la dissolution des parois cellulaires microbiennes (van Loon *et al.*, 2006b). D'autres protéines PR existent également. C'est le cas pour les peroxydases ou PR-9 qui sont impliquées aussi bien dans le métabolisme de l'auxine et la biosynthèse de l'éthylène que dans la formation de la lignine (Lagrimini *et al.*, 1987). Les PR-10, des N-glycosidases, quant à elles, sont capables d'inhiber la synthèse protéique chez les micro-organismes pathogènes (Stirpe *et al.*, 1992). A ces protéines PR, s'ajoutent également les défensines, les thionines et les LTP (pour "Lipid Transfer Protein"), respectivement PR-12, PR-13 et PR-14 qui possèdent des activités antifongiques, parfois antibactériennes voire même insecticides comme c'est le cas pour des défensines (Wijaya *et al.*, 2000 ; van Loon *et al.*, 2006b). Chez les monocotylédones, des protéines PR-15 et PR-16 ont également été caractérisées. Elles sont à l'origine d'H_2O_2 cytotoxique pour les agents pathogènes et peuvent induire indirectement les réactions de défenses de la plante (Donaldson *et al.*, 2001 ; Hu *et al.*, 2003). Enfin, les PR-17 ont été récemment détectées chez le tabac, le blé et l'orge infectés par des agents pathogènes. Elles contiennent des séquences ressemblant aux sites actifs des métalloprotéinases (Christensen *et al.*, 2002) mais leurs fonctions n'ont pas encore été complètement caractérisées (van Loon *et al.*, 2006b).

Tableau 1 : Les différents types de protéines PR d'après van Loon *et al*. (2006b).

Protéines PR	**Membre type**	**Activité enzymatique**	**Cible**
PR-1	Tabac PR-1a	Inconnue	Inconnue
PR-2	Tabac PR-2	β-1,3- glucanase	β-1,3-glucanes de la paroi
PR-3	Tabac P, Q	Chitinase de type I, II, III, IV, V, VI et VII	Chitine de la paroi
PR-4	Tabac R	Chitinase de type I, II	Chitine de la paroi
PR-5	Tabac S	Osmotine	Membrane cytoplasmique
PR-6	Tabac Inhibiteur I	Inhibiteur de protéinase	Protéinase
PR-7	Tomate P69	Endoprotéinase	Inconnue
PR-8	Concombre, chitinase	Chitinase de type VIII	Chitine de la paroi
PR-9	Tabac « lignin-forming peroxydase »	Peroxydase	Inconnue
PR-10	PR1 de persil	RNase	Inconnue
PR-11	Tabac chitinase de classe V	Chitinase de type I	Chitine de la paroi
PR-12	Radis Rs-AFP3	Défensine	Inconnue
PR-13	Arabidopsis THI2.1	Thionine	Inconnue
PR-14	Orge LTP4	Protéine de transfert de lipides	Inconnue
PR-15	Orge OxOa (germine)	Oxalate oxydase	Membrane cytoplasmique
PR-16	Orge OxOLP	Oxalate oxydase-like	Membrane cytoplasmique
PR-17	Tabac PRp27	Inconnue	Inconnue

Même si ces protéines PR sont synthétisées en réponse à l'agression par un agent pathogène, il est important de prendre en compte qu'elles sont également synthétisées lors de divers stress biotiques et abotiques voire même lors du développement des plantes (pour revue récente van Loon *et al.*, 2006b). Néanmoins, ce sont les mêmes types de protéines PR que l'on peut retrouver dans ces différentes situations (Broekaert *et al.*, 2000 ; Veronese *et al.*, 2003).

2.4.2. Les autres réponses de défenses

En plus de ces protéines PR, d'autres molécules de défenses sont synthétisées lors de l'infection de la plante par un agent pathogène. Ainsi, des inhibiteurs de polygalacturonases (PGIP ; Glinka et Protsenko 1998 ; de Lorenzo *et al.*, 2001), les lipoxygénases (LOX ; Feussner et Wasternack, 2002), les phénylalanine ammonia-lyases (PAL ; Fukasawa-Akada *et al.*, 1996), les glycoprotéines riches en hydroxyproline (HRGP ; Esquerré-Tugayé *et al.*, 1999), les protéines riches en glycine (Sachetto-Martins *et al.*, 2000) et également des produits d'autres gènes sont sécrétés en réponse à l'infection de la plante par un agent agresseur (pour revue van Loon *et al.*, 2006b).

La plupart de ces enzymes interviennent dans différents métabolismes secondaires et vont conduire à la production de différents composés qui, à leur tour, vont être impliqués dans les défenses de la plante. Des composés phénoliques issus du métabolisme secondaire tels que des terpénoïdes, des phénylpropanoïdes, des flavonoïdes, des isoflavonoïdes et des tanins vont être ainsi sécrétés (Bennett et Wallsgrove, 1994 ; van Etten *et al.*, 1994 ; Hammershmidt, 1999 ; Paiva, 2000). Ces molécules de faibles poids moléculaires peuvent former des barrières structurales ou sont toxiques sur les agents pathogènes, ce qui permet de restreindre leur progression (Jeandet *et al.*, 2007 ; Seppänen *et al.*, 2004 ; Serazetdinova *et al.*, 2005).

2.5. Les mécanismes de défense

Lors de l'infection de la plante par un agent agresseur, différents mécanismes, impliquant les composés de défenses que nous venons de citer, se mettent en place dans les tissus de la plante infectée (Jones et Dangl, 2006).

2.5.1. La réponse hypersensible ou HR

Une HR (pour “Hypersensitive Response”), se traduisant par une mort cellulaire programmée, peut intervenir dans le cas de l’interaction plante / agent pathogène (Watanabe et Lam, 2006). La HR est souvent associée à des niveaux élevés de SA, la synthèse de protéines PR, le renforcement des parois cellulaires ainsi que la production de composés antimicrobiens comme les phytoalexines (Hammond-Kosack et Jones, 1996 ; Ryals *et al.*, 1996). De multiples signaux secondaires tels que les FAO, le NO et le SA sont également essentiels pour l’activation et la mise en place de cette HR (Delledonne *et al.*, 2001 ; Torres *et al.*, 2002 ; Nawrath *et al.*, 2006). Ce mécanisme de défense est particulièrement efficace pour contrer l’invasion de certains agents pathogènes, notamment les champignons biotrophes. En revanche chez les nécrotrophes, la HR ne suffit pas à restreindre leur colonisation (Glazebrook, 2005).

2.5.2. Le renforcement des parois cellulaires

Lors d’une attaque d’un agent pathogène, les parois des cellules infectées subissent également des modifications : la paroi cellulaire se renforce. Ce renforcement est généralement associé à la formation de papilles et de dépôts de macromolécules observables en microscopie telles que des composés phénoliques fluorescents sous Ultra-Violets (UV) ou sous lumière bleue (Benhamou, 2006). Des polysaccharides tels que la callose (un polymère de β-1,3-glucanes) et des HRGP peuvent également s’accumuler. Ceci augmente la résistance de la plante et permet de bloquer le développement de l’agent infectieux (Benhamou, 2006).

2.5.3. La résistance locale acquise ou LAR

Enfin, par un phénomène d'induction se met en place une résistance locale ou LAR (pour “Local Acquired Resistance”). La LAR est induite par les cellules en état de mort cellulaire *via* la transmission des molécules signals SA, JA et ET et se met en place en marge de la zone de la HR (Dorey *et al.*, 1998). C’est une zone typique où est accumulé et synthétisé un grand nombre de molécules antimicrobiennes telles que des composés phénoliques et des protéines PR. La résistance est de ce fait très importante au niveau de cette zone locale, ce qui contribue à restreindre la progression des agents pathogènes (Cordelier *et al.*, 2003).

2.5.4. La résistance systémique acquise ou SAR

Suite à la LAR se met en place une résistance au niveau systémique : la SAR (pour "Systemic Acquired Resistance"). Ce phénomène de SAR a fait l'objet de diverses études et a pu être nommé de différentes façons. Ainsi en 1933, Chester évoquait une "immunité physiologique acquise" chez des plantes infectées par des agents pathogènes. Cependant, ce terme prête aujourd'hui à confusion car il n'y a pas de système immunitaire chez les plantes comparable à ce qui existe chez l'animal. La SAR a pu également être nommée quelques fois ISR (pour "Induced Systemic Resistance") puisqu'il s'agit d'une réponse induite (Kloepper *et al.*, 1992). Néanmoins, ce terme a ensuite été beaucoup plus utilisé pour les micro-organismes de la rhizosphère (van Loon et Bakker, 2005), comme nous pourrons le constater. Il est, cependant, important de prendre en compte que ces termes de SAR et d'ISR ont été déclarés synonymes par Hammerschmidt *et al.* (2001) et par Tuzun (2006). Dans cette introduction, la SAR sera définie comme type de résistance induite par les agents pathogènes (Kuć, 1995) et l'ISR comme résistance induite par des micro-organismes bénéfiques (Pieterse *et al.*, 1996 ; van Loon *et al.*, 1998 ; 2006b), ces deux termes étant considérés encore comme différents par de nombreux auteurs.

La SAR fait suite à l'infection d'agents pathogènes avirulents, lors d'expression de symptômes de maladies ou suite à l'application d'éliciteurs. Elle conduit à la protection systémique de la plante vis à vis de nouvelles infections (Ross, 1961 ; 1966). La SAR peut ainsi protéger les différents organes de la plante telles que la tige, les feuilles voire même les fleurs ou les fruits contre une attaque ultérieure par un phytopathogène. Son activité protectrice peut durer plusieurs semaines après le premier contact entre la plante et l'agent pathogène (pour revue van Loon *et al.*, 2006b).

Le phénomène de SAR est associé de nos jours à l'expression de différents gènes de défenses ainsi qu'à une signalisation impliquant le SA (Baker *et al.*, 1997 ; Métraux *et al.*, 2002).

Au niveau du signal impliqué dans la SAR, il est ainsi communément admis que cette résistance dépend majoritairement de l'accumulation du SA aussi bien au niveau local qu'au niveau systémique (Métraux *et al.*, 2002 ; Nawrath *et al.*, 2006). Néanmoins, le SA n'est peut être pas toujours transporté dans la plante (Verberne *et al.*, 2003). A l'heure actuelle, il existe même l'hypothèse selon laquelle un autre signal est transporté et induit, à distance, une production de SA (Durrant et Dong, 2004).

Le SA peut être un signal intervenant dans la synthèse de différentes protéines PR qui sont, ainsi, devenues des marqueurs de la SAR. Ceci inclut certaines isoformes des PR-1, PR-2, et PR-5 dont la synthèse dépend de la voie de biosynthèse du SA (van Loon *et al.*, 2006b). Il existe cependant des différences selon les espèces végétales étudiées. Si chez *A. thaliana*, la distinction entre les PR-1, PR-2, et PR-5 induites par la voie du SA et les PR-3, PR4 et PR-12 par la voie du JA / ET est claire (Thomma *et al.*, 2001), chez d'autres plantes, ce n'est pas identique. Chez le tabac par exemple, la synthèse des protéines PR-1, PR-2, PR-3 et PR-5 acides est tout d'abord régulée par le SA mais l'ET et / ou le JA peuvent également intervenir et être, de ce fait, des signaux à l'origine de leur biosynthèse (Niki *et al.*, 1998 ; Seo *et al.*, 2001).

2.6. La vigne et ses défenses

Toutes les réactions de défense et leurs mécanismes associés cités précedemment ont été décrits chez la vigne. Des barrières constitutives sont ainsi trouvées au niveau des feuilles, des baies et des racines. Le nombre de couches cellulaires qui joue un rôle de protection (Sarig *et al.*, 1998), ainsi que l'épaisseur et la composition de la cuticule (Hill *et al.*, 1981 ; Marois *et al.*, 1986 ; Percival *et al.*, 1993 ; Commenil *et al.*, 1997), la cire épidermique (Gabler *et al.*, 2003) et la densité des baies dans une grappe (Vail et Marois, 1991 ; Percival *et al.*, 1993) permettent ainsi de restreindre la progression des agents pathogènes.

Des composés chimiques et des enzymes constitutives participent également à la résistance de la vigne (Pezet *et al.*, 2004). Ainsi, des peroxydases et des phytoanticipines (composés chimiques de faibles poids moléculaires et issus du métabolisme secondaire) sont naturellement présentes au niveau de différents organes de la vigne. Ces défenses constitutives contribuent à la résistance contre divers agents pathogènes comme par exemple *P. viticola*, l'agent responsable du mildiou et *B. cinerea*, l'agent responsable de la pourriture grise (pour revue Pezet *et al.*, 2004).

Chez la vigne, il existe également des gènes de résistance (*R*) (Di Gaspero et Cipriani, 2003). Néanmoins, il n'existe pas de résistance "gène pour gène" pour la plupart des agents pathogènes de cette plante. Des PAMPs issus de l'interaction vigne / agent pathogène sont cependant reconnus par la plante et conduisent à l'induction de différentes réactions de défenses. C'est le cas des PAMPs issus de *B. cinerea* tels que l'endopolygalacturonase BcPG1 (Poinssot *et al.*, 2003 ; Vandelle *et al.*, 2006) ou des éliciteurs "botrycin" et "cinerein"

(Repka, 2006). Ces PAMPs sont à l'origine de changements de flux d'ions, de l'activation de protéines kinases et de phosphatases, ainsi que de la production de FAO et de NO (Poinssot *et al.*, 2003 ; Vandelle *et al.*, 2006).

Chez la vigne, différentes protéines PR ont également été identifiées et leurs gènes ont été séquencés. Depuis 1996, la littérature mentionne des glucanases (PR-2), des chitinases (PR-3), des osmotines (PR-5), des inhibiteurs de polygalacturonases (PR-6), des RNases (PR-10), des protéines de transfert de lipides (PR-14), et une PR-17 (Salzman *et al.*, 1998 ; Jacob *et al.*, 1999 ; Bézier, 2003 ; Goes da Silva *et al.*, 2005). Des analyses sérologiques ont également permis de mettre en évidence une peroxydase (PR-9) et une PR-1 (Repka *et al.*, 2000 ; 2001).

Ces protéines PR ou leurs gènes ont été identifiés soit lors du développement de la vigne tels que pour des PR-2 (Goes da Silva *et al.*, 2005), des chitinases (Derckel *et al.*, 1998), des PR-5 (Salzman *et al.*, 1998) et des PR-14 (Salzman *et al*., 1998), soit suite à la réponse de la plante à un agent pathogène virulent (*B. cinerea*, *E. necator*, *P. viticola*) ou avirulent (*Pseudomonas syringae* pv. pisi) comme les PR-2, PR-3, PR-5, PR10 et PR-17 (Derckel *et al.*, 1996 ; Renault *et al.*, 1996 ; Robinson *et al*., 1997 ; Robert *et al.*, 2001 ; Bézier, 2003). D'autres ont été identifiés après une blessure comme par exemple pour les chitinases (Derckel *et al*, 1998) ou après application d'éliciteurs comme nous pourrons le constater lors de cette introduction.

Parmi ces protéines ou leurs gènes associés, certains ont bien été étudiés au sein de notre laboratoire. C'est le cas notamment pour *VvGluC* (codant une PR-2 basique) et *VvChi4C* (codant une PR-3 acide) ou de leurs protéines qui présentent de fortes activités antimicrobiennes (Derckel *et al.*, 1996 ; 1998 ; Bézier *et al.*, 2002 ; 2007 ; Bézier, 2003). Une approche moléculaire par "Differential Display" a également permis d'identifier d'autres gènes codants des protéines PR ou des gènes régulés lors de l'infection de la vigne par *B. cinerea* (Bézier, 2003 ; Bézier *et al.*, 2007). Parmi ces gènes, on peut distinguer ceux nommés *Big* (pour "*Botrytis*-induced grapevine gene") et *Brg* (pour "*Botrytis*-repressed grapevine gene"). Certains d'entre eux ont permis d'identifier ou de ré-identifier *VvPR6*, *VvPR10.1* et *VvPR27* qui codent respectivement pour une PR-6, une PR-10 et une PR-17 (Bézier, 2003 ; Bézier *et al.*, 2007). Un autre de ces gènes est le gène *Vv102*. Ce gène ne présente pas encore d'homologie avec d'autres gènes connus chez d'autres plantes et sa fonction est encore inconnue. Néanmoins, ce gène peut quand même être classé comme gène codant une protéine

PR (F. Baillieul, communication personnelle) puisqu'il a été détecté comme induit par un agent pathogène (Bézier *et al.*, 2007).

La signalisation permettant la régulation des gènes de défense de la vigne (c'est à dire dépendant du SA , du JA ou de l'ET) n'est pas encore caractérisée comme chez *A. thaliana* ou chez le tabac. Les connaissances actuelles font état de la synthèse de PR-1 dépendante du SA et de PR-4, de la voie du JA (Hamiduzzaman *et al.*, 2005). Néanmoins, des analyses au sein de notre laboratoire ont révélé que l'induction du gène *Vv102* est majoritairement dépendante du SA, et celle de *VvPR6*, de la voie du JA (Névians, 2007). De plus, les expressions de *VvChi4C* et *VvGluC* sont régulées à la fois par le SA et le JA (Névians, 2007). Ces gènes présentent ainsi un intérêt particulier lors d'étude sur l'interaction entre la vigne et un micro-organisme puisque leur suivi permet de suggérer les voies de signalisation impliquées dans les mécanismes de défense en plus de décrire leur expression. En revanche, pour les gènes *VvPR10.1* et *VvPR27*, les signalisations n'ont pas encore été élucidées. Une étude préliminaire a permis cependant de démontrer que leurs expressions étaient régulées par le JA mais ceci semble dépendre du stress appliqué au niveau de la plante puisque le SA peut être également impliqué (F. Mazeyrat-Gourbeyre, communication personnelle).

Chez la vigne, d'autres molécules de défenses ou leurs gènes ont été également identifiés comme c'est le cas par exemple pour les PGIP, les LOX et les HRGP (Bézier, 2003 ; Bézier *et al.*, 2007).

L'accumulation de composés phénoliques tels que des flavonoïdes, des tanins et des phytoalexines (les stilbènes *trans*- et *cis*–resvératrol et ses produits d'oxydation α-, β-, δ-, γ- et ε-viniférine) est également retrouvée fréquemment chez la vigne, notamment suite à l'infection par un agent pathogène (Jeandet *et al.*, 1999 ; 2007). Comme nous avons pu le décrire précédemment, ces composés fluorescent sous lumière bleue ou sous U.V. Ils sont, de ce fait, particulièrement intéressants lors d'études microscopiques d'une interaction entre la plante et son environnement biotique puisqu'ils peuvent facilement identifiés, comme nous pourrons le constater lors de ces travaux de thèse.

Ainsi, différents mécanismes de défenses ont été caractérisés chez la vigne. Néanmoins, ces défenses ne sont pas suffisantes pour contrer le développement de certains agents pathogènes comme c'est le cas pour *B. cinerea* (Pezet *et al.*, 2004), d'où la nécessité de trouver d'autres méthodes de lutte.

3. Les alternatives à la résistance naturelle

Afin de contrer le développement des agents pathogènes, il existe de nos jours différentes méthodes de lutte pour pallier au manque de résistance naturelle des plantes. C'est le cas avec l'utilisation de micro-organismes bénéfiques. Néanmoins, l'utilisation de produits phytosanitaires, la transgénèse ainsi que l'élicitation ont également été bien étudiées (Leroux, 2004 ; Gilbert *et al.*, 2006 ; Garcia-Brugger *et al.*, 2006).

3.1. La lutte chimique et les produits phytosanitaires

La lutte chimique s'est révélée depuis toujours une méthode de lutte efficace et indispensable pour combattre les agents pathogènes (Leroux, 2003 ; Pezet *et al.*, 2004).

Depuis les années 1960, cette méthode de lutte implique surtout les fongicides. Ces produits sont actuellement utilisés chez diverses plantes cultivées, y compris la vigne. Pour cette dernière, jusqu'à 78300 tonnes de produits phytosanitaires ont été utilisées en France en 2005 (données UIPP). Ceci correspond à environ 50% des produits phytosanitaires utilisés dans notre pays.

Les produits phytosanitaires sont très actifs sur la vigne en particulier pour combattre les agents pathogènes. Néanmoins, ils présentent également beaucoup d'inconvénients. Leur coût est très élevé et certains produits présentent une toxicité aiguë ou chronique pour l'homme et les organismes non cibles (Leroux, 2004). De plus, les agents pathogènes peuvent devenir résistants à ces produits chimiques. Ceci a entraîné l'arrêt de certains fongicides (Panon *et al.*, 2006). Depuis 2002, plus de 160 matières actives dont une vingtaine de fongicides jugés trop toxiques sont par conséquent en cours de retrait. L'Organisation Européenne et Méditerranéenne pour la Protection des Plantes (OEPP) préconise même, pour une bonne pratique phytosanitaire, de limiter le nombre d'applications de chaque classe chimique et d'utiliser des fongicides ayant des modes d'actions biochimiques différents (Bulletin OEPP/EPP0, 2002). Ceci étant en accord avec la directive européenne 91/414/CEE concernant la mise sur le marché de nouveaux produits phytosanitaires.

3.2. Les plantes transgéniques

Une autre méthode de protection des plantes vis-à-vis des agresseurs consiste à modifier génétiquement les plantes en exprimant des gènes de défense issus d'autres organismes ou en sur-exprimant les gènes de défenses déjà existants (Cornelissen et Melchers, 1993 ; Shah, 1997 ; Punja, 2001 ; Gilbert *et al.*, 2006). De nombreuses stratégies visent ainsi à exprimer des gènes dont la protéine correspondante possède une activité antifongique comme c'est le cas pour des gènes codants des protéines PR ou d'autres molécules à fonction antimicrobienne (Cornelissen et Melchers, 1993). Une autre solution consiste à sur-exprimer des gènes à l'origine de signaux impliqués dans les voies de défenses ainsi que des gènes de résistance (Punja, 2001). Enfin, il est possible de transférer chez la plante des gènes codants des protéines capables d'inhiber une fonction nécessaire au déroulement du processus infectieux de l'agent pathogène (Cornelissen et Melchers, 1993).

Au niveau de la vigne, l'utilisation de la transgénèse a permis de conférer une protection contre différentes maladies (Kikkert, 1997 ; Yamamoto *et al.*, 2000 ; Coutos-Thévenot *et al.*, 2001 ; Legrand *et al.*, 2003 ; Ferreira *et al.*, 2004 ; Agüero *et al.*, 2005 ; Vidal *et al.*, 2006). Une sur-expression du gène *Mag-2* codant la magainin-2 s'est ainsi révélée efficace contre *Agrobacterium vitis* et *Erysiphe necator* (Vidal *et al.*, 2006). Les vignes ont également été transformées avec un gène codant une endochitinase d'origine fongique, ceci permettant de réduire l'infection de *B. cinerea* (Kikkert, 1997 ; Reisch *et al.*, 2003). De plus, une construction impliquant un gène chimère combinant un promoteur inductible (originaire de la luzerne) par l'agent pathogène et le gène de la stilbène synthase cloné à partir de la vigne a fait ses preuves pour protéger la plante contre la pourriture grise (Coutos-Thévenot *et al.*, 2001). Un autre exemple peut impliquer la sur-expression du gène codant les protéines inhibant les polygalacturonases de *B. cinerea*, ceci permettant à la plante d'être mieux protégée contre cet agent pathogène (Agüero *et al.*, 2005).

Il existe ainsi différentes possibilités de transgénèses permettant de combattre les agents pathogènes de la vigne et en particulier *B. cinerea*. Néanmoins, même si la directive européenne 2003/11/CE autorise la préparation de plants transgéniques de vigne sur tout le territoire de l'Union Européenne, la profession viti-vinicole est aujourd'hui réservée vis à vis d'un éventuel recours aux vignes génétiquement modifiées. Celles-ci pourraient, en effet, ternir l'image des vins français aussi bien sur notre propre territoire qu'au niveau international. De ce fait, des stratégies alternatives ont été envisagées.

3.3. Les éliciteurs en vue de déclencher les défenses des plantes

Les éliciteurs d'origines biotique ou abiotique sont de nos jours bien connus pour pouvoir induire des réactions de défense et permettre une protection de la plante contre ses agresseurs (Garcia-Brugger *et al.*, 2006).

3.3.1. Les éliciteurs d'origines biotiques

Il est communément admis que certains éliciteurs sont des PAMPs ou des MAMPs quand ils sont d'origines biotiques et peuvent être même des MIMPs (pour "Microbial-Induced Molecular Pattern") quand ils proviennent de la plante agressée (Mackey et MacFall, 2006). Ces produits peuvent être sécrétés par des micro-organismes ou dérivent des parois fongiques, bactériennes ou de la plante lors de l'interaction avec un agent pathogène (Côté et Hahn, 1994 ; Ebel et Costo, 1994) voire même d'algues marines (Bouarab *et al.*, 1999 ; Klarzinski *et al.*, 2000 ; Potin *et al.*, 1999).

De nos jours, différents éliciteurs existent. Des glycoprotéines (Felix *et al.*, 1999 ; Felbrich *et al.*, 2002 ; Poinssot *et al.*, 2003 ; Garcia-Brugger *et al.*, 2006), des oligosaccharides (Côté *et al.*, 1998 ; Klarzynski *et al.*, 2000 ; Rabea *et al.*, 2003), des composés lipidiques (Amborabé *et al.*, 2003 ; Zeidler *et al.*, 2004) ainsi que les molécules signals (JA, ET ou SA et ses dérivés analogues acide 2,6-dichloroisonicotinique ou INA et acide benzo (1, 2, 3) thiadiazole-7-carbothioïque S-méthyl-ester ou BTH ; Kachroo et Kachroo, 2007) peuvent éliciter des réponses de défenses.

Chez la vigne, certains de ces éliciteurs sont actuellement utilisés et permettent de protéger la vigne contre divers agents pathogènes, notamment *B. cinerea*. Parmi eux existe l'ergostérol, un stérol majeur des champignons, qui élicite des réponses de défense sur cultures cellulaires, ce qui se manifeste par l'expression du gène de la stilbène synthase, la synthèse de protéines de transfert de lipides, de facteurs de transcription ainsi que par une accumulation de resvératrol (Laquitaine *et al.*, 2006). Cette élicitation de la vigne par l'ergostérol permet une protection des plantules *in vitro* contre *B. cinerea* (Laquitaine *et al.*, 2006).

Chez la vigne le chitosane a également été étudié. Ce composé, issu de l'exosquelette de crustacés ou d'insectes, stimule la sécrétion de la LOX, de la PAL, de phytoalexines, de β-1,3-glucanase et de chitinases, ce qui induit une résistance des feuilles de vigne contre *B. cinerea* (Compant, 2002 ; Ait Barka *et al.*, 2004 ; Aziz *et al.*, 2006 ; Trotel-Aziz *et al.*, 2006).

La laminarine peut également éliciter les réactions de défense chez la vigne. Il a été ainsi montré que cet éliciteur, provenant de *Laminaria digitata*, induit un influx de calcium, une alcalinisation du milieu extracellulaire, un burst oxydatif, l'activation de deux MAP kinases, l'induction de gènes de défenses (*VvLox*, *VvPal*, *VvSts1*, *VvChi1b*, *VvChi3*, *VvChi4C*, *VvGlu1* et *VvPgip*) ainsi que la production de phytoalexines. La laminarine protège les feuilles de vigne contre *B. cinerea* en même temps qu'envers *P. viticola* (Aziz *et al.*, 2003).

Les molécules signals sont également utilisées chez la vigne. Par exemple, une application de SA sur la vigne stimule l'accumulation de protéines PR sur suspensions cellulaires (Busam *et al.*, 1997), ainsi que lors d'une application sur des racines, des tiges, des feuilles et des baies (Renault *et al.*, 1996 ; Derckel *et al.*, 1996 ; 1998).

Ainsi, différents composés d'origine biologique élicitent les réponses de défenses chez les plantes y compris sur la vigne et contribuent à restreindre le développement de maladies.

3.3.2. Les éliciteurs d'origines abiotiques

Des composés d'origines abiotiques sont également connus pour éliciter les réactions de défenses des plantes. Ces composés tels que le cuivre, le chlorure d'aluminium, le calcium et / ou les U.V. peuvent ainsi éliciter des réactions de défenses chez diverses plantes dont la vigne (Langcake et Pryce, 1977 ; Adrian *et al.*, 1996l ; Coulomb *et al.*, 1998 ; Bonomelli *et al.*, 2004 ; Borie *et al.*, 2004). Cette stratégie permet de diminuer l'infection causée par divers agents pathogènes, notamment *B. cinerea* (pour revue récente Elmer et Reglinski, 2006).

3.3.3. Le phénomène de potentialisation ou "priming"

Certains éliciteurs peuvent également agir comme potentialisateurs. Ce phénomène de potentialisation, également appelé "priming", correspond à la capacité de la plante à présenter des réactions de défenses plus intenses et plus rapides lors de l'attaque d'un agent pathogène (pour revues récentes Conrath *et al*., 2002 ; Conrath *et al*., 2006). Cet effet, tout d'abord décrit chez l'animal dès 1987 (Gifford et Lohman-Mathhes, 1987) a ensuite été étudié chez les plantes (Kauss *et al.*, 1992). Il a été ainsi décrit qu'un traitement au SA (Kauss *et al.*, 1992 ; Thulke et Conrath, 1998), ses dérivés INA et BTH (Katz *et al.*, 1998 ; 2002 ; Thulke et Conrath, 1998), des composés volatiles (Kessler *et al.*, 2006) voire même des micro-organismes bénéfiques (cf. chapitre suivant ; van Wees *et al.*, 1999 ; Ton *et al.*, 2007) peuvent potentialiser les défenses des plantes lors d'une infection par un agent pathogène.

Chez la vigne, la potentialisation peut être générée avec l'acide β-aminobutyrique (BABA ; Hamiduzzaman *et al.,* 2005). Le BABA n'induit pas de défense lorsqu'il est appliqué sur la vigne. Cependant, lors d'une infection ultérieure par *P. viticola*, les défenses sont potentialisées et permettent de protéger plus efficacement la plante contre son agresseur. Ceci se fait par le biais d'une formation de callose et de l'induction de gènes régulés par la voie du JA (*VvLox9* et *VvPR4* ; Hamiduzzaman *et al.,* 2005).

Ainsi, différentes stratégies telles que la lutte chimique, la transgénèse et l'élicitation peuvent pallier au manque de résistance naturelle des plantes. Néanmoins, une autre stratégie prometteuse correspond actuellement à la lutte biologique.

4. Les micro-organismes bénéfiques

Dans le cas qui nous intéresse, cette alternative consiste à utiliser des micro-organismes pouvant être antagonistes des agents pathogènes et / ou éliciteurs des plantes (Nowak et Schulaev, 2003). Néanmoins, il est important de prendre en compte que ces micro-organismes peuvent également stimuler directement la croissance des plantes. Du point de vue de l'agriculture, ces micro-organismes ont ainsi suscité un très grand intérêt (Morrissey *et al.*, 2004).

La plupart de ces micro-organismes dérivent de la rhizosphère, les 2 à 5 mm de sol entourant le système racinaire où des populations microbiennes sont en relation avec la plante (Hinsinger et Marschner, 2006). Certains de ces micro-organismes sont épiphytiques, c'est à dire qu'ils colonisent les surfaces racinaires, tandis que d'autres peuvent également entrer à l'intérieur des plantes et devenir ainsi des endophytes. Certains d'entre eux peuvent également former des symbioses remarquables avec leur hôte végétal (pour revue Gray et Smith, 2005). Néanmoins, il est nécessaire de clarifier certains de ces termes compte tenu des controverses relevées dans la littérature. J'entends ainsi par le terme "épiphyte", un micro-organisme capable de coloniser les surfaces de la plante comme décrit par Hallman *et al.* (1997). Pour le terme "endophyte", je sous-entends un micro-organisme capable d'entrer à l'intérieur d'une plante et d'y survivre au moins une partie de sa vie sans provoquer de symptômes apparents à son hôte végétal. Cette définition correspond à la définition de Hallmann (2001) pour les bactéries endophytiques et inclut également les micro-organismes

de nature fongique. Ceci exclut en revanche les autres définitions de de Bary (1866), Kado (1992) ainsi que celle de Quispel (1992) trop restrictives ou pouvant également inclure des agents pathogènes. Pour le terme de symbiose, je sous-entends la définition de de Bary (1879) qui se définit par une association entre deux partenaires d'espèces différentes. Ceci inclut une association de type commensalisme, neutralisme, mutualisme ou de type parasitisme (de Bary, 1879). Etant donné que l'interaction plante / agents pathogène peut être, ainsi, représentée dans ce terme de symbiose, j'utiliserai souvent le terme de symbiose de type mutualisme d'après la définition de Daida *et al.* (1996), c'est à dire une association à caractère constant et durable où des bénéfices sont partagés entre deux espèces différentes. Ceci exclut, par conséquent, les micro-organismes ne présentant un impact positif sur leur hôte que pendant un laps de temps limité. Cependant, ces derniers peuvent quand même être considérés comme symbiontes puisqu'ils restent au contact de la plante pour certains, mais deviennent neutres pour leur hôte végétal.

Parmi les micro-organismes bénéfiques, qu'ils soient épiphytiques ou endophytiques voire même à l'origine de symbioses remarquables (de type mutualisme), existent des champignons et des bactéries (Welbaum *et al.*, 2004).

4.1. Les PGPF ou champignons bénéfiques

4.1.1. Définition et propriétés

Des champignons peuvent stimuler les défenses de la plante et présenter une activité antagoniste envers différents phytopathogènes, ceci tout en stimulant directement la croissance de la plante (Whipps, 2001). Ces champignons bénéfiques ont été récemment appelés PGPF pour " Plant Growth-Promoting Fungi" (pour revue Bent, 2006). Ils peuvent être des champignons filamenteux (Harman *et al.*, 2004) voire même des levures également appelées PGPY pour "Plant Growth-Promoting Yeasts" (El-Tarabily et Sivasithamparam, 2006). Les PGPF peuvent être naturellement présents chez diverses plantes, aussi bien chez des plantes herbacées (Sinclair et Cerkauskas, 1996 ; Saikkonen et *al.*, 1998) que des plantes ligneuses (Arnold *et al.*, 2003), notamment la vigne (Nappi *et al.*, 1985 ; Cheng et Baumgartner, 2004 ; 2006). Chez ces plantes, les PGPF peuvent être épiphytiques et / ou endophytiques (Carroll, 1988 ; Clay, 1990 ; Ahlohm *et al.*, 2002 ; Faeth, 2002 ; Hahn *et al.*,

2003 ; Schardl *et al.,* 2004) et sont même quelquefois à l'origine de symbioses remarquables comme c'est le cas avec les mycorrhizes (Selosse *et al.,* 2004).

4.1.2. Mécanismes impliqués dans la protection et la stimulation de croissance des plantes

Divers mécanismes sont impliqués dans la protection et la stimulation de croissance de la plante lors de l'association bénéfique entre une plante et un PGPF. Ces micro-organismes peuvent agir par une interaction directe avec l'agent pathogène, ce qui protège la plante indirectement. Ceci peut se réaliser par le biais d'un mycoparasitisme et d'une sécrétion d'allélochimiques inhibiteurs (ce terme est utilisé en fonction de sa dernière définition, pour plus d'information voir IAS, 1998 ou Sturz et Christie, 2003), et / ou d'un phénomène de compétition pour l'espace et les nutriments (Whipps, 2001 ; Mauchline *et al.,* 2002 ; Howell, 2003), tout en fournissant des composés bénéfiques pour le développement de la plante (Harman *et al.,* 2004 ; El-Tarabily et Sivasithamparam, 2006).

Ces micro-organismes peuvent également stimuler les défenses des plantes, ce qui va aboutir à une résistance de la plante hôte contre les agents pathogènes (pour revue Bent, 2006). Des barrières structurales avec des appositions pariétales peuvent notamment se mettre en place et des phytoalexines ainsi que des protéines PR peuvent être ou non sécrétées suite à l'inoculation de la plante avec un PGPF. Cependant, la plupart des PGPF induisent une ISR à la place d'une SAR : il n'y a pas de HR, le SA n'est pas impliqué au niveau systémique mais est remplacé par le JA et l'ET. En outre, et souvent, il n'y a pas de protéines PR sans présence d'un agent pathogène (pour revue Harman *et al.,* 2006).

Diverses recherches ayant porté sur l'interaction plante-PGPF mentionnent un phénomène de potentialisation des défenses. C'est le cas lors de l'interaction entre la tomate et un champignon mycorrhizien, *Glomus mosseae*, ceci permettant une protection de la plante contre *Phytophthora parasitica* (Cordier *et al.*, 1998 ; Pozo *et al.*, 1999 ; 2002). Un autre exemple concerne des plants de concombres, pré-inoculés avec *Trichoderma asperellum* souche T203, puis ensuite confrontés à l'agent pathogène *Pseudomonas syringae* pv. *Lachrymans*. Les résultats ont montré que les défenses sont potentialisées suite à l'infection par la bactérie pathogène, ce qui permet une protection de la plante contre l'agent agresseur (Shoresh *et al*., 2005).

4.1.3. L'utilisation des PGPF sur la vigne

Chez la vigne, divers PGPF sont actuellement étudiés ou utilisés en vue de protéger cette plante contre les agents pathogènes, notamment *B. cinerea*. Il existe ainsi des champignons filamenteux appartenant aux genres *Trichoderma, Ulocladium, Pythium, Gliocladium* et *Epicoccum*, ainsi que des levures comme *Pichia* spp. et *Aureobasidium* spp. (pour revue récente Elmer et Reglinski, 2006). Bien que la plupart de ces champignons proviennent de la rhizosphère (et également de la spermosphère, de la phyllosphère, voire même de la carposphère pour certains d'entre eux), les études démontrant leur potentiel d'action envers les agents pathogènes (notamment *B. cinerea*) portent souvent sur une application aux sites d'infection et de colonisation des agents infectieux (Pezet *et al.*, 2004). Ainsi, ces micro-organismes sont généralement appliqués aux niveaux des déchets floraux ainsi qu'au niveau foliaire en vue de lutter directement sur le développement de l'agent pathogène (pour revue Elmer et Reglinski, 2006), sans tenir compte de leur niche écologique naturelle. Cependant, il existe des exceptions. Une étude a par exemple montré chez la vigne le potentiel d'une souche de *Pythium oligandrum* issue de la rhizosphère et d'un de ses MAMP, l'oligandrine, pour contrer le développement de la pourriture grise après application racinaire (Mohamed *et al.*, 2007).

4.2. Les PGPB ("Plant Growth-Promoting Bacteria") et les PGPR ("Plant Growth-Promoting Rhizobacteria")

4.2.1. Généralités

Parallèlement aux PGPF, des bactéries bénéfiques sont également utilisées pour protéger la plante contre les infections par les agents pathogènes (Schroth et Hancock, 1981 ; van Loon et Bakker, 2004 ; 2005). Certaines de ces bactéries peuvent de plus stimuler directement la croissance de leur hôte végétal et sont ainsi d'un grand intérêt pour l'agriculture (Glick, 1995 ; Welbaum *et al.*, 2004).

Ces micro-organismes bénéfiques ou PGPB (pour "Plant Growth-Promoting Bacteria" ; Bashan et Holguin, 1998) peuvent provenir de différentes niches écologiques telles que la rhizosphère, la spermosphère, la phyllosphère voire même de l'anthosphère ou de la carposphère. Néanmoins la plupart d'entre elles sont originaires de la rhizosphère comme décrit par Lorenz Hitlner dès 1904 (pour revue Hartmann, 2005). Elles sont de ce fait des rhizobactéries et ont ainsi été appelées PGPR pour "Plant Growth-Promoting Rhizobacteria"

(Kloepper et Schroth, 1978). Néanmoins, d'autres appellations telles que EPR ("Emergence-Promoting Rhizobacteria" ; Kloepper *et al.,* 1986), PGPB (Bashan et Holguin, 1998), "biocontrol-PGPB" (Bashan et Holguin, 1998) voire même YIB ("Yield-Increased Bacteria" ; Chen *et al.,* 1996) peuvent être rencontrées dans la littérature, ceci coïncidant avec leurs différentes actions sur les plantes.

Les PGPR peuvent ainsi présenter des activités antagonistes, induire une résistance systémique chez la plante, produire des composés stimulant la croissance des plantes, accélérer l'émergence des graines, induire une floraison précoce, augmenter la récolte de leur hôte végétal, voire même conférer au sol des propriétés minimisant les dégâts des plantes (pour revues Vessey, 2003 ; Welbaum *et al.*, 2004).

De nos jours, il est communément admis que ces PGPR colonisent les surfaces racinaires et sont ainsi épiphytiques. Néanmoins, la plupart d'entre elles peuvent également entrer à l'intérieur des racines et se propager au sein de leur hôte végétal (Gray et Smith, 2005). Certaines de ces PGPR endophytiques peuvent même former des symbioses remarquables avec les plantes comme c'est le cas avec les genres *Rhizobium* (pour revue récente Sessitsch *et al.,* 2002), et *Burkholderia* dans les nodules des Fabacées (Moulin *et al.,* 2001 ; Chen *et al.,* 2003 ; 2005 ; 2006) ou chez les plantes actinorhiziennes avec *Frankia* sp. (pour revue Schwencke et Caru, 2001).

4.2.2. Protection envers les agents pathogènes et stimulation de la croissance des plantes

Les PGPR (qu'elles soient des épiphytes et / ou des endophytes) influencent leur hôte végétal par différents mécanismes (Figure 10).

Ceci peut impliquer une stimulation directe de la croissance par le biais d'une biofertilisation et *via* la sécrétion d'autres composés augmentant la croissance de leur hôte végétal comme par exemple des sidérophores chélatant le fer, la production de vitamines, des phytohormones ou l'aminocyclopropane carboxylate (ACC) déaminase qui clive l'ACC, le précurseur de l'éthylène qui inhibe la croissance racinaire, ce qui permet en retour une augmentation de la croissance de la plante (pour revue de ces mécanismes, voir Glick *et al.,* 1998 ; Vessey *et al,* 2003 ; Sturz et Christie, 2003).

L'effet des PGPR peut provenir en outre de leur activité de bio-contrôle (van Loon et Bakker, 2005) comme dans le cas qui nous intéresse. Cette activité a été associée à différents

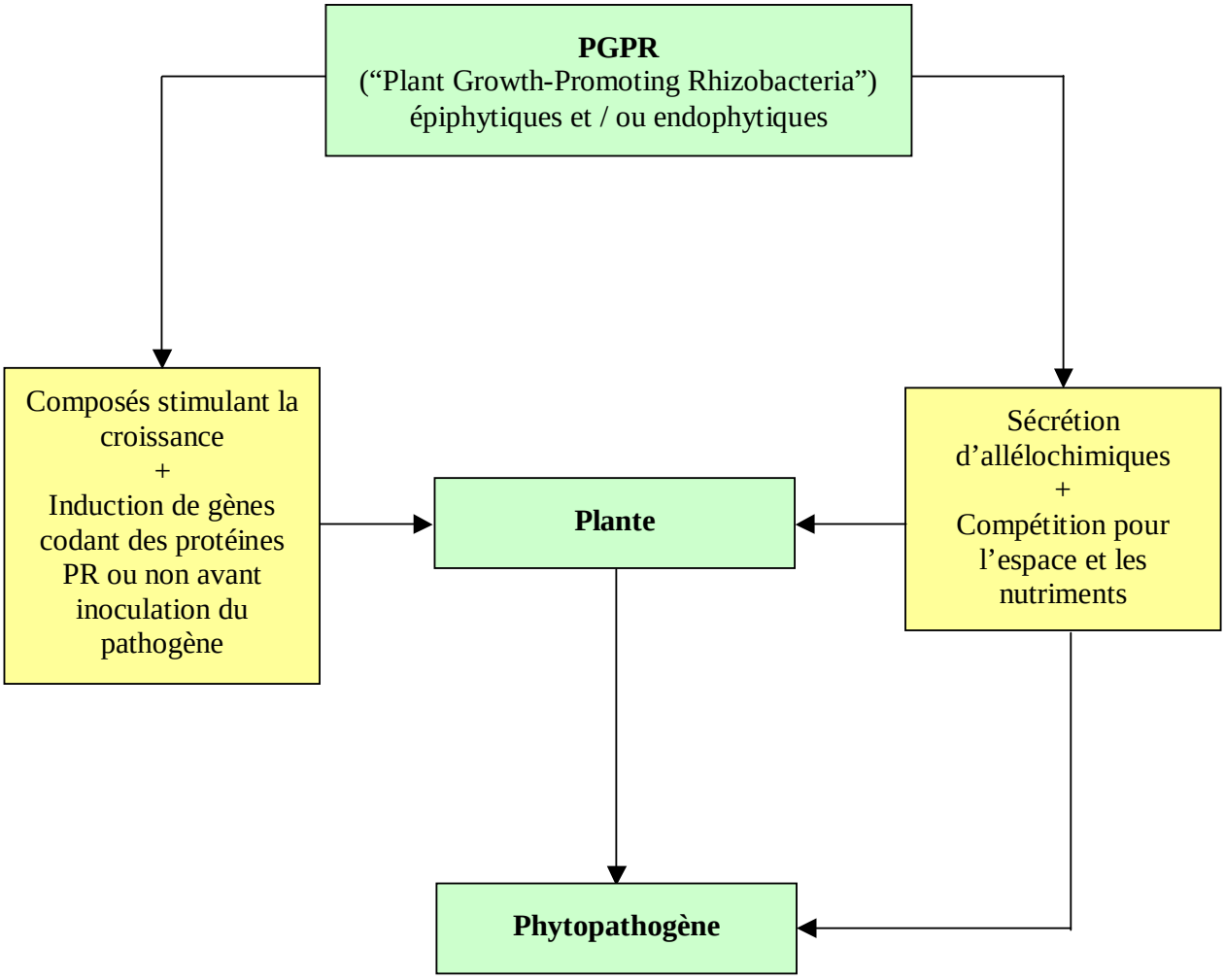


Figure 10 : Illustration des mécanismes d'action des PGPR épiphytiques et / ou endophytiques sur la plante et les agents pathogènes.

mécanismes. Le phénomène de compétence rhizosphèrique, une sécrétion d'allélochimiques pour une activité de bio-contrôle directe, et l'ISR induite par la bactérie ou par ses composés ont ainsi pu être décrits. Nous allons pouvoir le constater dans la revue "Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects" (Compant *et al.*, 2005a).

Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects

Stéphane Compant[1], Brion Duffy[2], Jerzy Nowak[3], Christophe Clément[1], and Essaïd Ait Barka[1]

Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cédex 2, France[1];

Agroscope FAW Wädenswil, Swiss Federal Research Institute for Fruit Protection, Viticulture, and Horticulture CH-8820 Wädenswil, Switzerland[2] ;

Department of Horticulture, Virginia Polytechnic Institute and State University, 0327-301 Saunders Hall, Blacksburg, VA 24060, USA[3]

Applied and Environmental Microbiology, 2005, 71: 4951-4959

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2005, p. 4951–4959 Vol. 71, No. 9
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.9.4951–4959.2005

MINIREVIEW

Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects

Stéphane Compant,[1] Brion Duffy,[2] Jerzy Nowak,[3] Christophe Clément,[1] and Essaïd Ait Barka[1]*

Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cedex 2, France[1]; Agroscope FAW Wädenswil, Swiss Federal Research Institute for Fruit Production, Viticulture, and Horticulture, CH-8820 Wädenswil, Switzerland[2]; and Department of Horticulture, Virginia Polytechnic Institute and State University, 0327-301 Saunders Hall, Blacksburg, Virginia 24060[3]

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. As agricultural production intensified over the past few decades, producers became more and more dependent on agrochemicals as a relatively reliable method of crop protection helping with economic stability of their operations. However, increasing use of chemical inputs causes several negative effects, i.e., development of pathogen resistance to the applied agents and their nontarget environmental impacts (44, 62). Furthermore, the growing cost of pesticides, particularly in less-affluent regions of the world, and consumer demand for pesticide-free food has led to a search for substitutes for these products. There are also a number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent (62). Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (44, 62, 136, 188).

There has been a large body of literature describing potential uses of plant associated bacteria as agents stimulating plant growth and managing soil and plant health (reviewed in references 63, 70, 143, 165, and 188). Plant growth-promoting bacteria (PGPB) (8) are associated with many, if not all, plant species and are commonly present in many environments. The most widely studied group of PGPB are plant growth-promoting rhizobacteria (PGPR) (82) colonizing the root surfaces and the closely adhering soil interface, the rhizosphere (82, 84). As reviewed by Kloepper et al. (84) or, more recently, by Gray and Smith (65), some of these PGPR can also enter root interior and establish endophytic populations. Many of them are able to transcend the endodermis barrier, crossing from the root cortex to the vascular system, and subsequently thrive as endophytes in stem, leaves, tubers, and other organs (10, 28, 65, 70). The extent of endophytic colonization of host plant organs and tissues reflects the ability of bacteria to selectively adapt to these specific ecological niches (65, 70). Consequently, intimate associations between bacteria and host plants can be formed (28, 70, 84) without harming the plant (70, 83, 84, 92, 191). Although, it is generally assumed that many bacterial endophyte communities are the product of a colonizing process initiated in the root zone (102, 165, 177, 188), they may also originate from other source than the rhizosphere, such as the phyllosphere, the anthosphere, or the spermosphere (70).

Despite their different ecological niches, free-living rhizobacteria and endophytic bacteria use some of the same mechanisms to promote plant growth and control phytopathogens (15, 46, 63, 70, 92, 165). The widely recognized mechanisms of biocontrol mediated by PGPB are competition for an ecological niche or a substrate, production of inhibitory allelochemicals, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens (15, 63, 66, 67, 97, 146) and/or abiotic stresses (reviewed in references 101 and 117). This review surveys the advances of plant-PGPB interaction research focusing on the principles and mechanisms of action of PGPB, both free-living and endophytic bacteria, and their use or potential use for the biological control of plant diseases.

COMPETITIVE ROOT COLONIZATION

Despite their potential as low-input practical agents of plant protection, application of PGPB has been hampered by inconsistent performance in field tests (167); this is usually attributed to their poor rhizosphere competence (153, 189). Rhizosphere competence of biocontrol agents comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable time period, in the presence of the indigenous microflora (95, 127, 189, 190). Given the importance of rhizosphere competence as a prerequisite of effective biological control, understanding root-microbe communication (6, 135), as affected by genetic (80, 118) and environmental (128) determinants in spatial (6) and temporal (23) contexts, will significantly contribute to improve the efficacy of these biocontrol agents.

* Corresponding author. Mailing address: Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cedex 2, France. Phone: 33-3-26-91-34-41. Fax: 33-3-26-91-34-41. E-mail: ea.barka@univ-reims.fr.

Competition for root niches and bacterial determinants directly involves root colonization. The root surface and surrounding rhizosphere are significant carbon sinks (143). Photosynthate allocation to this zone can be as high as 40% (34). Thus, along root surfaces there are various suitable nutrient-rich niches attracting a great diversity of microorganisms, including phytopathogens. Competition for these nutrients and niches is a fundamental mechanism by which PGPB protect plants from phytopathogens (50). PGPB reach root surfaces by active motility facilitated by flagella and are guided by chemotactic responses (41, 42, 112, 162, 171, 172). Known chemical attractants present in root exudates include organic acids, amino acids, and specific sugars (188). Some exudates can also be effective as antimicrobial agents and thus give ecological niche advantage to organisms that have adequate enzymatic machinery to detoxify them (reviewed in reference 6). The quantity and composition of chemoattractants and antimicrobials exuded by plant roots are under genetic and environmental control (6). This implies that PGPB competence highly depends either on their abilities to take advantage of a specific environment or on their abilities to adapt to changing conditions. As an example, *Azospirillum* chemotaxis is induced by sugars, amino acids, and organic acids, but the degree of chemotactic response to each of these compounds differs among strains (142). PGPB may be uniquely equipped to sense chemoattractants, e.g., rice exudates induce stronger chemotactic responses of endophytic bacteria than from non-PGPB present in the rice rhizosphere (5).

Bacterial lipopolysaccharides (LPS), in particular the O-antigen chain, can also contribute to root colonization (35). However, the importance of LPS in this colonization might be strain dependent since the O-antigenic side chain of *Pseudomonas fluorescens* WCS374 does not contribute to potato root adhesion (43), whereas the O-antigen chain of *P. fluorescens* PCL1205 is involved in tomato root colonization (35). Furthermore, the O-antigenic aspect of LPS does not contribute to rhizoplane colonization of tomato by the plant beneficial endophytic bacterium *P. fluorescens* WCS417r but, interestingly, this bacterial determinant was involved in endophytic colonization of roots (57).

It has also been recently demonstrated that the high bacterial growth rate and ability to synthesize vitamin B_1 and exude NADH dehydrogenases contribute to plant colonization by PGPB (35, 157). Another determinant of root colonization ability by bacteria is type IV pili, better known for its involvement in the adhesion of animal and human pathogenic bacteria to eukaryotic cells (69, 162, 163). The type IV pili also play a role in plant colonization by endophytic bacteria such as *Azoarcus* sp. (49, 162).

Root colonization and site-specific recombinase. Bacterial traits required for effective root colonization are subject to phase variation, a regulatory process for DNA rearrangements orchestrated by site-specific recombinase (36, 149, 174). In certain PGPB, efficient root colonization is linked to their ability to secrete a site-specific recombinase (36). Transfer of the site-specific recombinase gene from a rhizosphere-competent *P. fluorescens* into a rhizosphere-incompetent *Pseudomonas* strain enhanced its ability to colonize root tips (37).

Utilization of root exudates and root mucilage by PGPB. Since root exudates are the primary source of nutrients for rhizosphere microorganisms (143, 176), rhizosphere competence implies that PGPB are well adapted to their utilization (96). Despite the fact that sugars have often been reported as the major carbon source in exudates, the ability to use specific sugars does not play a major role in tomato root colonization (96). Similarly, although amino acids are present in root exudates, the bioavailability of amino acids alone is considered insufficient to support root tip colonization by auxotrophic mutants of *P. fluorescens* WCS365 (158). In contrast, Simons et al. (158) reported that amino acid synthesis is required for root colonization by *P. fluorescens* WCS365, indicating that amino acid prototrophy is involved in rhizosphere competence. In addition, PGPB regulate the rate of uptake of polyamines such as putrescine, spermine, and spermidine, since their high titer could retard bacterial growth and reduce their ability to competitively colonize roots (87). Root mucilage also offers a utilizable carbon source for PGPB (85) to use for the competitive colonization.

BIOCONTROL ACTIVITY MEDIATED BY THE SYNTHESIS OF ALLELOCHEMICALS

Offensive PGPB colonization and defensive retention of rhizosphere niches are enabled by production of bacterial allelochemicals, including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes, and detoxification enzymes (6, 63, 166).

Competition for iron and the role of siderophores. Iron is an essential growth element for all living organisms. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition (93). Under iron-limiting conditions PGPB produce low-molecular-weight compounds called siderophores to competitively acquire ferric ion (191). Although various bacterial siderophores differ in their abilities to sequester iron, in general, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (94, 122). Some PGPB strains go one step further and draw iron from heterologous siderophores produced by cohabiting microorganisms (19, 92, 94, 137, 186, 191).

Siderophore biosynthesis is generally tightly regulated by iron-sensitive Fur proteins, the global regulators GacS and GacA, the sigma factors RpoS, PvdS, and FpvI, quorum-sensing autoinducers such as *N*-acyl homoserine lactone, and site-specific recombinases (31, 141). However, some data demonstrate that none of these global regulators is involved in siderophore production. Neither GacS nor RpoS significantly affected the level of siderophores synthesized by *Enterobacter cloacae* CAL2 and UW4 (148). RpoS is not involved in the regulation of siderophore production by *Pseudomonas putida* strain WCS358 (86). In addition, GrrA/GrrS, but not GacS/GacA, are involved in siderophore synthesis regulation in *Serratia plymuthica* strain IC1270, suggesting that gene evolution occurred in the siderophore-producing bacteria (123). A myriad of environmental factors can also modulate siderophores synthesis, including pH, the level of iron and the form of iron ions, the presence of other trace elements, and an adequate supply of carbon, nitrogen, and phosphorus (52).

Antibiosis. The basis of antibiosis as a biocontrol mechanism of PGPB has become increasingly better understood over the past two decades (191). A variety of antibiotics have been

identified, including compounds such as amphisin, 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, and cyclic lipopeptides produced by pseudomonads (33, 40, 114, 115, 138) and oligomycin A, kanosamine, zwittermicin A, and xanthobaccin produced by *Bacillus*, *Streptomyces*, and *Stenotrophomonas* spp. (72, 81, 103, 104, 110). Interestingly, some antibiotics produced by PGPB are finding new uses as experimental pharmaceuticals (45, 75, 192), and this group of bacteria may offer an untapped resource for compounds to deal with the alarming ascent of multidrug-resistant human pathogenic bacteria.

Regulatory cascades of these antibiotics involve GacA/GacS or GrrA/GrrS, RpoD, and RpoS, *N*-acyl homoserine lactone derivatives (15, 21, 68, 131) and positive autoregulation (17, 151). Antibiotic synthesis is tightly linked to the overall metabolic status of the cell, which in turn is dictated by nutrient availability and other environmental stimuli (167), such as major and minor minerals, type of carbon source and supply, pH, temperature, and other parameters (11, 51, 52, 61, 78, 103, 104, 124, 125). Trace elements, particularly zinc, and carbon source levels influence the genetic stability/instability of bacteria, affecting their ability to produce secondary metabolites (53). It is important to note that many strains produce pallet of secondary antimicrobial metabolites and that conditions favoring one compound may not favor another (52). Thus, the varied arsenal of biocontrol strains may enable antagonists to perform their ultimate objective of pathogen suppression under the widest range of environmental conditions. For example, in *P. fluorescens* CHA0 biosynthesis of DAPG is stimulated and pyoluteorin is repressed in the presence of glucose as a carbon source. As glucose is depleted, however, pyoluteorin becomes the more abundantly antimicrobial compound produced by this strain (52). This ensures a degree of flexibility for the antagonist when confronted with a different or a changeable environment. Biotic conditions can also influence antibiotic biosynthesis (51, 54, 68, 116, 128). For example bacterial metabolites salicylates and pyoluteorin can affect DAPG production by *P. fluorescens* CHA0 (151). Furthermore, plant growth and development also influence antiobiotic production, since biological activity of DAPG producers is not induced by the exudates of young plant roots but is induced by the exudates of older plants, which results in selective pressure against other rhizosphere microorganisms (129). Plant host genotype also plays a significant role in the disease-suppressive interaction of plant with a microbial biocontrol agent, as demonstrated by Smith et al. (160, 161).

Lytic enzyme production. A variety of microorganisms also exhibit hyperparasitic activity, attacking pathogens by excreting cell wall hydrolases (26). Chitinase produced by *S. plymuthica* C48 inhibited spore germination and germ-tube elongation in *Botrytis cinerea* (58). The ability to produce extracellular chitinases is considered crucial for *Serratia marcescens* to act as antagonist against *Sclerotium rolfsii* (121), and for *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 to suppress *Fusarium oxysporum* f. sp. *cucumerinum*. It has been also demonstrated that extracellular chitinase and laminarinase synthesized by *Pseudomonas stutzeri* digest and lyse mycelia of *F. solani* (91). Although, chitinolytic activity appears less essential for PGPB such as *S. plymutica* IC14 when used to suppress *S. sclerotiorum* and *B. cinerea*, synthesis of proteases and other biocontrol traits are involved (77). The β-1,3-glucanase synthesized by *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 lyse fungal cell walls of *F. oxysporum* f. sp. *cucumerinum* (159). *B. cepacia* synthesizes β-1,3-glucanase that destroys the integrity of *R. solani*, *S. rolfsii*, and *Pythium ultimum* cell walls (59). Similar to siderophores and antibiotics, regulation of lytic enzyme production (proteases and chitinases in particular) involves the GacA/GacS (30, 60, 111, 147) or GrrA/GrrS regulatory systems (123) and colony phase variation (97).

Detoxification and degradation of virulence factors. Another mechanism of biological control is the detoxification of pathogen virulence factors. For example, certain biocontrol agents are able to detoxify albicidin toxin produced by *Xanthomonas albilineans* (9, 183, 194, 195). The detoxification mechanisms include production of a protein that reversibly binds the toxin in both *Klebsiella oxytoca* (183) and *Alcaligenes denitrificans* (9), as well as an irreversible detoxification of albicidin mediated by an esterase that occurs in *Pantoea dispersa* (194, 195). Several different microorganisms, including strains of *B. cepacia* and *Ralstonia solanacearum*, can also hydrolyze fusaric acid, a phytotoxin produced by various *Fusarium* species (169, 170). More often though, pathogen toxins display a broad-spectrum activity and can suppress growth of microbial competitors, or detoxify antiobiotics produced by some biocontrol microorganisms, as a self-defense mechanism against biocontrol agents (55, 152).

Recently, it has been discovered that certain PGPB quench pathogen quorum-sensing capacity by degrading autoinducer signals, thereby blocking expression of numerous virulence genes (47, 48, 105, 106, 113, 173). Since most, if not all, bacterial plant pathogens rely upon autoinducer-mediated quorum-sensing to turn on gene cascades for their key virulence factors (e.g., cell-degrading enzymes and phytotoxins) (181), this approach holds tremendous potential for alleviating disease, even after the onset of infection, in a curative manner.

Although biocontrol activity of microorgansims involving synthesis of allelochemicals has been studied extensively with free-living rhizobacteria, similar mechanisms apply to endophytic bacteria (92), since they can also synthesize metabolites with antagonistic activity toward plant pathogens (24). For example, Castillo et al. (20) demonstrated that munumbicins, antibiotics produced by the endophytic bacterium *Streptomyces* sp. strain NRRL 30562 isolated from *Kennedia nigriscans*, can inhibit in vitro growth of phytopathogenic fungi, *P. ultimum*, and *F. oxysporum*. Subsequently, it has been reported that certain endophytic bacteria isolated from field-grown potato plants can reduce the in vitro growth of *Streptomyces scabies* and *Xanthomonas campestris* through production of siderophore and antibiotic compounds (154). Interestingly, the ability to inhibit pathogen growth by endophytic bacteria, isolated from potato tubers, decreases as the bacteria colonize the host plant's interior, suggesting that bacterial adaptation to this habitat occurs within their host and may be tissue type and tissue site specific (164). Aino et al. (1) have also reported that the endophytic *P. fluorescens* strain FPT 9601 can synthesize DAPG and deposit DAPG crystals around and in the roots of tomato, thus demonstrating that endophyte can produce antibiotics in planta.

INDIRECT PLANT GROWTH PROMOTION THROUGH INDUCED SYSTEMIC RESISTANCE

Biopriming plants with some PGPB can also provide systemic resistance against a broad spectrum of plant pathogens. Diseases of fungal, bacterial, and viral origin, and in some instances even damage caused by insects and nematodes, can be reduced after application of PGPB (79, 135, 139, 146, 165).

Induced systemic resistance. Certain bacteria trigger a phenomenon known as ISR phenotypically similar to systemic acquired resistance (SAR). SAR develops when plants successfully activate their defense mechanism in response to primary infection by a pathogen, notably when the latter induces a hypersensitive reaction through which it becomes limited in a local necrotic lesion of brown, desiccated tissue (175). As SAR, ISR is effective against different types of pathogens but differs from SAR in that the inducing PGPB does not cause visible symptoms on the host plant (175). PGPB-elicited ISR was first observed on carnation (*Dianthus caryophillus*) with reduced susceptibility to wilt caused by *Fusarium* sp. (178) and on cucumber (*Cucumis sativus*) with reduced susceptibility to foliar disease caused by *Colletotrichum orbiculare* (187). Manifestation of ISR is dependent on the combination of host plant and bacterial strain (80, 175). Most reports of PGPB-mediated ISR involve free-living rhizobacterial strains, but endophytic bacteria have also been observed to have ISR activity. For example, ISR was triggered by *P. fluorescens* EP1 against red rot caused by *Colletotrichum falcatum* on sugarcane (182), *Burkholderia phytofirmans* PsJN against *Botrytis cinerea* on grapevine (2, 3) and *Verticllium dahliae* on tomato (156), *P. denitrificans* 1-15 and *P. putida* 5-48 against *Ceratocystis fagacearum* on oak (18), *P. fluorescens* 63-28 against *F. oxysporum* f. sp. *radicis-lycopersici* on tomato (109) and *Pythium ultimum* and *F. oxysporum* f. sp. *pisi* on pea roots (12), and *Bacillus pumilus* SE34 against *F. oxysporum* f. sp. *pisi* on pea roots (13) and *F. oxysporum* f. sp. *vasinfectum* on cotton roots (29).

Determinants of ISR. The ability to act as bioprotectants via ISR has been demonstrated for both rhizobacteria and bacterial endophytes, and considerable progress has been made in elucidating the mechanisms of plant-PGPB-pathogen interaction. Several bacterial traits (i.e., flagellation and production of siderophores and lipopolysaccharides) have been proposed to trigger ISR (73, 88, 90, 175, 179), but there is no compelling evidence for an overall ISR signal produced by bacteria (67). It has recently been reported that volatile organic compounds may play a key role in this process (135, 145). For example, volatiles secreted by *B. subtilis* GBO3 and *B. amyloquefaciens* IN937a were able to activate an ISR pathway in *Arabidopsis* seedlings challenged with the soft-rot pathogen *Erwinia carotovora* subsp. *carotovora* (144). A major distinction often drawn between ISR and SAR is the dependence of the latter on the accumulation of salicylic acid (SA) (128). Some PGPB do trigger an SA-dependent signaling pathway by producing nanogram amounts of SA in the rhizosphere (38, 39). However, the majority of PGPB that activate ISR appear to do so via a SA-independent pathway involving jasmonate and ethylene signals (128, 133). ISR is associated with an increase in sensitivity to these hormones rather than an increase in their production, which might lead to the activation of a partially different set of defense genes (71, 134).

Defense mechanisms of ISR-mediated by PGPB. PGPB-triggered ISR fortifies plant cell wall strength and alters host physiology and metabolic responses, leading to an enhanced synthesis of plant defense chemicals upon challenge by pathogens and/or abiotic stress factors (117, 139). After inoculation of tomato with endophytic *P. fluorescens* WCS417r, a thickening of the outer tangential and outermost part of the radial side of the first layer of cortical cell walls occurred when epidermal or hypodermal cells were colonized (57). In *Burkholderia phytofirmans* PsJN-grapevine interaction, a host defense reaction coinciding with phenolic compound accumulation and a strengthening of cell walls in the exodermis and in several cortical cell layers was also observed during endophytic colonization of the bacterium (28). The type of bacterized plant response induced after challenge with a pathogen resulted in the formation of structural barriers, such as thickened cell wall papillae due to the deposition of callose and the accumulation of phenolic compounds at the site of pathogen attack (13, 14, 109). Biochemical or physiological changes in plants (139) include induced accumulation of pathogenesis-related proteins (PR proteins) such as PR-1, PR-2, chitinases, and some peroxidases (76, 100, 109, 126, 139, 182). However, certain PGPB do not induce PR proteins (73, 132, 139, 180) but rather increase accumulation of peroxidase, phenylalanine ammonia lyase, phytoalexins, polyphenol oxidase, and/or chalcone synthase (25, 120, 139, 178). Recent evidence indicates that induction of some of these plant defense compounds (e.g., chalcone synthase) may be triggered by the same *N*-acyl homoserine lactones that bacteria use for intraspecific signaling (99). The revelation that some PGPB genes involved in antibiotic biosynthesis (e.g., *phlD*) are highly homologous with some plant genes involved in defense (e.g., chalcone synthase) (4, 7) raises the intriguing but as yet unexplored possibility that the products of these DeVriesien-like pangens may have interspecies activity benefiting plant protection, in addition to their currently known functions.

CONCLUSIONS AND FUTURE PROSPECTS TO MAKE BETTER USE OF PGPB

Research into the mechanisms of plant growth promotion by PGPB have provided a greater understanding of the multiple facets of disease suppression by these biocontrol agents. Still, most of the focus has been on free-living rhizobacterial strains, especially to *Pseudomonas* and *Bacillus*. Much remains to be learned from nonsymbiotic endophytic bacteria that have unique associations and apparently a more pronounced growth-enhancing effect on host plants (6, 22, 29, 135).

Revelations about the mechanisms of PGPB action open new doors to design strategies for improving the efficacy of biocontrol agents (107, 108, 184). Identification of key antimicrobials produced by superior agents, such as 2,4-diacetylphloroglucinol, can be exploited for streamlining strain discovery by targeting selection of new isolates that carry relevant biosynthetic genes (193). Determination of the role of edaphic parameters favorable for disease suppression, particularly those that stimulate antibiotic production and activity, can be exploited by targeting inoculants for soils that are more likely to support biocontrol. For example, amending soils or growth substrates with minerals such as zinc or priming inoculants with

media amendments during fermentation (51, 53, 125) can be very effective. Similarly, modulation of the rhizosphere bacteria consortia can be accomplished by soil aeration, hydrogenation, and delivery of molasses, sugars and by appropriate crop rotations (reviewed in reference 188).

Identifying different mechanisms of action facilitate the combination of strains, bacteria with bacteria or bacteria with fungi, to hit pathogens with a broader spectrum of microbial weapons (32, 56, 80, 89, 98, 119, 130, 140, 150). Along this same line, biotechnology can be applied to further improve strains that have prized qualities (e.g., formulation ease, stability, or otherwise exceptionally suited to plant colonization) by creating transgenic strains that combine multiple mechanisms of action (27, 74, 168). For example, transforming the 1-aminocyclopropane-1-carboxylic acid deaminase gene, which directly stimulates plant growth by cleaving the immediate precursor of plant ethylene (64) into *P. fluorescens* CHAO, not only increases plant growth but can also increase biocontrol properties of PGPB (185). Continued work with endophytic bacteria also holds potential for developing biocontrol agents that may be self-perpetuating by colonizing hosts and being transferred to progeny much as is the case with associative nitrogen-fixing PGPB on sugarcane (16) or the nonsymbiotic endophyte bacterium *Burkholderia phytofirmans* PsJN (117, 155).

ACKNOWLEDGMENTS

This study was supported by a grant from Europôl-Agro (Reims, France). Additional support for B. Duffy was provided by the Swiss Federal Office of Agriculture (project 04.24.3.3).

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Ainsi, les PGPR restreintes au niveau des surfaces racinaires ou endophytiques peuvent présenter diverses caractéristiques permettant de réduire le développement des agents pathogènes. Bien que ces phénomènes ont été développés dans la publication précédente, certains d'entre eux nécessitent cependant d'être mieux développés dans cette introduction, compte tenu de leur importance dans ce travail de thèse. C'est le cas, notamment, des phénomènes de compétence rhizosphèrique et de colonisation épi- et endophytique par ces micro-organismes, ainsi que les réponses de défenses induites chez la plante et également le phénomène de résistance systémique. Une compréhension de ces phénomènes permet de mieux cibler le type de micro-organisme pouvant être utilisé chez la vigne ainsi que ces conséquences bénéfiques sur la plante, comme nous allons pouvoir le contaster.

4.2.3. La compétence rhizosphèrique et la colonisation épiphytique des PGPR

Une des particularités intéressantes des PGPR réside tout d'abord dans leur compétence rhizosphèrique. Ceci correspond à leur capacité à coloniser la rhizosphère, les surfaces racinaires, à y survivre et y proliférer pendant une période considérable en présence ou non d'une microflore existante (Parke, 1991 ; Whipps, 1997 ; Lugtenberg et Dekkers, 1999 ; Benizri *et al.*, 2001 ; Bloemberg et Lugtenberg, 2001 ; Compant *et al.*, 2005a). Cette compétence rhizosphèrique est un pré-requis pour que les PGPR puisse présenter des effets sur leur plante hôte. En effet, une absence d'effet des PGPR, que ce soit dans un cas de bio-contrôle ou de stimulation directe de la croissance de la plante, a souvent été associée à une faible compétence des PGPR à coloniser la rhizosphère et les surfaces racinaires (Thomashow, 1996). Ceci a conduit à la recherche de PGPR présentant une forte compétence rhizosphèrique (Compant *et al.*, 2005a).

Diverses études ont permis de constater que les PGPR colonisent la rhizosphère puis la surface racinaire (rhizoplan) de leur hôte végétal où sont présentes des molécules exsudées par la plante (sucres, acides aminés, acides organiques, vitamines, mucilage…), nécessaires à leur développement (Bais *et al.*, 2006). Les PGPR peuvent ainsi se retrouver au niveau de zones riches en ces macromolécules et coloniser, de ce fait, la zone d'émergence des racines secondaires, la zone pilifère, la zone d'élongation, l'apex racinaire ainsi que leurs portions de sols associés (Hansen *et al.*, 1997 ; Benizri *et al.*, 2001 ; Humphris *et al.*, 2005). Au niveau de ces zones, les PGPR sont attirées chimiotactiquement par ces sources de nutrition puis s'attachent ensuite aux surfaces racinaires notamment par le biais de poils bactériens et de lectines (Bashan et de Bashan, 2005). Cependant, il est important de prendre en compte que

les molécules exsudées par la plante attirent également d'autres micro-organismes. Ainsi, des différences peuvent survenir entre la colonisation des surfaces racinaires en conditions gnotobiotiques (environnements contrôlés où le seul micro-organisme présent est celui inoculé) et en conditions non stériles, ceci étant fonction de l'activité directe de bio-contrôle des PGPR. De plus, des différences physiologiques des plantes cultivées dans ces deux conditions interviennent également. Par exemple, des plantules *in vitro* et des plantes *in vivo* n'exsudent pas quantitativement de la même façon les exsudats racinaires (Benizri *et al.*, 2001). Ceci a de plus une incidence sur le potentiel des PGPR à présenter un effet sur la plante puisqu'il y a moins de sources de nutrition pour ces micro-organismes et par conséquent leurs effets sont moins importants.

Ainsi, des différences peuvent survenir lors d'études en conditions gnotobiotiques ou *in vivo* et souvent, certains de ces micro-organismes, dits symbiotiques, ne présentent même pas d'effets sur la plante en conditions naturelles ou leur effets sont limités (Bashan et de Bashan, 2005). De plus, certains d'entre eux disparaissent rapidement de la rhizosphère ou des surfaces racinaires, en particulier lors de l'inoculation de plantes non naturellement hôtes. Ceci coïncide avec l'équilibre microbiologique s'étant mis en place depuis l'apparition des plantes et est due également aux conditions stressantes régnantes au niveau de la rhizosphère (Compant *et al.,* 2005a ; Bashan et de Bashan, 2005). Ceci a amené à la recherche de PGPR ayant une très forte compétence rhizosphérique mais également de PGPR pouvant entrer à l'intérieur de leur hôte végétal, lieu où elles peuvent être relativement protégées des conditions extérieures (Nowak et Shulaev, 2003 ; Compant *et al.*, 2005a).

4.2.4. La colonisation endophytique de la plante par les PGPR

Les effets des PGPR endophytiques sur la plante sont souvent plus prononcés que ceux des bactéries restreintes au niveau des surfaces racinaires (Conn *et al.,* 1997 ; Chanway *et al.*, 2000). En effet, lors de la colonisation endophytique, les PGPR internes ont une interaction plus proche avec les cellules végétales que les PGPR non endophytiques. Elles stimulent, de ce fait, d'avantage les défenses et la croissance de la plante (Nowak et Shulaev, 2003). Enfin, elles sont mieux protégées face aux stress biotiques et abiotiques que les épiphytes bactériens et interagissent ainsi plus longtemps avec leurs hôtes (Hallmann *et al.,* 1997). Les PGPR endophytiques ont ainsi fait l'objet d'un très grand intérêt en agriculture (Sturz *et al.,* 2000).

4.2.4.1. L'étude de la colonisation endophytique de la plante par les PGPR, un pré-requis avant leur utilisation

La plupart des PGPR peut coloniser leur hôte végétal de façon endophytique (Hallman *et al.*, 1997 ; Sturz *et al.*, 2000 ; Hallman, 2001 ; Lodewyckx *et al.*, 2002 ; Nowak et Shulaev, 2003 ; Gray et Smith, 2005 ; Rosenblueth et Martínez-Romero, 2006). Cependant, peu d'études portent sur la colonisation endophytique de la plante par les PGPR en comparaison aux études concernant leurs utilisations. Pourtant, le devenir des PGPR endophytiques au sein de la plante, ainsi que les mécanismes associés à leur colonisation méritent d'être étudiés. Tout d'abord, d'un point de vue fondamental, afin d'analyser et de comprendre où se situent les bactéries, pourquoi elles se retrouvent dans certaines parties de la plante et quels sont les mécanismes de défense de la plante associés à leur colonisation. Ensuite, d'un point de vue appliqué, car le consommateur peut demander si ces micro-organismes sont présents dans les parties de la plante destinées à sa consommation. Ainsi, l'étude de la colonisation de la plante par les PGPR endophytiques apparaît comme un pré-requis avant leur utilisation sur des plantes cultivées (Nowak et Shulaev, 2003).

4.2.4.2. La pénétration au sein de la plante par les PGPR endophytiques

D'après la littérature, la colonisation endophytique des PGPR au niveau racinaire peut se réaliser de différentes façons (Figure 11). Tout d'abord, la zone d'émergence des racines secondaires constitue une ouverture naturelle permettant l'entrée des PGPR à l'intérieur de la plante (Reinhold-Hurek et Hurek, 1998). Ensuite, les blessures provoquées par des facteurs biotiques, comme par exemple les agents pathogènes, peuvent également permettre l'entrée des PGPR (Lodewyckx *et al.*, 2002). Une autre possibilité consiste en la sécrétion par ces micro-organismes d'enzymes dégradant les parois cellulaires de la plante telles que des cellulases, des endoglucanases, des polygalacturonases et des xylanases (Hallman, 2001 ; Lodewyckx *et al.*, 2002 ; Gyaneshwar *et al.*, 2001). Cette sécrétion enzymatique est cependant de faible intensité et n'est pas une sécrétion continue qui détruirait les tissus de la plante. Elle permet ainsi juste à certaines PGPR endophytiques d'entrer à l'intérieur de leur hôte végétal (pour revue Rosenblueth et Martínez-Romero, 2006).

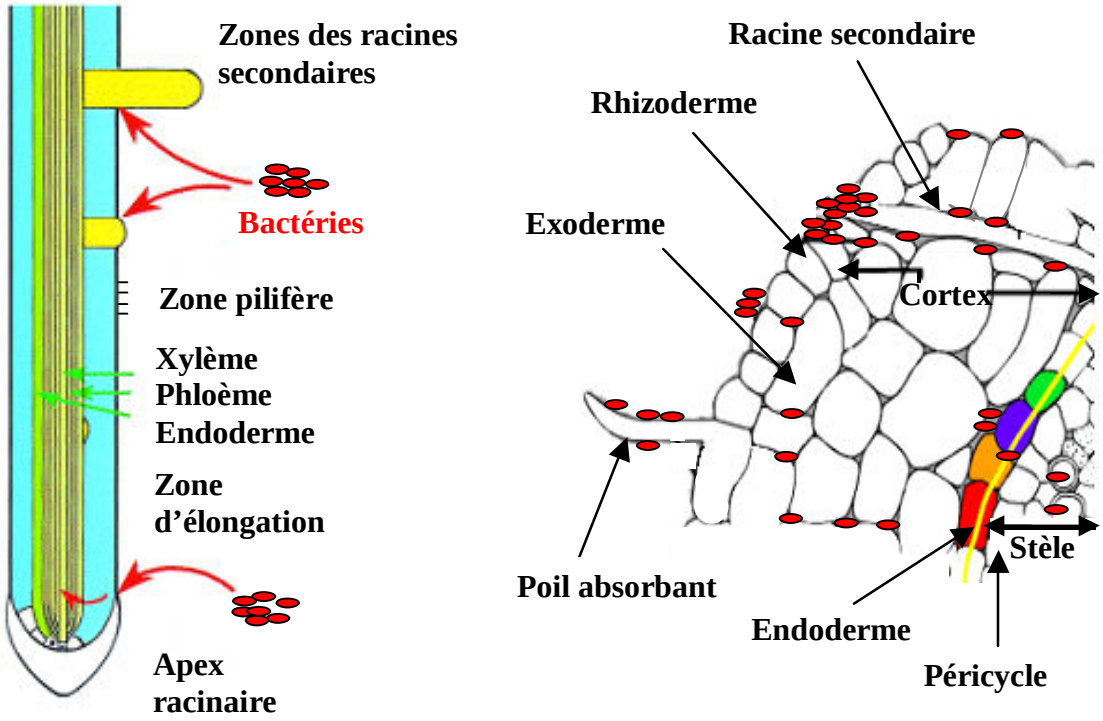

Figure 11 : Illustration des sites possibles d'infection et de colonisation des racines par des PGPR endophytiques montrés par une section longitudinale et transversale d'une racine, modifié d'après Reinhold-Hurek et Hurek (1998).

4.2.4.3. La propagation des PGPR endophytiques à l'intérieur de la plante

Suite à leur pénétration dans les racines, certaines PGPR endophytiques vont se propager dans différents tissus de leur hôte (Whipps, 2001 ; Rosenblueth et Martínez-Romero, 2006 ; Sørensen et Sessitsch, 2006). La colonisation affecte tout d'abord le cortex racinaire par la voie intercellulaire et atteint ensuite la barrière de l'endoderme (Sprent et James, 1995 ; Reinhold-Hurek et Hurek, 1998 ; Hallmann, 2001). Cette barrière peut être partiellement détruite par certaines PGPR (James *et al.*, 2002). Néanmoins, des cellules de l'endoderme sont détruites lors du développement des racines secondaires (Hallmann, 2001), ce qui facilite la progression des PGPR endophytiques jusqu'au cylindre central. Les endophytes bactériens peuvent alors atteindre les vaisseaux conducteurs du xylème (Figures 11 et 12 a-b) et les utiliser pour se propager jusqu'aux parties aériennes de la plante (Sprent et James, 1995 ; James *et al.*, 2002 ; Cocking, 2003). Cependant, il est important de prendre en compte que cette migration des bactéries endophytiques *via* les vaisseaux du xylème a fait l'objet de beaucoup de discussions. Il a ainsi été rapporté par certains botanistes que les endophytes ne se propagent pas par le xylème, mais plutôt par l'apoplaste de ces vaisseaux, la lumière du xylème étant utilisée, selon eux, seulement par les agents pathogènes (McCully, 2001). Cependant, la migration des bactéries endophytiques par la lumière du xylème a bien été prouvée et diverses publications citent, ainsi, une propagation des endophytes bactériens par cette voie de colonisation (pour revue Hallmann, 2001).

4.2.4.4. Les différences de colonisation de la plante par les PGPR endophytiques

Toutes les PGPR endophytiques ne colonisent pas leur hôte de la même façon. Une spécificité de colonisation se met ainsi en place entre la plante hôte et le micro-organisme (Rosenblueth et Martínez-Romero, 2006). Certains micro-organismes sont ainsi cantonnés aux racines, voire même aux tubercules, tandis que d'autres vont se retrouver dans la tige et dans les feuilles (Kobayashi et Palumbo, 2000 ; Whipps, 2001).

Différentes études ont ainsi permis de démontrer la colonisation endophytique des PGPR, notamment chez des hôtes naturels, comme par exemple *Pantoea* sp. et *Ochrobacterium* sp. qui colonisent les racines de riz (Verma *et al.*, 2004), *Azorhizobium caulinodans* colonisant le xylème racinaire d'Arabidopsis (Cocking, 2003) et *Herbaspirillum seropedicae* Z67 (James *et al.*, 2002) ainsi que *Serratia marcescens* (Gyaneshwar *et al.*, 2001) qui colonisent le riz, des racines jusqu'aux parties aériennes. Des autres exemples concernent

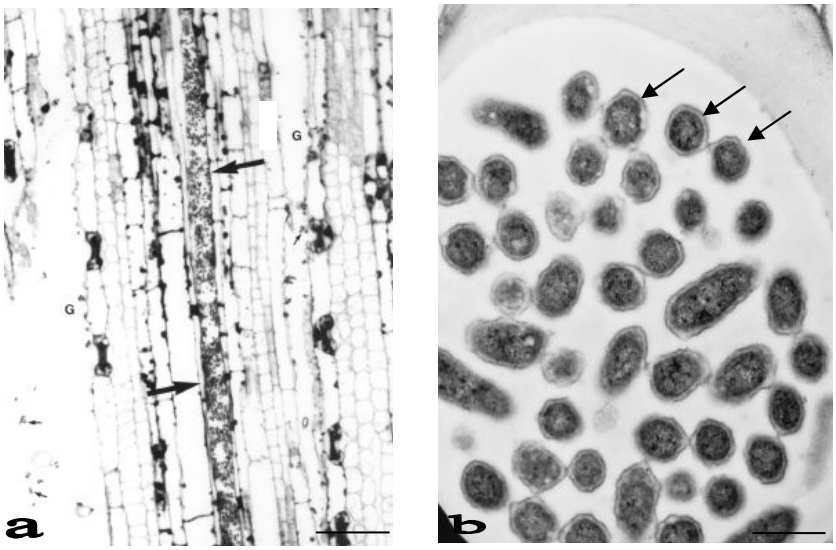

Figure 12 : Microphotographies de PGPR endophytiques (*Serratia marcescens*) dans les vaisseaux du xylème racinaire de *Oriza sativa* d'après Gyaneshwar *et al.* (2001). Barres d'échelles : (a) 30 µm et (b) 1 µm. Les flèches indiquent la présence de bactéries endophytiques dans les vaisseaux du xylème.

également *Azoarcus* sp. souche BH72, par exemple, qui colonise des plantes herbacées (Hurek *et al.*, 1994), ou encore *B. megaterium* souche C4, colonisateur endophytique du maïs, des racines jusqu'aux parties aériennes (Liu *et al.*, 2006).

En parallèle, il est important de prendre en compte qu'il existe des différences de colonisation pour des plantes sauvages ou cultivées. Par exemple, *Herbaspirillum* sp. souche B501 colonise, chez le riz sauvage, les graines et les parties aériennes. En revanche, chez le riz cultivé, il n'y a pas de colonisation endophytique par cette PGPR (Elbeltagy *et al.*, 2001).

Compte tenu des variations de colonisations des PGPR endophytiques dans leurs hôtes, il apparaît, ainsi, nécessaire de caractériser la colonisation des PGPR endophytiques chez diverses plantes, en particulier lors d'une application d'une PGPR sur un nouvel hôte végétal. Ceci permet de déterminer les sites de colonisation par ces micro-organismes et de connaître, ainsi, leurs niches écologiques au sein de la plante.

4.2.4.5. La colonisation des inflorescences par les endophytes bactériens

Des études sur les inflorescences ont permis de constater que les endophytes bactériens peuvent se retrouver dans les fleurs, au niveau de l'ovule et des fruits (Mundt et Hinkle, 1976 ; Misaghi et Donndelinger, 1990). Ceci pourrait sous-entendre une transmission des bactéries endophytiques par les graines. Cependant, la plupart des fleurs, des fruits et des graines ne contiennent pas ou peu de bactéries endophytiques (pour revue Hallmann, 2001). La colonisation des différents organes des inflorescences par les bactéries endophytiques est néanmoins possible. Elle mérite, cependant, d'être plus approfondie, en particulier avec les PGPR endophytiques utilisées en agriculture, car aucune étude n'a porté sur la colonisation de ces parties de la plante par ces micro-organismes bénéfiques. En effet, la plupart des études ont porté sur la détection des endophytes dans ces organes. Néanmoins, il est important de prendre en compte que tous les endophytes bactériens ne sont pas des PGPR puisque certains d'entre eux peuvent présenter un impact neutre sur la plante (Hallmann, 2001). Ainsi, suite à la colonisation par des PGPR, rien ne semble avoir été démontré concernant les inflorescences. Pourtant, certaines PGPR endophytiques sont utilisées sur des espèces cultivées. Il apparaît, donc, intéressant d'étudier si ces PGPR peuvent se retrouver dans les organes des inflorescences, en particulier chez les plantes comme la vigne où l'organe récolté est le fruit.

Afin d'étudier la colonisation des PGPR endophytiques, il faut pouvoir visualiser ces micro-organismes et les différencier de la microflore existante. De plus, des composés de la plante peuvent souvent être confondus avec des bactéries. Il apparaît, de ce fait, primordial de les identifier également sous conditions gnotobiotiques (Hallmann, 2001). Ceci est possible, notamment, grâce à des marqueurs moléculaires comme par exemple le gène *gfp* (pour "Green Fluorescent Protein") codant la protéine verte fluorescente ou ses dérivés *egfp, cgfp* et *ygfp* (Larrainzar *et al.*, 2005) ou à des marqueurs chromogéniques comme le gène *gusA* codant la protéine GUS ou β-glucuronidase conférant une coloration bleue à la bactérie après ajout d'un substrat (Gamalero *et al.*, 2003 ; Jansson, 2003).

4.2.5. Les défenses induites et la résistance systémique associée aux PGPR

Une des propriétés intéressantes des PGPR, qu'elles soient épiphytiques et / ou endophytiques, réside dans leur capacité à induire ou non des réactions de défense lors de leur colonisation (Figure 13). Ces réactions de défense peuvent être à l'origine d'une résistance systémique ou ISR conférant une protection de la plante vis à vis des agents pathogènes (Compant *et al.*, 2005a ; van Loon et Bakker, 2005 ; van Loon, 2007).

4.2.5.1. Reconnaissance des PGPR

Lors de l'interaction plante / PGPR, une reconnaissance entre l'hôte végétal et le micro-organisme bénéfique peut se mettre en place, comme nous l'avons décrit pour les agents pathogènes avirulents. Néanmoins, il n'existe pas, à l'heure actuelle, de données concernant des gènes d'avirulence chez les PGPR. Cependant, des éliciteurs généraux de type MAMPs ont pu être mis en évidence lors d'études portant sur les déterminants moléculaires de l'ISR (van Loon et Bakker, 2005). C'est le cas pour les LPS (van Peers et Schippers, 1992 ; Leeman *et al.*, 1995 ; van Wees *et al.*, 1997 ; Reitz *et al.*, 2002 ; Meziane *et al.*, 2005), les sidérophores (Maurhofer *et al.*, 1994 ; Leeman *et al.*, 1996 ; Audenaert *et al.*, 2002 ; Meziane *et al.*, 2005), le composé volatile 2,3-butanediol (Ryu *et al.*, 2004) ou des antibiotiques comme le 2,4-diacetylphloroglucinol (DAPG ; Iavicoli *et al.*, 2003 ; Siddiqui et Shaukat, 2003 ; Weller *et al.*, 2004) et la pyocyanine (Audenaert *et al.*, 2002) provenant des PGPR. Ces composés sont nécessaires à l'ISR et peuvent donc être considérés comme des MAMPs (van Loon et Bakker, 2005).

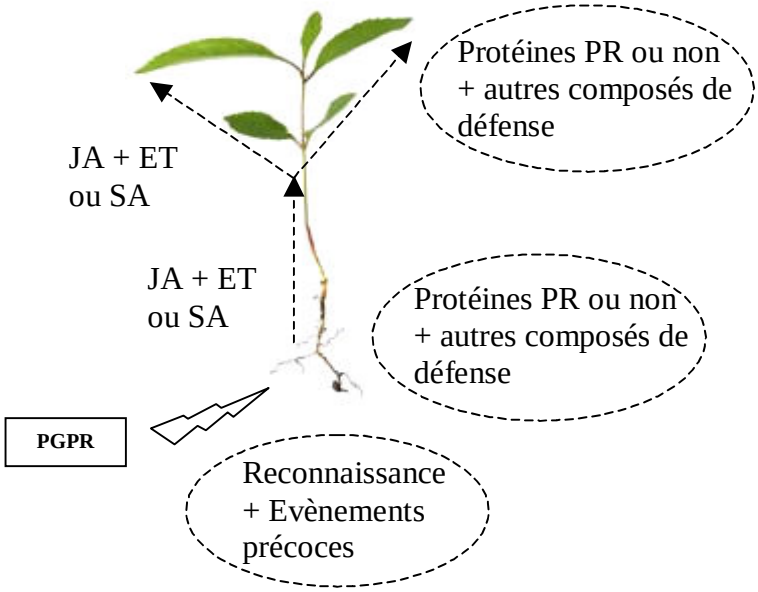

Figure 13 : Illustration des différents phénomènes intervenants lors de l'interaction entre des PGPR et des plantes.

4.2.5.2. Signalisation

Lors de l'interaction plante-PGPR, une signalisation intracellulaire puis intercellulaire se met en place.

Des événements précoces comme un influx de Ca^{2+}, la production d'H_2O_2 et de NO ont ainsi pu être mis en évidence chez le tabac avec les LPS de la PGPR endophytique *Burkholderia cepacia* (Gerber *et al.*, 2004). En outre, il a été démontré que le sidérophore pyocheline et l'antibiotique pyocyanine induisent la production d'H_2O_2 au niveau des racines du riz (de Vleesschauwer *et al.*, 2006).

Au niveau de la signalisation intercellulaire, une accumulation de SA a pu être constatée dans les racines lors de l'interaction entre diverses PGPR et leurs plantes hôtes. C'est notamment le cas chez la tomate ou le haricot avec *Pseudomonas aeruginosa* 7NSK2 (de Meyer et Höfte, 1997). Toutefois, il est généralement admis qu'il n'y a pas de production de SA lors de l'interaction entre une plante et une PGPR (pour revue van Loon et Bakker, 2005).

Au niveau systémique, le SA ne semble pas non plus impliqué mais il serait remplacé par l'ET et le JA (Pieterse *et al.*, 2001 ; 2007 ; van Loon *et al.*, 2006a ; Pieterse et van Loon, 2007). Il existe néanmoins des exceptions. Une augmentation de SA au niveau systémique a ainsi été constatée après application racinaire de l'endophyte *P. fluorescens* souche CHAO sur le tabac (Maurhofer *et al.*, 1994) ou de *P. aeruginosa* souche 7NSK2 sur la tomate (de Meyer *et al.*, 1999), ce qui souligne que des différences peuvent survenir lors de l'interaction entre les plantes et les PGPR.

4.2.5.3. Induction ou non de gènes codants des protéines PR et potentialisation

Diverses publications indiquent que lors de l'interaction plante-PGPR il n'y a pas expression de gènes codants des protéines PR aux niveaux local et systémique. C'est le cas pour *P. fluorescens* souche WCS417r qui n'induit pas l'expression des gènes *PR-1, PR-2, PR-3, PR-4 et PR-5* chez *Raphanus sativus* (Hoffland *et al.*, 1995) ni des gènes *PR-1, PR-2*, et *PR-5* chez *A. thaliana* (Pieterse *et al.*, 1996), ni la synthèse de protéines PR-1 et de chitinases chez *Lycopersicon esculentum* (Duijff *et al.*, 1998). En revanche, ces défenses sont potentialisées lors de l'infection ultérieure de la plante par un agent pathogène comme dans le cas de l'infection d'*A. thaliana* par *Pseudomonas syringae* pv. tomato (van Wees *et al.*, 1999). De ce fait, il a été décrit que la colonisation de la plante par les PGPR n'est

généralement pas associée avec l'accumulation de protéines PR en l'absence d'un agent pathogène (van Loon *et al.*, 1998). En accord avec cette proposition, Ongena *et al.* (2005) ont montré qu'il n'y a pas d'expression de *PR-1* chez la tomate ni de *PR-8* chez le concombre après inoculation de *Bacillus subtilis* souche M4.

Néanmoins, d'autres études ont démontré l'induction locale et systémique de gènes codants des protéines PR lors de l'interaction entre la plante et une PGPR, qu'elle soit épiphytique et / ou endophytique (Tableau 2). Au niveau local, Léon-Kloosterziel *et al.* (2005) ont ainsi montré l'accumulation d'une PR-5 (dont l'expression dépend de l'éthylène) dans le xylème d'*A. thaliana* suite à l'application de la PGPR épiphytique *P. fluorescens* souche WCS417r. L'activation d'un promoteur de *PR-1* a pu être également démontré dans les racines de tabac suite à l'inoculation de diverses PGPR (Park et Kloepper, 2000). Une autre étude concerne la PGPR endophytique *Azoarcus* sp. BH72. Cette bactérie induit l'accumulation, au niveau racinaire, d'une PR-10 (dépendante du JA) chez *Oryza sativa* cv. IR36 suivant la colonisation endophytique par la bactérie ainsi qu'une PR-1 chez *Oriza sativa* cv. IR42, un cultivar moins compatible à la colonisation endophytique de la plante par la PGPR (Miché *et al.*, 2006). Enfin, la PGPR endophytique *Paenibacillus polymyxa* induit l'expression du gène *PR-1* dans les racines d'*A. thaliana* (Timmusk et Wagner, 1999).

L'induction de gènes codants des protéines PR, suite à l'application racinaire de PGPR, a également été démontrée au niveau systémique. Il a ainsi été montré que chez le tabac, *P. fluorescens* souche CHAO induit l'induction de gènes codant une PR-1, une β-1,3-glucanase et une endochitinase au niveau foliaire (Maurhofer *et al.*, 1994). Ryu *et al.* (2004) ont également démontré que des transcripts de *Pdf1-2* (dépendant du JA) mais pas de *PR-1* (dépendant du SA) s'accumulent au niveau systémique chez *A. thaliana* suite à l'application de *Serratia marcescens* souche 90-166. Un autre exemple concerne l'application de *Pseudomonas thivervarlensis* souche MLG45 qui induit l'expression systémique de gènes codants des chitinases chez *A. thaliana* (Cartieaux *et al.*, 2003).

Ces données récentes contredisent le fait admis que les PGPR n'induisent pas l'expression de gènes codants des protéines PR (Pieterse *et al.*, 1996 ; van Loon *et al.*, 1996). Cependant, des analyses ont parfois été réalisées après inoculation de fortes concentrations bactériennes, ce qui peut rendre les PGPR toxiques pour la plante et induire, de ce fait, la synthèse de protéines PR sans même provoquer de symptômes apparents (pour revue van Loon et Bakker, 2005). Néanmoins, d'après la littérature, certaines PGPR peuvent quand

Tableau 2 : Exemples de types d'associations PGPR-plante et leurs inductions de gènes codant des protéines PR ou leurs accumulations.

PGPR-plante hôte	Type d'interaction	Réponses de défense	Références
Azoarcus sp. BH72-*Oriza sativa*	Endophytisme	PR-1 ou PR-10, fonction du type de cultivar de riz	Miché *et al.*, 2006
Bacillus subtilis souche M4-*Lycopersicon esculentum*	Epiphytisme	Pas d'induction de *PR-1*	Ongena *et al.*, 2005
Bacillus subtilis souche M4-*Cucumis sativus*	Epiphytisme	Pas d'induction de *PR-8*	Ongena *et al.*, 2005
Paenibacillus polymyxa-*Arabidopsis thaliana*	Endophytisme	Induction de *PR-1*	Timmusk *et al.*, 1999
Pseudomonas fluorescens souche CHAO-*Nicotiana glutinosa /N. tabacum*	Endophytisme	PR-1, β-1,3-glucanase, endochitinase	Maurhofer *et al.*, 1994
P. fluorescens souche FPT9601-T5-*A. thaliana*	Endophytisme	Induction de gènes codant des PR-2, PR-3 et PR-4	Wang *et al.*, 2005
P. fluorescens souche WCS417r-*A. thaliana*	Epiphytisme	Rien excepté une PR-5 dans le xylème des racines	Léon-Kloosterziel *et al.*, 2005
P. fluorescens souche WCS417r-Lycopersicon esculentum	Endophytisme	Pas de PR-1 ni de chitinase	Duijff *et al.*, 1998
P. fluorescens souche WCS417r- *Raphanus sativus*	Epiphytisme, (endophytisme non déterminé)	Pas d'induction des gènes codant des PR-1, PR-2, PR-3, PR-4 et PR-5	Hoffland *et al.*, 1995
Pseudomonas thivarlensis souche MLG45 - *A. thaliana*	Epiphytisme, (endophytisme non déterminé)	Chitinase	Cartieaux *et al.*, 2003
Serratia marcescens souche 90-166-*A. thaliana*	Epiphytisme (endophytisme non déterminé)	Pas de *PR-1* mais *Pdf*1-2	Ryu *et al.*, 2004

même induire la synthèse de protéines PR même à de faibles densités bactériennes. Une spécificité plante / PGPR peut ainsi exister, ce qui expliquerait les différences concernant l'induction ou non de gènes codants des protéines PR. Il serait intéressant, de ce fait, d'étudier si une expression de ces gènes est déclenchée au cours d'autres interactions, en particulier lors de l'association *Vitis vinifera* L.-*B. phytofirmans* souche PsJN, comme dans le cas qui nous intéresse.

4.2.5.4. Les autres réponses de défenses des plantes induites par les PGPR

D'autres mécanismes de défense peuvent être également déclenchés au niveau de la plante après inoculation de PGPR. C'est le cas avec *Pseudomonas putida* BTP1 chez le haricot qui est à l'origine d'une accumulation de lipoxygénases ou d'hydroperoxyde lyases (Ongena *et al.,* 2004).

Chez les PGPR endophytiques, des renforcements pariétaux, un dépôt de callose et l'accumulation de composés phénoliques ont été également caractérisés comme lors de l'interaction entre l'endophyte *P. fluorescens* 63-28 chez le pois (Benhamou *et al.,* 1996b) ou chez la tomate (M'Piga *et al.,* 1997). Ces modifications locales forment des barrières structurales permettant de restreindre la colonisation de pathogènes des genres *Fusarium* ou *Pythium* (Benhamou *et al.,* 1996a ; M'Piga *et al.,* 1997). D'autres PGPR endophytiques telles que *Bacillus pumilus* souche SE 34 (Benhamou *et al.,* 1998), *P. fluorescens* souche WCS417r chez la tomate (Duijff *et al.,* 1997) et même *Serratia plymuthica* souche RIGC4 chez le concombre (Figure 14 ; Benhamou *et al.,* 2000) peuvent également induire la formation de barrières structurales au niveau racinaire. Néanmoins, pour les autres endophytes, nous ne savons pas à l'heure actuelle si ces barrières peuvent être formées. Il apparaît, de ce fait, intéressant, de déterminer si ces événements peuvent se mettre en place lors de nouvelles associations plantes-PGPR endophytiques. Ceci pourrait accroître nos connaissances sur les réponses de défenses induites par les PGPR endophytiques.

4.2.5.5. La résistance systémique induite par les PGPR

L'interaction entre la plante et une PGPR est à l'origine d'une résistance systémique (Figure 15) qui fait suite à l'induction ou à la préparation des mécanismes de défense que nous avons pu décrire précédemment (van Loon *et al.,* 2006b).

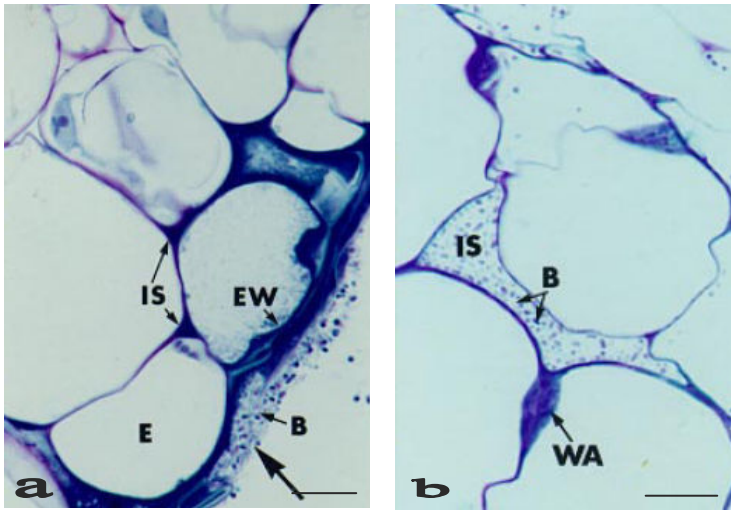

Figure 14 : Microphotographies de sections transversales d'une racine de concombre inoculée avec *Serratia plymuthica* souche RIGC4 et observée au microscope optique montrant (a) la présence de bactéries au niveau du rhizoderme (flèche), responsables d'un épaississement (EW) de l'épiderme (E) ainsi que (b) les bactéries dans un espace intercellulaire (IS), au niveau cortical, responsables d'une apposition pariétale (WA) dans les cellules adjacentes. Barres d'échelle : 10 µm. D'après Benhamou *et al.* (2000).

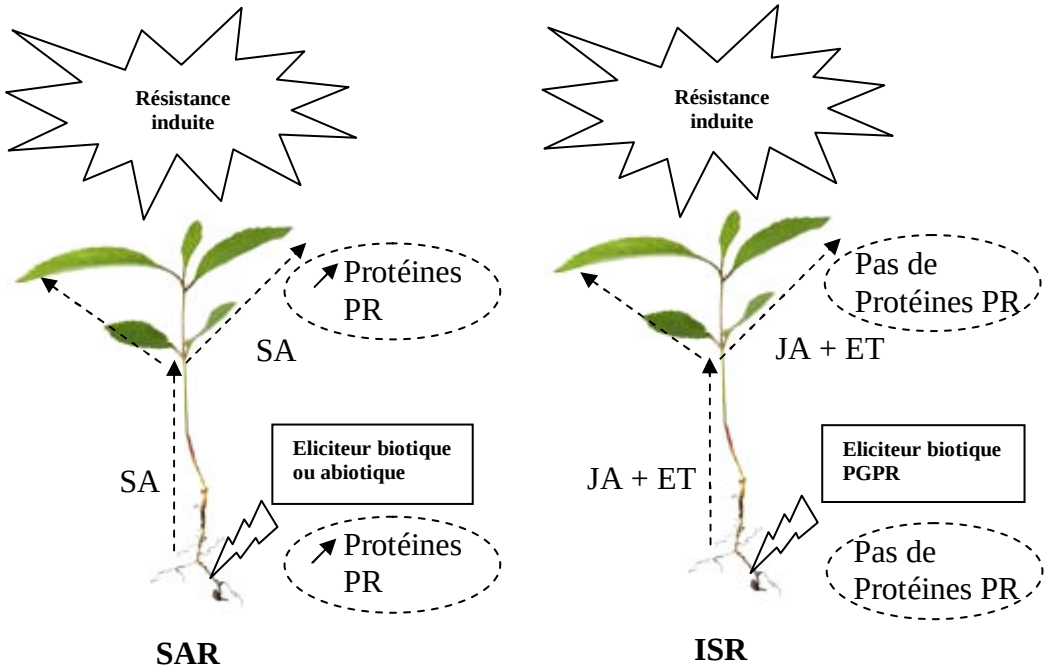

Figure 15 : Illustration des phénomènes généraux de la résistance systémique acquise (SAR) et de la résistance systémique induite (ISR). Modifiée d'après Vallad et Goodman (2004) et en accord avec le modèle de van Loon *et al.* (2006b).

L'ISR induite par les PGPR (que ce soit des épiphytes et / ou des endophytes) s'est montrée efficace pour contrer l'infection de différents pathogènes tels que les virus, bactéries et champignons (pour revues Compant *et al.,* 2005a ; Ton *et al,* 2006). Ce phénomène permet ainsi une protection de la plante au niveau de différents organes : les feuilles, les fleurs et les fruits (Hoffland *et al.,* 1996), comme nous avons pu le décrire dans le cas de la SAR.

L'ISR induite par les PGPR présente ainsi des similitudes avec la SAR. Cependant, l'ISR permet de protéger la plante à un niveau plus faible que la SAR car les mécanismes de défense ne sont pas induits avec la même intensité (Hoffland *et al.,* 1996). De plus, dans le cas général, l'ISR est caractérisée comme n'étant pas associée à la synthèse de protéines PR et est dépendante au niveau systémique de la voie de synthèse du JA et de l'ET (Pieterse *et al.,* 1996), contrairement à la SAR qui passe par la synthèse de protéines PR et qui est dépendante du SA au niveau systémique (Figure 15 ; Ton *et al.,* 2006 ; van Loon, 2007). Néanmoins, il peut y avoir des exceptions comme nous avons pu le décrire précédemment. Ainsi, des PGPR ont été caractérisées comme inductrices d'une SAR à la place d'une ISR (pour revue van Loon et Bakker, 2005).

Récemment, une étude a également montré que l'application de l'endophyte *P. fluorescens* souche FPT9601-T5 chez *A. thaliana* induit à la fois les mécanismes de l'ISR et de la SAR (Wang *et al.,* 2005). Cette PGPR induit ainsi l'expression de gènes codants des protéines PR, mais à une faible intensité, et cette induction est dépendante du JA et de l'ET au niveau systémique. Il semblerait intéressant de confirmer que ce même type de réponse peut survenir lors d'autres interactions plante / PGPR endophytique, en particulier lors de l'interaction entre la vigne et *B. phytofirmans* souche PsJN et même au niveau des inflorescences, organes cibles de *B. cinerea*.

4.2.6. Les PGPR épiphytiques et / ou endophytiques et la vigne

Au début de ce travail de doctorat, peu d'études avaient porté sur l'interaction entre la vigne et des PGPR (Khmel *et al.,* 1998 ; Ait Barka *et al.,* 2000 ; 2002 ; Thomma, 2004 ; 2006 ; Trotel-Aziz *et al.,* 2003 ; 2004). Certaines de ces études avaient porté sur des micro-organismes qui ont même été isolés comme naturellement endophytiques chez la vigne (Tableau 3). Néanmoins, tous les endophytes de la vigne n'ont pas encore été caractérisés comme PGPR, à l'exception de *Brevibacillus* sp., qui peut stimuler la croissance des plantes et former des populations endophytiques dans des plantules *in vitro* de vigne (Thomma, 2006)

Tableau 3 : Les différentes espèces de bactéries endophytiques originaires de la vigne.

Espèce endophytique	**Organes d'origine**	**Références**
Acinetobacter lwoffii	racines	Trotel-Aziz *et al.*, 2003
Bacillus fastidiosus	tige (sève)	Bell *et al.*, 1995
Bacillus pumilus	culture *in vitro* (désinfection)	Thomas, 2004
Brevibacillus sp.	culture *in vitro* (désinfection)	Thomas, 2006
Comamonas terrigena	tige (sève)	Bell *et al.*, 1995
Enterobacter agglomerans	tige (sève)	Bell *et al.*, 1995
E. cloacae	tige (sève)	Bell *et al.*, 1995
Klebsiella ozaenae	tige (sève)	Bell *et al.*, 1995
K. pneumoniae	tige (sève)	Bell *et al.*, 1995
K. terrigena	tige (sève)	Bell *et al.*, 1995
Moraxella bovis	tige (sève)	Bell *et al.*, 1995
Pantoea agglomerans	feuilles	Trotel-Aziz *et al.*, 2003
Pseudomonas cichorii	tige (sève)	Bell *et al.*, 1995
P. corrugata	tige (sève)	Bell *et al.*, 1995
P. fluorescens	tige	Trotel-Aziz *et al.*, 2003
P. marginalis	tige (sève)	Bell *et al.*, 1995
P. putida	tige (sève)	Bell *et al.*, 1995
P. syringae	tige (sève)	Bell *et al.*, 1995
Pseudomonas spp.	tige (sève)	Bell *et al.*, 1995
Rahnella aquatilis	tige (sève)	Bell *et al.*, 1995
Rhodococcus luteus	tige (sève)	Bell *et al.*, 1995
Straphylococcus spp.	tige (sève)	Bell *et al.*, 1995
Xanthomonas campestris	tige (sève)	Bell *et al.*, 1995

ou encore différentes souches des genres *Acinetobacter, Pseudomonas* et *Pantoea* issues du vignoble champenois qui protègent la vigne contre *B. cinerea* (Trotel-Aziz *et al.,* 2003 ; 2004 ; Magnin-Robert *et al.,* 2007).

En vue de lutter contre *B. cinerea,* plusieurs études avaient montré le potentiel de bactéries bénéfiques chez la vigne au début de ces travaux de thèse. C'est le cas pour différentes souches des genres *Bacillus* (Ferreira *et al.*, 1990 ; Krol, 1998 ; Paul *et al.*, 1997 ; 1998 ; Esterio *et al.*, 2000), *Pseudomonas* (Krol, 1998) et *Serratia* (Whitman et Stewart, 1998). Pourtant, la plupart de ces travaux ont porté sur une protection des baies en post-récolte ou sur des feuilles et, de ce fait, ne concerne pas des PGPR, ou bien ne prend pas en compte leur niche écologique naturelle. En vue de lutter contre *B. cinerea* avec des PGPR, et en respectant leurs niches écologiques, les seuls travaux effectués portent sur *Burkholderia phytofirmans* souche PsJN (Ait Barka *et al.,* 2000 ; 2002), isolée d'une plante autre que la vigne (cf. paragraphe 4.2.7.), ainsi que sur des bactéries des genres *Acinetobacter, Pseudomonas, Bacillus* et *Pantoea* issues du vignoble champenois (Magnin-Robert *et al.,* 2007).

Au sein de notre laboratoire, la PGPR endophytique *B. phytofirmans* souche PsJN, avait déjà fait l'objet de quelques études (Ait Barka *et al.*, 2000 ; 2002). Cependant, le mode de vie épi- et endophytique de cette PGPR en conditions gnotobiotiques et *in vivo,* ainsi que l'induction de défenses et d'une résistance systémique protégeant la vigne contre *B. cinerea* n'ont été que superficiellement abordés.

4.2.7. Le genre *Burkholderia*, l'espèce *B. phytofirmans* et la souche PsJN

De nos jours, le genre *Burkholderia* suscite un intérêt croissant. Ce genre bactérien a été originellement connu pour ses espèces pathogènes de l'homme, des animaux ou des plantes. A ce jour, il apparaît néanmoins que de nombreuses espèces de *Burkholderia* ne sont pas pathogènes et peuvent même être bénéfiques pour leur hôte végétal (pour plus d'informations cf. Annexe 1). C'est le cas notamment pour *B. phytofirmans*.

B. phytofirmans est présente au niveau de divers écosystèmes naturels et cultivés. Elle a ainsi été trouvée en association avec le riz en Corée (Muthukumarasamy *et al.*, 2007), des plantes herbacées en Hollande (Salles *et al.,* 2006) et avec la sphaigne en Russie (Belova *et al.*, 2006). Néanmoins, la plupart des études sur cette espèce bactérienne a porté sur la souche

PsJN, cette souche étant à l'origine de l'identification de l'espèce *B. phytofirmans* (Sessitsch *et al.,* 2005).

B. phytofirmans souche PsJN (Sessitsch *et al.,* 2005) était préalablement nommée *Pseudomonas* sp. souche PsJN et a été isolée des tissus internes de racines d'oignon infectées par l'agent mycorrhizien *Glomus vesiculiferum* (Frommel *et al.,* 1991 ; Nowak *et al*., 1995). *B. phytofirmans* souche PsJN peut établir des populations endophytiques à l'intérieur des plantes (Frommel *et al.,* 1991 ; Pillay et Nowak, 1997 ; Ait Barka *et al.,* 2000 ; Sessitsch *et al.,* 2005). Elle a été ainsi détectée comme endophyte notamment dans les racines et les tiges de plants de pomme de terre (Frommel *et al.,* 1991) et de tomate (Pillay et Nowak, 1997), ainsi que dans les racines de pois chiches (Sessistch *et al.,* 2005) et les feuilles de vigne après inoculation d'explants de tige (Ait Barka *et al.,* 2002). Cette souche bactérienne peut également être transmise par multiplication clonale des plantules *in vitro* bactérisées sans besoin de ré-inoculation, comme c'est le cas avec la pomme de terre (Frommel *et al.,* 1991) ou la vigne (Ait Barka *et al.,* 2002). *B. phytofirmans* souche PsJN présente également une activité antagoniste contre différents phytopathogènes comme par exemple *B. cinerea* (Ait Barka *et al.,* 2000), tout en pouvant induire une certaine résistance des plantes face aux maladies (Figure 16 ; Frommel *et al.,* 1991 ; Sharma et Nowak, 1998 ; Ait Barka *et al.,* 2000), notamment la pourriture grise foliaire causée par *Botrytis cinerea* (Figure 17 ; Ait Barka *et al.,* 2000 ; 2002). Parallèlement, la souche PsJN est bien connue pour stimuler directement la croissance de diverses plantes non hôtes comme c'est le cas pour la pomme de terre, la tomate, le concombre, la pastèque et la vigne (Frommel *et al.,* 1991 ; Nowak *et al.,* 1995 ; 1998 ; Bensalim *et al.,* 1998 ; Ait Barka *et al.,* 2000 ; 2002). Cette stimulation est associée à de hauts niveaux d'ACC déaminases sécrétées par la bactérie (Sessitsch *et al.,* 2005), ainsi qu'à l'activité d'une quinolinate phosphoribosyl transférase bactérienne (Wang *et al*., 2006).

B. phytofirmans souche PsJN présente ainsi un potentiel intéressant pour l'agriculture. Néanmoins, différents points concernant cette bactérie avaient besoin d'être clarifiés, en particulier chez la vigne, où elle se retrouve au niveau foliaire après inoculation d'explants de tige et confère une résistance contre l'agent pathogène *B. cinerea*. Tout d'abord, les mécanismes précis de pénétration et de colonisation de cette plante par la bactérie méritaient d'être étudiés, ceci étant un pré-requis avant son utilisation. Ensuite, l'induction de défenses

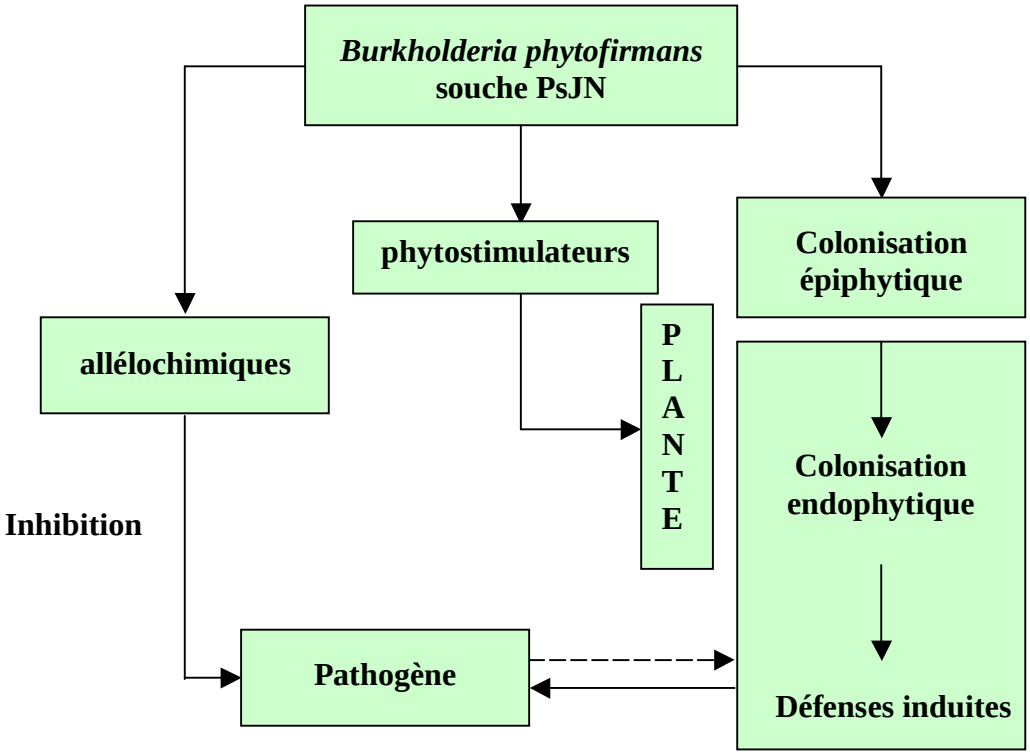

Figure 16 : Illustration énumérant les différentes propriétés de *B. phytofirmans* souche PsJN.

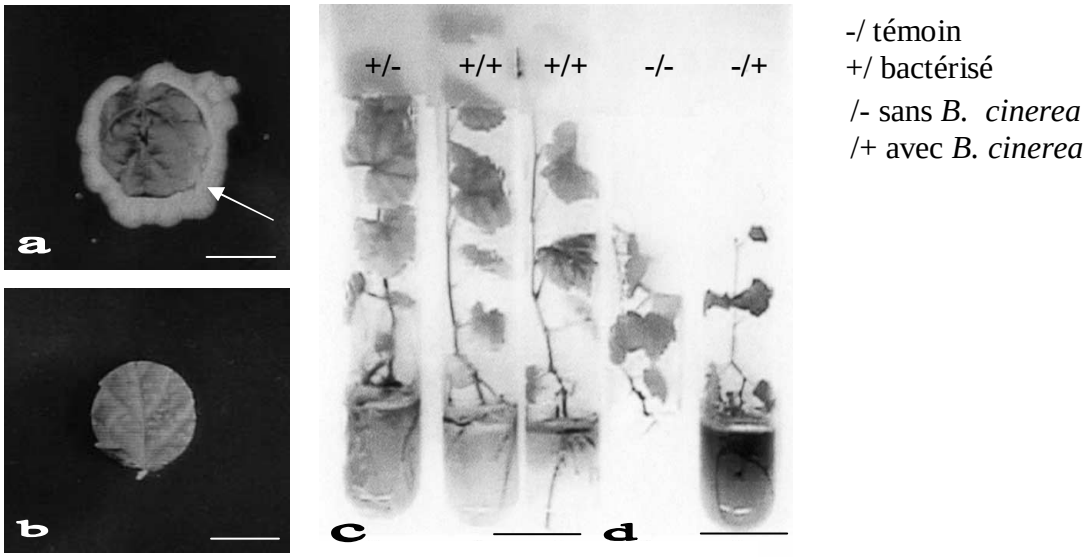

Figure 17 : Photographies de plantules *in vitro* de vigne ou de leurs feuilles, bactérisées ou non avec *Burkholderia phytofirmans* souche PsJN. (a et b) Portions de feuilles issues de plantules montrant la formation de colonies (flèche) sur une feuille issue d'une plantule bactérisée après dépôt sur un milieu nutritif. (c et d) Plantules *in vitro* (c) témoins et (d) bactérisées montrant l'augmentation de la croissance et la protection envers *B. cinerea*. D'après Ait Barka *et al*. (2002). Barres d'échelle : (a et b) 1cm et (c et d) 2,5 cm.

associées à ce phénomène de colonisation ainsi que la résistance systémique induite contre *B. cinerea* méritaient d'être approfondies.

Objectifs de la thèse

Compte tenu du potentiel bénéfique de l'interaction *B. phytofirmans* souche PsJN-*V. vinifera* L., il apparaissait nécessaire de mieux comprendre la nature de cette association (en particulier sur le cépage 'Chardonnay' sur lequel avaient porté des études précédentes ; Ait Barka *et al.,* 2000 ; 2002). De ce fait nous avons décidé :

1/ de caractériser la colonisation épi- et endophytique de la vigne cultivée *in vitro* par *B. phytofirmans* souche PsJN afin de déterminer la localisation de la bactérie et les mécanismes de défenses déclenchés par cette colonisation (Figure 18a). Pour cela nous avons utilisé des plantules *in vitro* ainsi que des souches transformées avec les gènes *gfp* et *gusA*, en même temps que les souches sauvages (Chapitre 2). Les plantules *in vitro* nous ont permis de travailler en conditions gnotobiotiques et les bactéries modifiées, de pouvoir localiser la colonisation de *B. phytofirmans* souche PsJN ;

2/ d'étudier la colonisation endophytique de la vigne par *B. phytofirmans* souche PsJN *in vivo* par le biais de boutures fructifères et des souches génétiquement modifiées (Figure 18 b). Les boutures fructifères permettent de travailler avec des inflorescences et sont un modèle plus proche de la plante entière. Ceci permet de déterminer si la bactérie peut coloniser également les organes reproducteurs, organes cibles de *B. cinerea* et de très grande importance chez la vigne (Chapitre 3). Pour cela nous avons utilisé des boutures fructifères présentant des bourgeons préfloraux, des fleurs ou des fruits ;

3/ d'étudier l'induction d'événements précoces et de gènes de défenses au niveau des racines et des parties aériennes de la vigne après inoculation par *B. phytofirmans* souche PsJN (Figure 18c). Pour cela nous avons étudié l'induction d'un "burst" oxydatif, d'un "burst" nitrique, ainsi que l'induction de gènes de défense principalement ceux codants des protéines PR (*VvGluC, VvChi4C, VvPR6, VvPR10.1, VvPR27* et *Vv102),* dont l'expression dépend des voies de signalisation du SA, du JA ou des deux, sur des boutures fructifères présentant des bourgeons préfloraux (Chapitre 4). L'étude de l'expression des gènes nous permet de caractériser s'il y a induction de protéines PR ou non lors de la colonisation de la vigne par *B. phytofirmans* souche PsJN, d'appréhender la signalisation dont ils dépendent et de déterminer si des marqueurs de SAR ou d'ISR interviennent ainsi lors de cette interaction.

Afin d'étudier une réponse locale et systémique de la vigne à la colonisation bactérienne, nous avons choisi d'étudier ces phénomènes sur des boutures fructifères et non sur des plantules *in vitro*, ceci étant fonction de la différence de rapidité de colonisation de la vigne par *B. phytofirmans* souche PsJN décrites dans les chapitres 2 et 3 ;

4/ de caractériser les effets protecteurs de la vigne envers *B. cinerea*. Les plantules *in vitro* étant rapidement colonisées par la bactérie (Chapitre 2) et compte tenu du fait que cette dernière possède une activité inhibitrice envers ce pathogène (Ait Barka *et al.*, 2000) et peut être présente dans les feuilles (Chapitre 2), ceci ne permettait pas de caractériser la résistance systémique contre *B. cinerea* comme énoncé par van Loon *et al*. (1998). En effet, les bactéries PGPR doivent être absentes du site d'infection du phytopathogène et, ceux-ci doivent être, par conséquent, séparés afin d'étudier l'ISR (van Loon *et al.*, 1998). Notre attention a de ce fait porté sur les inflorescences et ceci avant même leur colonisation par *B. phytofirmans* souche PsJN au niveau des fruits (caractérisée dans le chapitre 3). De plus, cette étude a été réalisée après les réponses de défenses induites analysées lors du stade de bourgeons préfloraux (Chapitre 4), la résistance des plantes nécessitant quelques jours avant de se mettre en place (van Loon *et al.*, 2006b). Ainsi, le phénomène de résistance a été caractérisé au stade de floraison (Figure 18d). Cette démarche a permis de démontrer, d'un point de vue fondamental, l'ISR induite par *B. phytofirmans* souche PsJN contre l'infection florale causée par *B. cinerea* (Chapitre 4).

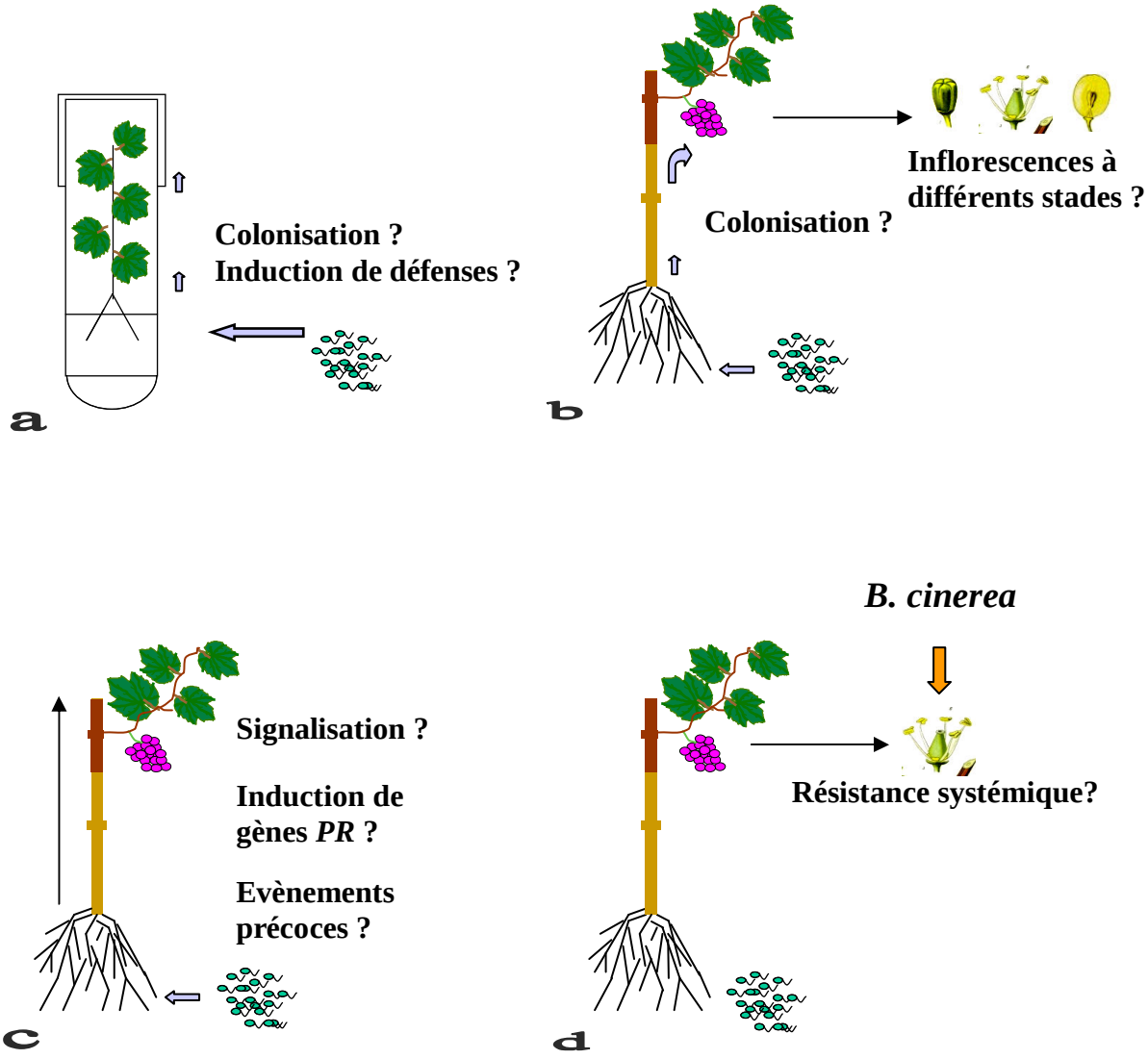

Figure 18 : Illustration des objectifs de la thèse. Etudier la colonisation de la vigne à partir de plantules *in vitro* (a) ou de boutures fructifères (b) par *B. phytofirmans* souche PsJN, et analyser (a) les défenses suite au phénomène de colonisation sur plantules *in vitro* puis (c) sur boutures fructifères tels que les événements précoces, l'induction de gènes codants des protéines PR, leur signalisation et enfin déterminer (d) la résistance systémique induite chez la vigne par *B. phytofirmans* souche PsJN envers *B. cinerea.*

Chapitre 2

Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium, *Burkholderia* sp. strain PsJN.

Stéphane Compant[1], Birgit Reiter[2], Angela Sessitsch[2], Jerzy Nowak[3], Christophe Clément[1], and Essaïd Ait Barka[1]

Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cédex 2, France[1];

Department of Bioresources, ARC Seibersdorf research GmbH, A-2444 Seibersdorf, Austria[2];

Department of Horticulture, Virginia Polytechnic Institute and State University, 0327-301 Saunders Hall, Blacksburg, VA 24060, USA[3]

Applied and Environmental Microbiology, 2005, 71: 1685-1693

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2005, p. 1685–1693 Vol. 71, No. 4
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.4.1685–1693.2005

Endophytic Colonization of *Vitis vinifera* L. by Plant Growth-Promoting Bacterium *Burkholderia* sp. Strain PsJN

Stéphane Compant,[1] Birgit Reiter,[2] Angela Sessitsch,[2] Jerzy Nowak,[3] Christophe Clément,[1] and Essaïd Ait Barka[1]*

Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, Reims, France[1]; *Department of Bioresources, ARC Seibersdorf Research GmbH, Seibersdorf, Austria*[2]; *and Department of Horticulture, Virginia Polytechnic Institute and State University, Blacksburg, Virginia*[3]

Received 10 June 2004/Accepted 25 October 2004

Patterns of colonization of *Vitis vinifera* L. cv. Chardonnay plantlets by a plant growth-promoting bacterium, *Burkholderia* sp. strain PsJN, were studied under gnotobiotic conditions. Wild-type strain PsJN and genetically engineered derivatives of this strain tagged with *gfp* (PsJN::*gfp*2x) or *gusA* (PsJN::*gusA*11) genes were used to enumerate and visualize tissue colonization. The rhizospheres of 4- to 5-week-old plantlets with five developed leaves were inoculated with bacterial suspensions. Epiphytic and endophytic colonization patterns were then monitored by dilution plating assays and microscopic observation of organ sections. Bacteria were chronologically detected first on root surfaces, then in root internal tissues, and finally in the fifth internode and the tissues of the fifth leaf. Analysis of the PsJN colonization patterns showed that this strain colonizes grapevine root surfaces, as well as cell walls and the whole surface of some rhizodermal cells. Cells were also abundant at lateral root emergence sites and root tips. Furthermore, cell wall-degrading endoglucanase and endopolygalacturonase secreted by PsJN explained how the bacterium gains entry into root internal tissues. Host defense reactions were observed in the exodermis and in several cortical cell layers. Bacteria were not observed on stem and leaf surfaces but were found in xylem vessels of the fifth internode and the fifth leaf of plantlets. Moreover, bacteria were more abundant in the fifth leaf than in the fifth internode and were found in substomatal chambers. Thus, it seems that *Burkholderia* sp. strain PsJN induces a local host defense reaction and systemically spreads to aerial parts through the transpiration stream.

In both natural and managed ecosystems, plant-associated bacteria play a key role in host adaptation to a changing environment (17, 56). Interactions between plants and beneficial bacteria can have a profound effect on crop health and yield and soil quality (27, 56). These microorganisms can presensitize plant cell metabolism, so that upon exposure to stress the presensitized or primed plants are able to respond more quickly and more efficiently than nonprimed plants and thus can better withstand the challenge (7, 63). The mechanisms by which beneficial microbes support plant growth and health include increasing nutrient availability, improving soil structure, inducing plant defense mechanisms, producing antibiotics, outcompeting pathogens, and providing growth-stimulating substances or enzymes (5, 14, 27, 30, 62). Despite the beneficial action of the microorganisms on plants, application of such microorganisms in the field is often hampered by inconsistent performance (57).

The ability to colonize roots has been considered the major factor that determines inoculum efficacy both for crop yield enhancement and for disease control (53, 64). This has led to an emphasis on selection of plant-beneficial bacteria that are rhizosphere competent (i.e., beneficial bacteria that effectively colonize the root system) (43). In addition, there is ample evidence that bacteria can also colonize internal tissues and thrive as endophytes in roots and/or shoots and leaves (8, 13, 17, 30, 65). Endophytic bacteria may be of particular interest as they have the advantage of being relatively protected from the competitive, high-stress environment of the soil (55, 65). Moreover, plant growth promotion is often greater when it is induced by endophytes rather than by bacteria restricted to the rhizosphere and the root surface (4, 6). Therefore, a better understanding of the epi- and endophytic bacterial colonization patterns and the survival of introduced inocula both in the rhizosphere and in planta is a critical prerequisite for the development of effective ways to deliver and manage inocula.

Burkholderia sp. strain PsJN (unpublished results), which was originally designated *Pseudomonas* sp. strain PsJN (11), is an effective plant growth-promoting bacterium that was isolated as a contaminant from *Glomus vesiculiferum*-infected onion roots (38). This bacterium promotes the growth of potatoes (11), vegetables (37), and grapevines (1) via reduction of the level of the inhibitory hormone ethylene by a high level of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase that is secreted (unpublished results). Strain PsJN also shows biocontrol activity against gray mold since it can effectively protect against in vitro and in vivo growth of *Botrytis cinerea* (2). Furthermore, during clonal multiplication of potato (40) and grapevine (1) via nodal explants taken from stock plants preinoculated with PsJN, the bacteria are transmitted through successive subcultures of plantlets with no reinoculation. This

* Corresponding author. Mailing address: Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cédex 2, France. Phone and fax: 33-3-26-91-34-41. E-mail: ea.barka@univ-reims.fr.

bacterium has been detected in roots and stems of potatoes (11) and tomatoes (42), as well as in grapevine leaves, following in vitro culture (1) and in grapevine roots under hydroponic conditions (unpublished results). However, the colonization pattern and the method used for translocation from the rhizosphere to internal plant tissues need to be clarified.

Recently, molecular techniques based on whole-cell hybridization methods have been used to detect and enumerate microorganisms in situ and on plant surfaces (3, 50, 67). Tagging with green fluorescent protein (GFP) (12, 58, 59) and β-glucuronidase (GUS) (47, 51, 66) gene markers has been particularly useful in following bacterial infection pathways for determination of tissue and organ colonization.

Thus, the objectives of this study were to construct *gfp*- and *gusA*-marked derivatives of *Burkholderia* sp. strain PsJN and to determine the epi- and endophytic patterns of colonization of *Vitis vinifera* L. under gnotobiotic conditions by using wild-type strain PsJN or genetically marked derivatives of this strain.

MATERIALS AND METHODS

Fluorescent labeling and GUS labeling of *Burkholderia* sp. strain PsJN. PsJN was tagged with the *gfp* and *gusA* marker genes by using mini-Tn*5* systems, which form stable genomic insertions in a variety of bacteria (59, 60, 61, 67), according to the protocol described by Unge et al. (61). Briefly, wild-type strain PsJN was grown in King's B medium (26) in 5-ml cultures at 20°C until the optical density at 600 nm was 0.7. The bacterial cells were then pelleted by centrifugation (3,000 × *g*, 10 min, 4°C), washed three times with ice-cold distilled water, and resuspended in 500 μl of ice-cold glycerol. To each 100-μl cell suspension, 200 ng of delivery plasmid DNA was added; the plasmid used was either pUT*gfp*2x (61), in which two copies of the marker gene were constitutively expressed, or pCAM111 (67), in which *gusA* was under control of the *ptac* promoter. The mixture was then incubated for 15 min on ice and subsequently electroporated with a Gene Pulser Plus pulse controller (Bio-Rad, Richmond, Calif.) by using settings of 2.5 kV, 200 Ω, and 25 μF. Transformants carrying the *gfp* marker were selected on King's B medium containing 50 μg of kanamycin per ml (pUT*gfp*2x) or 50 μg of spectinomycin per ml (pCAM111). Colonies and cells of the *gfp*-marked strain were examined by using a fluorescence stereomicroscope (model MZ FLIII; Leica, Heerbrugg, Switzerland) equipped with a GFP 1 filter (Leica) and by using an optical microscope (model BH2; Olympus, Tokyo, Japan) equipped with a UV light source (BH2-RFL-T3; Olympus) and a 495-nm fluorescent filter (BP495; Olympus). The *gusA*-marked strain was grown for 4 days at 37°C on King's B medium amended with 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt; Sigma, St. Louis, Mo.) per ml. Then the bacteria were examined by using an optical stereomicroscope (model SZ-CTV; Olympus) and an optical microscope (model BH2; Olympus).

Determination of bacterial growth and inoculum preparation. The *gfp*- and *gusA*-marked strains and the wild-type strain were separately grown in King's B medium as described by Pillay and Nowak (42). Each bacterial inoculum was transferred to 100 ml of King's B liquid medium containing the appropriate antibiotic in a 250-ml Erlenmeyer flask and incubated at 20°C on a shaker (150 rpm) for 48 h. Bacteria were collected by centrifugation (4,500 × *g*, 15 min) and washed twice with phosphate-buffered saline (pH 6.5) (PBS). The concentration of the inoculum was then adjusted to approximately 3×10^8 CFU/ml with PBS, based on the optical density at 600 nm, and was confirmed by plate counting as described by Pillay and Nowak (42).

Transformant stability and bacterial growth comparison. Transformant stability was determined by growing derivatives in King's B liquid medium without selection pressure for over 10 generations (doubling times) and then plating a dilution series on King's B medium with or without the appropriate antibiotic. Five replicates were included for each treatment. Furthermore, the colony and cell morphologies and growth patterns of the genetically derivatives were compared to those of the PsJN wild-type strain in King's B medium (26), Luria-Bertani medium, and M9 minimal medium with 0.4% glucose (49).

Plant material, growth conditions, and inoculation. Disease-free plantlets of *V. vinifera* L. cv. Chardonnay (i.e., plantlets free of visible bacterial or fungal contamination) were propagated by using nodal explants in 25-mm-diameter test tubes containing 15 ml of Martin medium (32). The cultures were grown in a growth chamber under white fluorescent light (200 μmol 52 $m^2 \cdot s^{-1}$) with a 16-h photoperiod at 26°C (constant temperature).

Plant inoculation was monitored by spreading 200-μl aliquots of the bacterial inocula in PBS (wild-type strain PsJN and *gfp*- and *gusA*-marked strains) or PBS (control) on the surface of Martin medium in new test tubes under gnotobiotic conditions. Five-week-old rooted plantlets with five developed leaves were then delicately transferred into new test tubes previously inoculated with bacteria so that only the roots were in contact with the bacterial inoculum. The plantlets were then incubated in the culture chamber as described above.

Preparation of plant samples for determination of the plant growth-promoting effects. The effects of wild-type strain PsJN and the *gfp*- and *gusA*-marked strains on plant growth were compared to the effects of the control (PBS) 15 days after rhizosphere inoculation. Plantlets were removed from the growth medium, and shoot and root fresh weights and lengths were determined as growth parameters. There were four replicates for each treatment, and each replicate contained root and aerial systems from five plantlets.

Preparation of plant samples for bacterial enumeration. The rhizoplane and endophytic colonization of roots, shoots, and leaves by the *gfp*-marked strain was determined by plate counting, and bacterial colonies were counted by using a fluorescence stereomicroscope (model MZ FLIII; Leica) with a GFP 1 filter (Leica). Roots, the fifth internode, and the fifth leaf were sampled separately from 0 to 144 h after rhizosphere inoculation (see Fig. 2). For each plant part three samples were combined and weighed. Three or four replicates of three independent plating assays were used to determine the average colonization value. The lower limit of detection was between 0 and −1 log CFU/g (fresh weight).

(i) Rhizoplane colonization. Plantlets were removed from the agar, and roots were gently rinsed in sterile distilled water. The samples were then ground with a pestle in sterile Eppendorf microcentrifuge tubes containing 1 ml of PBS for approximately 1 min and shaken for 1 h (200 rpm) at the ambient temperature. The homogenates were vortexed for 5 s, 10-fold serially diluted, and cultured on King's B medium plates supplemented with kanamycin (50 μg/ml). Bacterial colonies were counted after 3 days of incubation at 30°C. Rhizoplane colonization by the *gfp*-marked strain was determined by subtracting the bacterial counts after surface sterilization from the total *gfp* bacterial counts determined without surface sterilization.

(ii) Endophytic colonization. To determine endophytic populations of the *gfp*-marked strain, samples were surface sterilized with 70% ethanol for 5 min (roots) or 3 min (fifth internode and fifth leaf), followed by 1% commercial bleach and a 0.01% Tween 20 solution for 1 min, and then washed three times in distilled water (1 min each time). The samples were then ground and handled as described above in order to determine the microbial populations inside surfaced-sterilized roots, internodes, and leaves. The bacterial colonies were counted after 3 days of incubation at 30°C.

Evaluation of surface sterilization methods for monitoring rhizoplane and endophytic populations. To determine the efficacy of the surface sterilization procedure, samples of sterilized roots, fifth internodes, and fifth leaves were taken 96 h after inoculation of the rhizosphere with the *gfp*-marked strain. The samples were observed with an epifluorescence microscope or placed on King's B medium plates containing kanamycin (50 μg/ml) and incubated for 1 min prior to crushing. The samples were then removed, and the plates were incubated at 30°C as described above. In addition, the wash solution from the last rinse was cultured on King's B medium plates amended with 50 μg of kanamycin per ml to determine the efficiency of sterilization.

In parallel, the fifth internodes and the fifth leaves taken from two sets of 10 plantlets were used without surface sterilization to determine if epiphytic colonization of these aerial plant parts occurred after rhizosphere inoculation.

Microscopy of rhizoplane colonization by PsJN. To determine colonization of the rhizoplane by the *gfp*-marked strain, approximately 20 plantlets were examined with the fluorescent stereomicroscope and the epifluorescence microscope, as describe above, 96 h after rhizosphere inoculation. Root surfaces were observed and photographed with an automatic photographic system (PM-CBSP; Olympus) or a numerical camera (C-4040; Olympus).

Similarly, 96 h after inoculation with the *gusA*-marked strain, 20 plantlets were used to localize the tagged strain. The *gusA*-tagged bacteria were stained by using the procedure described by Jefferson et al. (24). Fresh plant organs were individually immersed in the GUS staining solution in a desiccator connected to a pump. A vacuum was applied for 1 min to facilitate penetration of the substrate into the plant tissues. After 20 h of incubation at 37°C, the plant tissues were immersed in an ethanol bath. Then the samples were examined with a microscope and photographed as described above.

Microscopy of endophytic colonization by PsJN. Fresh plant organs (roots, fifth internodes, and fifth leaves) removed from six plantlets inoculated with either wild-type strain PsJN, genetically derivatives of this strain (*gfp*- or *gusA*-marked strain), or a control (PBS) were collected 96 h after inoculation. Samples

were then prepared for microscopy analysis as described by Gognies et al. (15), with some modifications. Briefly, plant organs were fixed for 24 h at room temperature in 2% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.24) with 2% (wt/vol) sucrose and 0.1% (vol/vol) Tween 20. After three rinses (5 min each) with the phosphate buffer containing 2% (wt/vol) sucrose, samples were fixed for 4 h in 1% (wt/vol) osmium tetroxide in phosphate buffer with 2% (wt/vol) sucrose. The samples were then dehydrated in an alcohol series, transferred to acetone, and embedded in araldite. Semithin sections (thickness, 1 μm) for different treatments were cut with a microtome (model Jung RM2055; Leica, Nussloch, Germany), collected on glass slides, stained with 0.1% toluidine blue, examined with a microscope (model BH2; Olympus), and photographed as described above.

In parallel, hand-cut transverse sections of different parts (roots, fifth internodes, and fifth leaves) of 20 plantlets inoculated with either PBS (control) or the *gfp*-marked strain were used to detect host defense reactions by visualizing autofluorescence and endophytic colonization by epifluorescence microscopy and were photographed as described above.

Additionally, samples taken from 20 plantlets inoculated with the *gusA*-marked strain were used to assess colonization of the fifth internode and leaf internal tissue after staining with the GUS substrate as described above. This method allowed us to visualize bacteria inside plant tissues without obtaining tissue sections.

Plate assays for endoglucanase and endopolygalacturonase activities. Enzyme activities were determined by the method of Reinhold-Hurek et al. (44), with some modifications. Briefly, plates containing KW (Kim-Wimpenny) solid medium (25), with or without D-glucose and with either 0.2% carboxymethyl cellulose (CMC) or 0.5% polygalacturonic acid, were spot inoculated with PsJN and incubated at 30°C for 3 days. Then the cells were removed from the plates, and the CMC-containing plates were stained with Congo red (0.1%) for 30 min; this was followed by several washes with 1 M NaCl to improve the contrast (44). Similarly, the polygalacturonic acid-containing plates were stained with ruthenium red (0.1%) and washed with 1 M NaCl (33). Endoglucanase (CMC-degrading cellulase) activity was determined by the appearance on a red background of clear yellowish halos around the points where the bacterium was inoculated (44). Endopolygalacturonase activity was determined by the appearance of intense purple-red halos on a colorless background at the point where the bacterium was inoculated (33).

Statistical analysis. Population densities estimated by using CFU were subjected to logarithmic transformation before data analysis (31). Data for plant growth and bacterial enumeration were statistically analyzed by using Student's *t* test.

RESULTS

Construction and stability of the *gfp*- and *gusA*-marked strains and comparison with the wild-type strain. The colony and cell morphologies and the growth patterns of the *gfp* and *gusA* genetic derivatives of *Burkholderia* sp. strain PsJN on King's B medium, Luria-Bertani medium, and M9 minimal medium (supplemented with 0.4% glucose) were similar to those of the wild-type strain (data not shown). Colonies and cells of the *gfp*-marked strain were strongly fluorescent under UV light, whereas the *gusA*-marked strain exhibited a blue color after application of the GUS substrate (data not shown). No white colonies appeared after several restreakings on King's B medium with or without the appropriate antibiotic, indicating that integrations of the mini-transposons into the bacterial chromosome were stable.

Comparison of the plant growth-promoting effects of *gfp*- and *gusA*-marked strains and the wild-type strain on grapevine plantlets. All inoculated plantlets survived rhizosphere bacterization and performed better that nonbacterized plantlets. Fifteen days postinoculation (p.i.) no significant differences ($P < 0.05$) in plantlet growth among the PsJN::*gfp*2x, PsJN::*gusA*11, and the wild-type treatments were found (Fig. 1). However, significant increases ($P < 0.05$) in the relative fresh weights of roots and aerial parts, as well as the lengths of aerial parts, were observed for bacterized treatments compared to the nonbacterized control (Fig. 1A, B, and D). Root length was the only parameter that was not significantly different for the bacterized and control cultures ($P > 0.05$) (Fig. 1C).

Efficacy of surface sterilization protocols. To ensure that the endophytic colonization values determined in this study reflected only the numbers of cells in the interior of plant tissues, a specific surface sterilization method was developed. This sterilization method should have killed and/or washed away the surface bacteria while the internal bacteria survived. First, root samples were examined 96 h p.i. by epifluorescence microscopy for GFP-containing cells remaining on the plant surface after surface sterilization. No bacterial cells were ever observed when this technique was used. Second, no bacterial growth was observed after 3 days on King's B solid medium when surface-sterilized roots were added. Third, either no colonies or only a few (<10) colonies were observed 3 days after inoculation on plates inoculated with the last wash solution after surface sterilization. In the very few cases in which the last wash yielded colonies with GFP, the number of such colonies represented less than 0.001% of that the root endophytic populations occurring at 96 h p.i. Based on the results for these three controls, the endophytic colonization values presented here reflected only the numbers of cells within the tissues, as reported by Dong et al. (8). Thus, the surface sterilization treatment was efficient and could be used to determine the endophytic populations of root internal tissues.

Similar to the root tests, no bacterial colonies were found on stems and leaves in poststerilization wash tests performed with King's B medium amended with 50 μg of kanamycin per ml 3 days after plating. Furthermore, no bacterial colonies were observed on King's B medium amended with 50 μg of kanamycin per ml 3 days after we added the surface-sterilized aerial plant parts prior to crushing. Thus, the surface sterilization treatment was efficient and could be used to determine the populations of internal tissues of aerial plant parts.

In addition, PsJN::*gfp*2x cells were not found on the fifth internode and fifth leaf surfaces from zero time to 96 h after rhizosphere inoculation, as determined by fluorescence microscopy. Furthermore, there was no difference in the total PsJN::*gfp*2x bacterial populations colonizing aerial parts when samples taken from surface-sterilized and nonsterilized plantlets were compared (data not shown). This finding further demonstrated that the inner tissues of aerial organs could not have been contaminated by surface-colonizing bacteria.

Root surface colonization. The rhizoplane of grapevine plantlets was rapidly colonized by PsJN::*gfp*2x cells immediately after rhizosphere inoculation. The PsJN::*gfp*2x population peaked at 24 h p.i. and then remained stable at 9.15 log CFU/g (fresh weight) (Fig. 2A).

Microscopic observations of roots 96 h after PsJN::*gfp*2x inoculation revealed green fluorescence on both primary and secondary roots. PsJN::*gfp*2x cells congregated in high numbers at the sites of lateral root emergence on plantlets (Fig. 3A and B). At 96 h p.i., PsJN::*gfp*2x cells were also found close to the cell walls of the rhizodermis (Fig. 3C), as well as on the whole outline of some rhizodermal cells (Fig. 3D).

In the experiment with PsJN::*gusA*11 no blue color was detected on roots taken from nonbacterized plantlets after incubation in the GUS substrate (Fig. 3E). However, GUS expres-

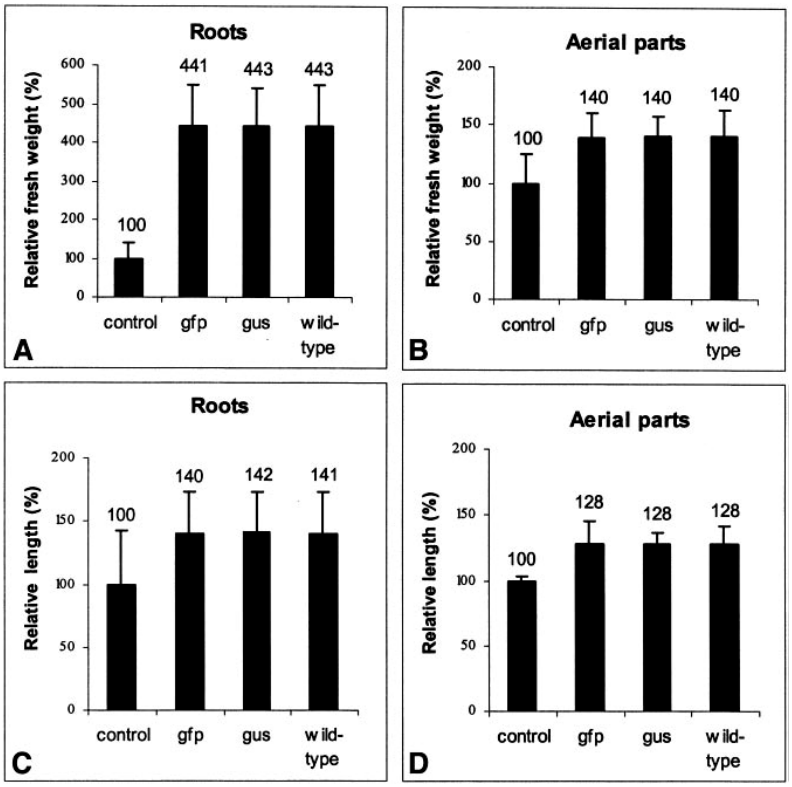

FIG. 1. Comparison of the plant growth-promoting effects of the PsJN::*gfp*2x, PsJN::*gusA*11, and wild-type PsJN strains on *V. vinifera* L. cv. Chardonnay plantlets 15 days after rhizosphere inoculation. (A and B) Percentages of relative fresh weight of the root system (A) and aerial parts (B). (C and D) Percentages of relative lengths of the root system (C) and aerial parts (D). The bars indicate means, and the error bars indicate standard deviations.

sion was detected on root tips at 96 h p.i. when the PsJN::*gusA*11 strain was applied (Fig. 3F and G).

Endophytic colonization of roots and host defense reactions. Colonization of the root interior by PsJN::*gfp*2x cells occurred between 1 and 3 h p.i., and the population remained at a level of 6.85 log CFU/g (fresh weight) for more than 48 h p.i. (Fig. 2B). Rhizodermis and endodermis layers, as well as xylem vessels of primary roots of control plantlets, exhibited yellow autofluorescence at 96 h p.i. (Fig. 3H) as a result of phenolic compounds in the cell wall which fluoresced under UV light (20). Yellow autofluorescence was also observed in primary roots of plantlets inoculated with PsJN::*gfp*2x (Fig. 3I). However, several cortical cell layers exhibited additional yellow fluorescence after inoculation with PsJN::*gfp*2x (Fig. 3I) compared to the nonbacterized control treatment (Fig. 3H). This enhancement indicated that there was accumulation of phenolic material corresponding to a host defense reaction. Furthermore, host defense reactions, which were correlated with a strengthening of some cell walls in the exodermis as well as cortical cells, were also observed in resin-embedded roots (Fig. 3J).

Due to the large bacterial population that developed on the rhizoplane, bacterial colonization of root internal tissues could not be confirmed microscopically by using hand-cut sections of plantlets inoculated with PsJN::*gfp*2x because cells could have been introduced from the external root surface during sample preparation, as reported by Shishido et al. (54). However, microscopic analyses of resin-embedded roots demonstrated that cortical cells (Fig. 3J and K), the endodermis (Fig. 3L), and xylem vessels (Fig. 3M) were colonized by bacterial cells in an inter- and/or intracellular colonization pattern, in contrast to control samples, in which no bacteria were observed (data not shown).

Endophytic colonization of stems. No PsJN::*gfp*2x cells were detected in the fifth internode before 72 h p.i. Then the first bacteria appeared, the highest level was reached 84 h p.i., and the population remained at a level of 5.85 log CFU/g (fresh weight) (Fig. 2C).

No blue color was observed in the fifth internode of nonbacterized plantlets after incubation with the GUS substrate (Fig. 4A). However, a blue color appeared in the center of the fifth internode 96 h p.i. in the plantlets inoculated with the PsJN::*gusA*11 strain (Fig. 4B).

Green autofluorescence (epidermis), red autofluorescence (parenchyma), and yellow autofluorescence (vascular system) were detected in the fifth internode 96 h p.i. both in nonbacterized plantlets (Fig. 4C) and in PsJN::*gfp*2x-bacterized plantlets (Fig. 4D). Microscopic observations at high magnifications from the epidermis to xylem vessels revealed that PsJN::*gfp*2x cells or wild-type PsJN cells were found only in xylem vessels

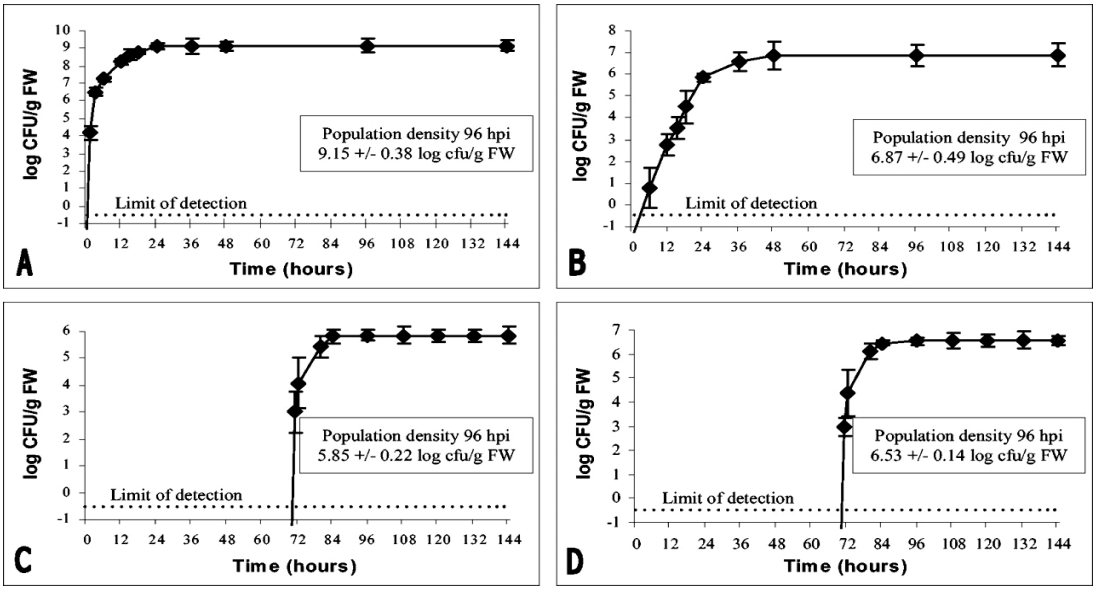

FIG. 2. Population dynamics of the PsJN::*gfp*2x strain for different parts of *V. vinifera* L. cv. Chardonnay plantlets after rhizosphere inoculation. (A) Rhizoplane; (B) root internal tissues; (C) fifth internode internal tissues; (D) fifth leaf internal tissues. The error bars indicate the standard deviations for mean log-transformed bacterial population sizes. The values are means and standard deviations. FW, fresh weight.

(Fig. 4E and F), in contrast to nonbacterized samples, in which no bacteria were observed (data not shown).

Endophytic colonization of leaves. No PsJN::*gfp*2x cells were detected in the fifth leaf until 72 h p.i. The highest population density occurred 84 h p.i., and the stationary-phase level was 6.53 log CFU/g (fresh weight) (Fig. 2D). The endophytic colonization of the fifth leaf was significantly greater than the endophytic colonization of the fifth internode ($P < 0.001$).

No blue color was observed for samples taken from nonbacterized plantlets after incubation with the GUS substrate (Fig. 4G). However, in the test conducted with the PsJN::*gusA*11 construct, a blue color was found in the primary and secondary veins 96 h p.i. (Fig. 4H).

As reported above for the fifth internode, the same autofluorescence was observed in the fifth leaf at 96 h p.i. both after control treatment and after PsJN::*gfp*2x treatment (Fig. 4I and J). PsJN::*gfp*2x or wild-type PsJN cells were found only in xylem vessels (Fig. 4K and L), in contrast to nonbacterized samples, in which no bacteria were observed (data not shown).

Interestingly, PsJN::*gfp*2x cells were not found on the abaxial surface of the fifth leaf regardless of the time after inoculation. However, when a little pressure was applied by hand to a leaf placed between a glass slide and a coverslip, bacterial cells were observed around stomata 96 h p.i. (Fig. 4M), which indicates that they were present in substomatal chambers. This was confirmed in the PsJN::*gusA*11 treatment, in which blue bacterial cells were detected by leaf transparency under stomata at 96 h p.i. (Fig. 4N).

Analysis of cell wall-degrading enzyme activities. Qualitative plate assays demonstrated that PsJN secreted endoglucanase (Fig. 5A) and endopolygalacturonase (Fig. 5B) activities.

DISCUSSION

The present study clearly demonstrated that *Burkholderia* sp. strain PsJN can form sustaining endophytic populations in roots, stems, and leaves of *V. vinifera* L. plantlets. Following rhizosphere inoculation, colonization of the grapevine plantlets progressed in distinguishable stages. The initial step consisted of rhizoplane colonization of grapevine plantlets by PsJN, although this could be expected as the bacterial inoculum was in contact with the root system. Despite this, the presence of bacteria was not uniform on the entire root surface. The highest bacterial concentrations appeared on both primary and secondary roots, as well as at the base of the lateral roots and at root tips. Furthermore, PsJN cells were often closely attached to the rhizodermal cell walls and could follow the whole outline of epidermal cells. Hansen et al. (18) described a similar colonization behavior for *Pseudomonas fluorescens* DF57 on barley roots, suggesting that there is a common pattern for rhizoplane colonization by different bacteria. The extensive colonization of the rhizoplane can be explained by root exudate effects (for a review see reference 41).

Irrespective of the mode of action, a key feature of all plant-beneficial bacteria is efficient colonization of root surfaces (65). After this initial colonization step, certain bacteria are able to enter roots through cracks at root emergence sites (crack entry process) and/or by passing through root tips (root tip pathway) (reviewed in reference 45) or through the middle

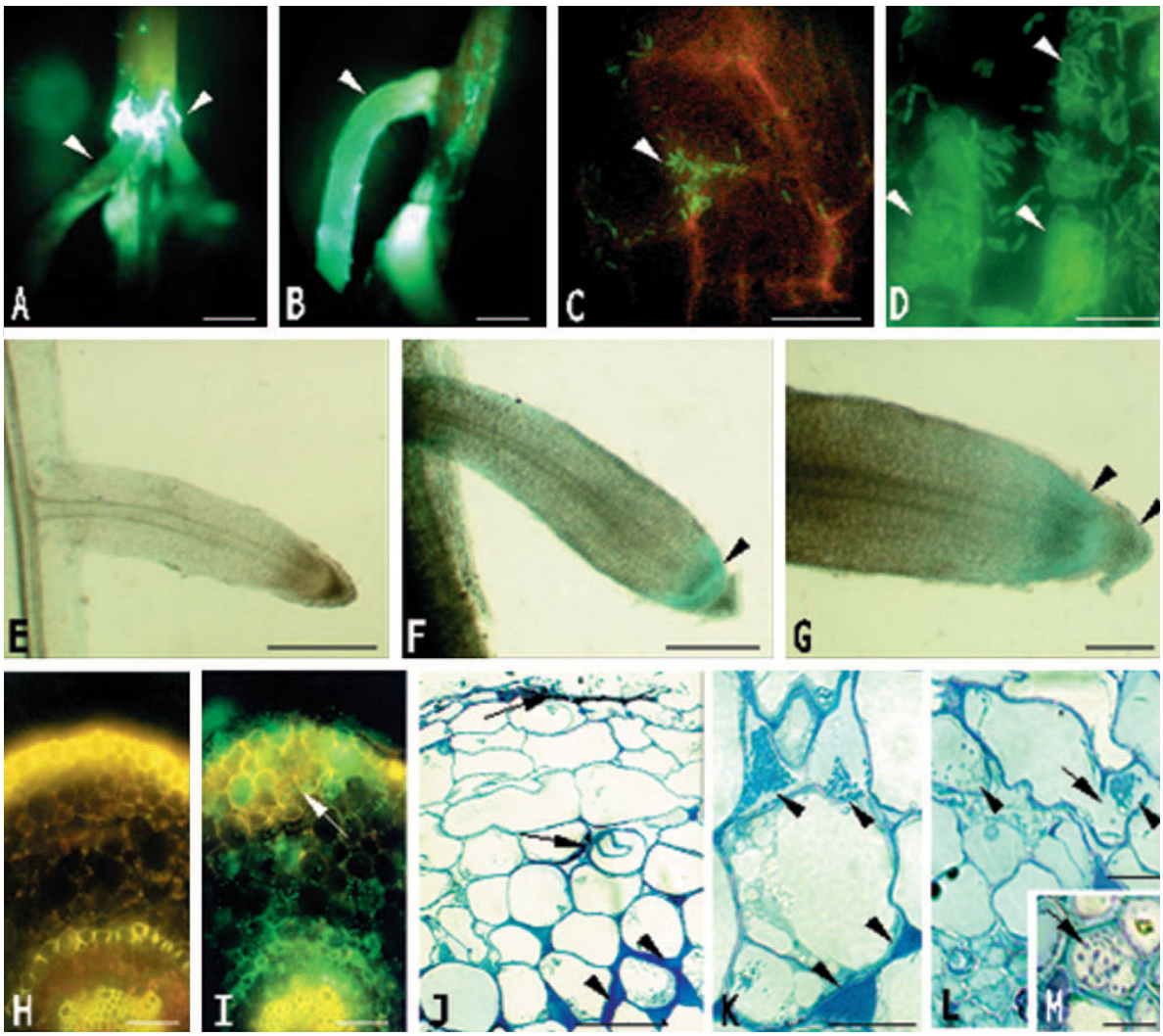

FIG. 3. Microphotographs of rhizoplane and root internal tissues of *V. vinifera* L. cv. Chardonnay plantlets after rhizosphere inoculation with *Burkholderia* sp. strain PsJN. (A and B) Fluorescence stereomicroscope images of roots after inoculation of PsJN::*gfp*2x, showing strong fluorescence at the site of emergence of lateral roots of the primary root (A) and on a lateral root due to *gfp*-marked cells (A and B) (arrowheads). (C and D) Epifluorescence microscope images of rhizoplane, showing *gfp*-marked cells close to the cell walls (C) or around the whole outline of some rhizodermis cells (D) (arrowheads). (E to G) Light microscope images of a secondary root after PBS inoculation (E) and after inoculation with PsJN::*gusA*11 (F and G), showing a blue color due to *gusA*-marked cells at the root tips (arrowheads). (H and I) Epifluorescence microscope images of primary root internal tissues of *V. vinifera* L. cv. Chardonnay plantlets after treatment with PBS (H) or after inoculation of the rhizosphere with PsJN::*gfp*2x (I), showing yellow fluorescence in several cortical cell layers corresponding to a host defense reaction (arrow). (J to M) Light microscope images of resin-embedded primary roots after inoculation with PsJN, showing host defense reactions (arrows) in the exodermis and cell wall of a cortical cell (J), intercellular colonization of cortical cells (J and K) (arrowheads), a break in the endodermis (arrow) caused by PsJN (L) (arrowheads), and PsJN in xylem vessels (M) (arrows). (A and B) Bars = 500 μm; (C) bar = 25 μm; (D) bar = 10 μm; (E and F) bars = 250 μm; (G) bar = 125 μm; (H and I) bars = 100 μm; (J, K, and L) bars = 50 μm; (M) bar = 20 μm.

lamella of the epidermal layer (19). In our study, the presence of PsJN at lateral root emergence sites suggested that crack entry colonization occurred in grapevine plantlets, similar to the phenomenon previously observed with the same strain and potato (39). Moreover, the occurrence of a blue color at root tips after PsJN::*gusA*11 inoculation also supports the possibility of entry via root tips. Furthermore, PsJN cells colonized rhizodermal cells, the inter- and intracellular spaces of cortical cells, the endodermis, and xylem vessels. This indicated that this bacterium can invade root internal tissues by passing between epidermal and cortical cells and can permeate the central cylinder by breaking the endodermis barrier. This conclusion is supported by the production of the cell wall-degrading enzymes endoglucanase and endopolygalacturonase.

It has been reported that the production of cell wall-degrading enzymes by endophytic bacteria is usually linked to localized host

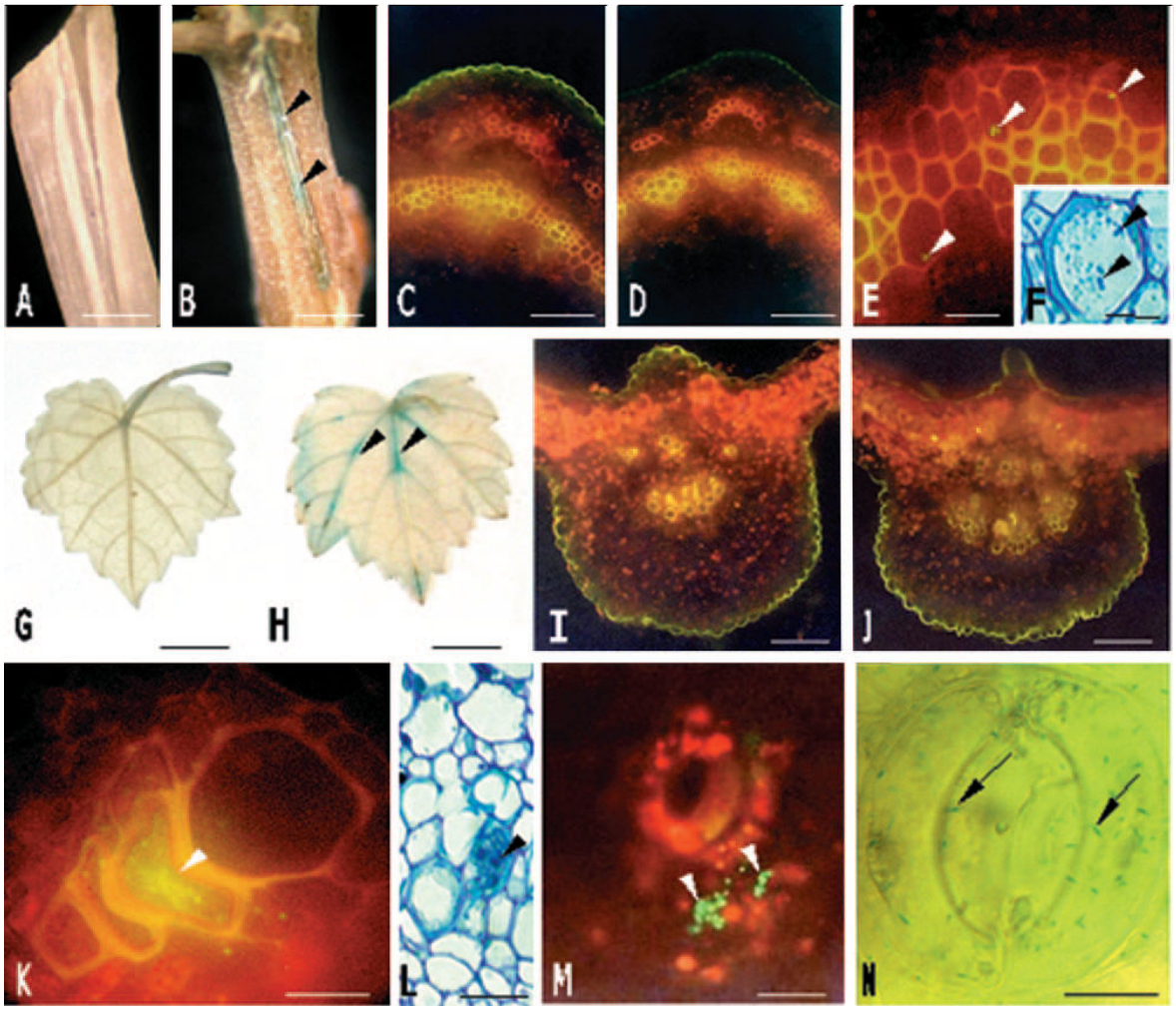

FIG. 4. Photographs of the fifth internode and leaf internal tissues of *V. vinifera* L. cv. Chardonnay plantlets after rhizosphere inoculation with PsJN. (A and B) Light stereomicroscope images of the fifth internode cut longitudinally with a razor after treatment with PBS (A) or after inoculation with PsJN::*gusA*11 (B), showing the blue color (arrowheads). (C to E) Epifluorescence microscope images of hand-cut sections of the fifth internode after treatment with PBS (C) or after inoculation with PsJN::*gfp*2x (D and E), showing PsJN::*gfp*2x cells in xylem vessels (E) (arrowheads). (F) Light microscope image of resin-embedded stem after inoculation with wild-type strain PsJN, showing PsJN cells in xylem vessels (arrowheads). (G and H) Light micrographs of the fifth leaf after treatment with PBS (G) or after inoculation with PsJN::*gusA*11 (H), showing the blue color in veins due to *gusA*-marked cells (arrowheads). (I to K) Epifluorescence microscope images of hand-cut sections of the fifth leaf after treatment with PBS (I) or after inoculation with PsJN::*gfp*2x (J), showing PsJN::*gfp*2x cells in xylem vessels (K) (arrowhead). (L) Light microscope image of resin-embedded leaf after inoculation with wild-type strain PsJN, showing PsJN cells in xylem vessels (arrowheads). (M and N) Epifluorescence (M) and light (N) microscope images of the fifth leaf after inoculation of PsJN::*gfp*2x (M), showing bacteria exiting from stomata (arrowheads), or after inoculation of PsJN::*gusA*11 (N), showing blue cells (arrows) under stomata. (A and B) Bars = 500 μm; (C and D) bars = 150 μm; (E) bar = 30 μm; (F) bar = 30 μm; (G and H) bars = 500 μm; (I and J) bars = 150 μm; (K) bar = 20 μm; (L) bar = 50 μm; (M) bar = 10 μm; (N) bar = 5 μm.

plant defense (17). In the present study, we also observed a host defense reaction that coincided with localized accumulation of phenolic compounds in several cortical cells following colonization by PsJN. This is not surprising since it is well established that phenolic compound accumulation is associated with a plant defense mechanism (35). We concluded that PsJN cells can induce a host defense response in roots of grapevines. Strengthening of the cell walls in the exodermis, as well as in some cortical cells, was also observed, as reported previously for tomato root colonization by *P. fluorescens* strain WCS417r (9).

Following colonization of the root interior, PsJN colonizes stems and leaves. Other studies have also detected endophytes within aerial plant parts, including stems, leaves, and flowers (28, 34, 46, 52). It has been suggested that bacteria can be transported in xylem vessels through the transpiration stream (16, 19, 22, 23, 54) or by colonizing intercellular spaces from

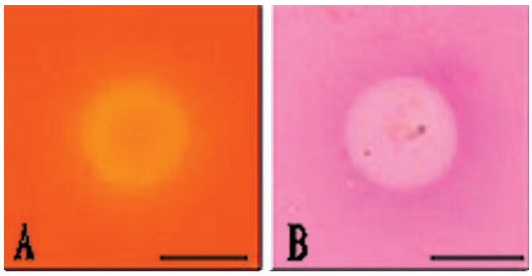

FIG. 5. Photographs of endoglucanase (A) and endopolygalacturonase (B) activities of PsJN. Bars = 3 mm.

roots to aerial parts (10, 16). Our results demonstrate that PsJN systemically spreads to aerial parts through the transpiration stream from root xylem vessels.

In this study, we observed significantly higher numbers of PsJN cells in leaves than in stems. Similar results were reported for the colonization of tomato plants by *P. fluorescens* SE34r (70). The differences between stem and leaf populations that were observed can be explained by accumulation of bacterial cells in the leaf, which can be considered a sink, whereas the stem serves only for transition. However, in this study we also detected PsJN in subtomatal chambers of grapevine leaves because PsJN cells exited from stomata after a little pressure between a glass slide and a coverslip was exerted on a leaf. Furthermore, PsJN was not detected on the leaf surface but was found inside substomatal chambers with *gus*-tagged bacteria after rhizosphere inoculation. This demonstrated that PsJN cells can reach substomatal chambers of leaves after spreading within the plant. Substomatal chambers, interstices, and trichomes are preferred habitats for bacterial survival and multiplication due to their relatively protected hydrophilic environments (29, 36, 68). Thus, stems and leaves that are colonized differently may also be explained by considering substomatal chambers of grapevines microhabitats for PsJN where multiplication may occur. However, the lack of PsJN cells in substomatal chambers in resin-embedded leaves may indicate that there was a low number of bacteria in this habitat which could not be detected. Furthermore, multiplication of endophytic bacteria inside plant tissues is difficult to demonstrate (17), and work is needed to confirm this hypothesis. Despite this, all rhizosphere bacteria are not capable of establishment in this habitat. Several studies have shown that endophytic populations of strains of various nonpathogenic bacterial species cannot become established following infiltration into leaves (48, 69). To our knowledge, only James et al. (21, 23) have reported colonization of substomatal chambers by plant-beneficial bacteria, but there was the possibility of epiphytic bacterial propagation on the phylloplane (21, 23) and/or spreading within the plant (23). Our finding of PsJN cells in substomatal chambers after an initial root colonization step confirmed that substomatal chambers can be colonized by bacteria after they spread within the plant. Such knowledge could ultimately lead to a better understanding of the plant-endophytic bacterium interactions. Although our experiments were based on early events, an agar-based system and substomatal chamber colonization by PsJN need to be demonstrated by using a soil-based system or hydroponic conditions under which PsJN can form endophytic populations in grapevine (unpublished results).

ACKNOWLEDGMENTS

This work was supported by a Ph.D. grant from Europôl-Agro (Reims, France).

We thank Richard E. Veilleux (Department of Horticulture, Virginia Polytechnic Institute and State University, Blacksburg) for a critical review of the manuscript.

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Les travaux présentés dans l'article du chapitre 2 démontrent une colonisation épi- et endophytique des plantules *in vitro* de vigne par *B. phytofirmans* souche PsJN, ainsi que l'induction de la synthèse de composés de défense en réponse à la propagation bactérienne. Cette étude a été réalisée avec deux souches modifiées génétiquement, l'une étant transformée avec le gène *gfp*, l'autre avec le gène *gusA*, ainsi que par le biais de l'utilisation de la souche de type sauvage. Ces souches ont été considérées comme identiques dans cet article par le biais de différents tests et nous ont permis de visualiser la colonisation bactérienne et d'être sûr que c'était bien la souche PsJN que l'on observait.

Tout d'abord les résultats obtenus ont permis de montrer que *B. phytofirmans* souche PsJN colonise les surfaces racinaires de plantules *in vitro* après inoculation de la rhizosphère. La bactérie PsJN a ainsi été localisée au niveau de l'émergence des racines secondaires et des extrémités racinaires, ainsi que sur toute la surface des cellules rhizodermiques des racines primaires.

Suite à la colonisation épiphytique du rhizoplan, *B. phytofirmans* souche PsJN a été retrouvée à l'intérieur des racines. Cette propagation bactérienne intra-racinaire peut s'expliquer par un passage *via* les extrémités racinaires ou par les ouvertures naturelles. Néanmoins, elle peut provenir également de la sécrétion bactérienne d'enzymes dégradant les parois cellulaires de la plante. En effet, nous avons montré que *B. phytofirmans* souche PsJN sécrète des cellulases et des endopolygalacturonases. Ces enzymes ou leurs produits de dégradation peuvent de plus être à l'origine de l'induction de composés de défense par la plante comme suggéré par James *et al.* (2002). Suite à la pénétration de la souche PsJN, nous avons détecté une accumulation de composés phénoliques au niveau de l'exoderme et des cellules corticales, ainsi que des renforcements pariétaux, ce qui démontre que des mécanismes de défense sont induits chez la plante en réponse à la colonisation bactérienne.

Lors de ce travail, nous avons également mis en évidence une progression de la souche PsJN jusqu'à l'endoderme. Compte tenu de nos résultats, il apparaît que cette barrière est détruite à certains endroits par la souche PsJN. Ceci lui permet de progresser jusqu'au cylindre central et d'atteindre les vaisseaux conducteurs du xylème. Des réactions de défense sous la forme d'épaississements pariétaux se mettent en place dans le parenchyme des vaisseaux du xylème suite à la propagation endophytique de la bactérie PsJN (Figure 19a et b ; Annexe 2), bien que nous ne les avons pas mentionnées dans l'article du chapitre 2. Ceci démontre, de plus, que le phénomène de colonisation endophytique de la vigne par *B. phyto-*

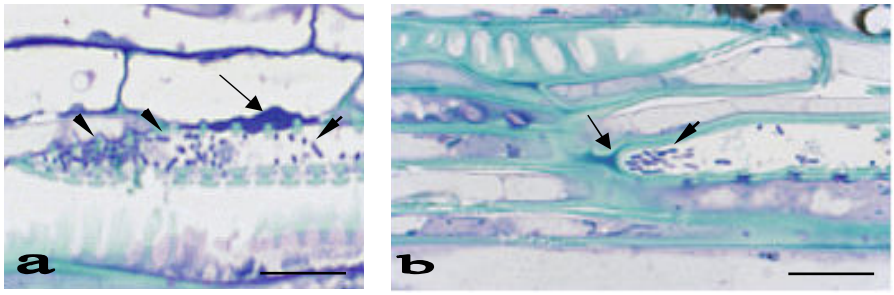

Figure 19 : Microphotographies optiques après coloration au bleu de toluidine du xylème de racine d'une plantule *in vitro* de vigne inoculée avec *B. phytofirmans* souche PsJN montrant la présence d'appositions pariétales (flèches) au niveau des cellules du parenchyme du xylème, suite à la propagation de la bactérie (têtes de flèches). Barres d'échelle : (a et b) 15 µm.

firmans souche PsJN est associé à des réponses de défense de son hôte jusqu'au niveau du xylème.

Suite à l'invasion des vaisseaux du xylème racinaire, la bactérie a ensuite été détectée dans le xylème de la tige et des feuilles, 72 heures après inoculation. Nous avons également montré la présence de nombres différents de populations dans ces organes végétatifs aériens. En effet, les sous-populations endophytiques de la souche PsJN sont plus importantes dans les feuilles que dans la tige. De façon intéressante, la bactérie endophytique est présente dans les chambres sous-stomatiques des feuilles. Il est possible que la bactérie s'y multiplie compte tenu du fort taux d'humidité régnant dans ce lieu de colonisation (Lindow, 1991). Ceci pourrait expliquer les différences de populations de la souche PsJN dans la tige et dans les feuilles. Ce lieu privilégié de colonisation suggère également que la colonisation bactérienne est favorisée par le phénomène d'évapo-transpiration de la plante *via* la voie du xylème. Des résultats similaires avaient été obtenus chez le riz avec *Herbaspirillum seropedicae* Z67 (James *et al.*, 2002). Néanmoins, lors de cette étude, la présence des bactéries dans les chambres sous-stomatiques foliaires pouvait également provenir d'une contamination du phylloplane (James *et al.*, 2002). Dans notre étude, cependant, aucune contamination foliaire ne fut détectée. Ainsi, il apparaît que *B. phytofirmans* souche PsJN se propage au sein de la plante par le phénomène d'évapotranspiration de la plante *via* les vaisseaux du xylème. Toutefois, il est également possible que la bactérie PsJN se propage d'elle même au sein des vaisseaux de la plante puisque cette souche bactérienne est flagellée (Sessitsch *et al.*, 2005).

Il est possible que des enzymes dégradant les parois cellulaires de la plante soient sécrétées par la bactérie au niveau des parties aériennes de la plante. Ceci pourrait faciliter la progression de la bactérie jusqu'au niveau des feuilles en migrant d'un vaisseau de xylème à un autre. Néanmoins, il a été décrit que les vaisseaux du xylème de la vigne permettent la migration de micro-organismes sans besoin de sécrétion enzymatique (Thorne *et al.*, 2006). De ce fait, il est possible que la sécrétion d'enzymes dégradant les parois cellulaires par la bactérie n'intervienne qu'au niveau racinaire et au non au niveau aérien, même si celà devra faire l'objet d'une étude approfondie.

Ainsi, nous avons pu démontrer, en conditions gnotobiotiques, que *B. phytofirmans* souche PsJN colonise les surfaces racinaires, pénètre dans les racines et se propage de façon systémique dans les parties aériennes des plantules *in vitro* de vigne par les vaisseaux du xylème, tout en induisant des réactions de défense lors de sa propagation.

Ait Barka *et al.* (2002) avaient déjà détecté la souche PsJN au niveau foliaire. Néanmoins, des explants nodulaires avaient été utilisés lors de cette étude. Ainsi, la bactérie pouvait être directement en contact avec les vaisseaux conducteurs et se propager rapidement jusqu'au niveau foliaire. Dans notre étude, nous avons utilisé des plantules enracinées afin d'étudier la colonisation bactérienne à partir de la rhizosphère jusqu'aux parties aériennes. Nous avons ainsi respecté les niches microbiologiques naturelles de la souche PsJN et étudié son phénomène de colonisation. De plus, nous avons utilisé des souches transformées pour étudier le phénomène de colonisation de la vigne par la souche PsJN. Ceci a permis d'être sûr que la souche observée était bien la souche PsJN.

Compte tenu de nos résultats obtenus en conditions gnotobiotiques, nous nous sommes ensuite interrogés si *B. phytofirmans* souche PsJN pouvait coloniser des plantes *ex vitro*, et se retrouver, en particulier, au niveau des inflorescences, organes de très grande importance chez la vigne.

Chapitre 3

Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: from the rhizosphere to inflorescence tissues

Stéphane Compant[1], Hervé Kaplan[2], Essaïd Ait Barka[1], Angela Sessitsch[3], Jerzy Nowak[4], and Christophe Clément[1]

Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cédex 2, France[1];

INSERM, Unité 514, IFR 53, UFR Pharmacie, Université de Reims Champagne-Ardenne, 51092 Reims Cédex, France[2] ;

Department of Bioresources, ARC Seibersdorf research GmbH, A-2444 Seibersdorf, Austria[3];

Department of Horticulture, Virginia Polytechnic Institute and State University, 0327-301 Saunders Hall, Blacksburg, VA 24060, USA[4]

Révision soumise à FEMS Microbiology Ecology

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Stéphane Compant[1], Hervé Kaplan[2], Essaïd Ait Barka[1*], Angela Sessitsch[3], Jerzy Nowak[4] & Christophe Clément[1]

[1]Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cédex 2, France; [2]IFR 53, UFR Pharmacie, Université de Reims Champagne-Ardenne, 51092 Reims Cédex, France; [3]Department of Bioresources, Austrian Research Centers GmbH, A-2444 Seibersdorf, Austria; [4]Department of Horticulture, Virginia Polytechnic Institute and State University, 0327-301 Saunders Hall, Blacksburg, VA 24060, USA

Correspondence

Essaïd Ait Barka, Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cédex 2, France. Tél./Fax : +33-326913441; e-mail: ea.barka@univ-reims.fr

Received 21 May 2007; revised 06 September 2007

Editor : Christoph Tebbe

Keywords

Burkholderia phytofirmans strain PsJN; grape; microflora, inflorescence; vitaceae

Abstract

The colonization pattern of *Vitis vinifera* L. by *B. phytofirmans* strain PsJN was determined using grapevine fruiting cuttings with emphasis on putative inflorescence colonization under non-sterile conditions. Two week-old rooted plants harbouring flower bud initials, grown in non-sterile soil, were inoculated with PsJN::*gfp*2x. Plant colonization was subsequently monitored at various times after inoculation with plate counts and epifluorescence and/or confocal microscopy. Strain PsJN was chronologically detected on the root surfaces, in the endorhiza, inside grape inflorescence stalks, not inside pre-flower buds and flowers but rather as an endophyte inside young berries. Data demonstrated low endophytic populations of strain PsJN in inflorescences organs, *i.e.* grape stalks and immature berries with inconsistency among plants for bacterial colonization of inflorescences. Nevertheless, endophytic colonization of inflorescences by strain PsJN was substantial for some plants. Microscopic analysis revealed PsJN as a thriving endophyte in inflorescence organs after the colonization process. Strain PsJN was visualized colonizing the root surface, entering the endorhiza and spreading to grape inflorescence stalks, pedicels and then to immature berries through xylem vessels. In parallel to these observations, a natural microflora was also detected on and inside plants, demonstrating the colonization of grapevine by strain PsJN in the presence of other microorganisms.

Introduction

Interaction between plants and beneficial bacteria can have a profound effect on plant health, growth, development, yield as well as on soil quality (Welbaum *et al*., 2004; Compant *et al.*, 2005a; Haas and Défago, 2005; Unno *et al*., 2005). Plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978) or plant growth-promoting bacteria (PGPB) (Bashan and Holguin, 1998) can colonize the plant interior and thrive as endophytes in various organs without harming their host (James and Olivares, 1998; Lodewyckx *et al.*, 2002; Berg *et al.*, 2004; Compant *et al*., 2005b; Rosenblueth and Martínez-Romero, 2006). Bacterial endophytes have been found in different vegetative parts of plants, such as roots, tubers, stem and leaves (Hallmann, 2001; Compant *et al.*, 2005b; Gray and Smith, 2005). A few studies have also reported bacterial endophytes in reproductive organs, such as flowers and fruits. This has been demonstrated mainly through the isolation of endophytes from their natural plant hosts (Samish *et al.*, 1961; Misaghi and Donndelinger, 1990; Hallmann, 2001; Bacon and Hinton, 2006). However, the colonization process of reproductive organs by endophytic bacteria, particularly by some PGPR, is unknown. A understanding of this process in plants of agricultural importance could potentially be used for enhancing plant production (Lodewyckx *et al.*, 2002; Welbaum *et al.*, 2004; Compant *et al.*, 2005a).

One of these endophytic PGPR, *Burkholderia phytofirmans* strain PsJN (Sessitsch *et al.*, 2005), has been isolated from surface sterilized onion roots. Strain PsJN has been able to differentially promote plant growth of non-natural hosts in addition to lowering biotic and abiotic stresses. This bacterium can also thrive as an endophyte inside various plant hosts, including tomato (Pillay and Nowak, 1997; Sharma and Nowak, 1998), potato (Frommel *et al.*, 1991; Bensalim

et al., 1999), and grape (Ait Barka *et al*., 2000; 2002; 2007; Compant *et al.*, 2005b). With grapevine, strain PsJN can form epiphytic as well as endophytic populations with *in vitro* plantlets (Compant *et al.*, 2005b). This was monitored mainly *via* the use of a *gfp* derivative of strain PsJN that allowed visualization of the bacterium both on and inside plants. Use of this tagged strain on grapevine plantlets has demonstrated bacterial colonization of the root surface and subsequent entrance into the endorhiza mainly through the 'root tip way', lateral root cracks or between rhizodermal cells *via* cell-wall degrading enzyme secretions. Finally, strain PsJN colonized stem and leaves through xylem vessels, before thriving as an endophyte inside substomatal chambers of leaves after using the plant transpiration stream (Compant *et al.*, 2005b). Prior analysis of the colonization patterns of grapevine by strain PsJN was conducted under gnotobiotic conditions. The epi- and endophytic colonization behaviour of strain PsJN in plants, grown in non-sterile soil, has not been elucidated. Furthermore, nothing was known on inflorescence colonization by strain PsJN. However, in grapevine, inflorescences are of particular interest and putative reproductive organs colonization by strain PsJN needed to be studied.

Therefore, the objective of the present study was to investigate epi- and endophytic colonization of grapevine by *B. phytofirmans* strain PsJN in plants grown in non-sterile soil with emphasis on putative inflorescence colonization, and to compare this pattern with the previous study on the colonization of *in vitro* plantlets.

Materials and methods

Bacterial strains, growth conditions and inoculum preparation

The *gfp*-marked strain PsJN::*gfp*2x (Compant *et al.*, 2005b) was grown in 100 ml Luria-Bertani liquid medium in 250 ml Erlenmeyer flask and incubated at 25°C, on a shaker (150 rpm) for 48h as described by Compant *et al*. (2005b). Bacteria were collected by centrifugation (4500*g*; 10min) and washed twice with sterile phosphate buffer, pH 6.5 (PBS). The bacterial concentration of the inoculum was then adjusted with PBS, based on optical density (600 nm) and confirmed with plate counting (Pillay and Nowak, 1997).

Plant material and growth conditions

Three-node cuttings (20 cm long) of *Vitis vinifera* L. cv. Chardonnay were cane pruned from 6-year-old plants in 2005, 2006 and 2007 at the 'Moët et Chandon' vineyard (Epernay, France). Fruiting cuttings were then prepared according to Lebon *et al.* (2005) with the following modifications: the cuttings were treated with 0.05% Cryptonol® during 4 hours and the distal node was covered with grafting wax (Agrochemie®, Germany) containing fungicide (0.1% Oxiquinoleine) and plant growth regulators (0.00175% 2,5-dichlorobenzoïc acid). Cuttings were then stored in the dark at 4°C for at least for 2 weeks. Before use and after 15h of hydratation at 28°C, the two proximal nodes were removed and the cuttings were immersed for 30s in indole-3-butyric acid solution (2 g/L) to promote rhizogenesis. Each cutting was then placed in a plastic pot (9x9x10 cm) filled with one part of clay balls and four parts (v/v) of potting soil (special Gramoflor, Gramoflor GmbH & Co. KG, Vechta, Germany) that was not sterilized in order to allow the growth of a natural microflora. The potted cuttings were irrigated daily with tap water and incubated in a growth chamber at 25/20°C day/night temperature, 16 h photoperiod, under 500 $\mu mol.s^{-1}.m^{-2}$ (Lumilux cool white L30W/840 and Fluora L30W/77, Osram, Germany) and 70% relative humidity. To avoid the beginning of vegetative growth and to facilitate development of inflorescence, we removed leaves daily according to Lebon *et al.* (2005). More than 400 fruiting cuttings were planted as only 25 to 75% of potted plants developed inflorescences.

Plant inoculation and growth conditions

Fruiting cuttings with a rooting system and fully developed inflorescences were then inoculated with either $5x10^7$ or $5x10^8$ CFU of PsJN::*gfp*2x g^{-1} of soil at stage 57 of the BBCH scale (last step of pre-flower buds development before anthesis, according to Meyer, 2001).

Preparation of plant samples for bacterial enumeration

The rhizoplane and endophytic colonizations of roots and inflorescences by the *gfp*-marked strain were determined by plate counting of bacterial colonies under a fluorescence stereomicroscope (model MZ FLIII; Leica, Heerbrugg, Switzerland) equipped with a GFP 1 filter (Leica, Switzerland) after inoculation with $5x10^7$ or $5x10^8$ CFU g^{-1} of soil. Roots, stalks, and reproductive organs, such as pre-flower buds, flowers or young berries, were sampled at different time intervals (Table 1). Five replicates of ten independent plating assays were used to determine the average colonization patterns. Base level of detection was between 0 and –1 $\log_{10}$ CFU g^{-1} of Fresh Weight (FW) for each plant organs.

Rhizoplane colonization

Root surface colonization by PsJN was determined at different times after soil inoculation. Plants were removed from the potting soil and roots were rinsed (4 times) with sterile distilled water. Root samples (1 g each) were ground in a mortlar and pestle, containing 1 mL of PBS. The homogenates were serially diluted in microtiter plates, and 100 µL aliquots plated on solid LB medium containing cycloheximide (100 $\mu g.mL^{-1}$) to inhibit fungal growth and kanamycin (100 $\mu g.mL^{-1}$; PsJN::*gfp*2x is resistant to kanamycin). Bacterial colonies were then counted under UV light 3 days after incubation at 30°C. Rhizoplane colonization by the *gfp*-marked strain was determined by subtracting bacterial counts after surface sterilization from the total *gfp*-tagged bacteria enumerated without surface sterilization, as described in Compant *et al.* (2005b).

Endophytic colonization

To determine endophytic populations of the *gfp*-marked strain, we surfaces sterilized roots, grape stalks, pre-flower buds and/or flowers or young berries with 70% ethanol for 5 min, followed by a bath of 1% commercial bleach and 0.01% Tween 20 solution for 1 min. The samples were then washed three times with distilled water (1 min each) and ground and handled as described above. Bacterial colonies were counted under UV light and confirmed under a fluorescence stereomicroscope, 3-5 after incubation at 30°C.

Evaluation of surface-sterilization methods

To determine the efficacy of the surface-sterilization procedure, we detached roots from five plants 1 week after soil inoculation with 5.10^8 CFU g^{-1} of soil, surface sterilized as described before and observed them at 200x and 1000x under an optical microscope (model BH2; Olympus, Tokyo, Japan) equipped with a UV light source (BH2-RFL-T3; Olympus) and a 495-nm fluorescent filter (BP495; Olympus). Surface sterilized roots were placed on LB plates amended with 100 µg.mL^{-1} of kanamycin and 100 µg.mL^{-1} cycloheximide during 1 min prior to crushing. Additionally, 100 µL aliquots of the last bath of surface-sterilization were plated on LB plates amended with 100 µg.mL^{-1} of each antibiotic and bacterial colonies were counted to be sure of the surface-sterilization procedure.
To confirm systemic colonization from the rhizosphere to inflorescence tissues, we sampled ten plants 6 weeks after soil inoculation with 5.10^8 CFU g^{-1} of soil. Inflorescences were used without surface-sterilization and ground in a mortlar and pestle containing 1 mL of PBS. The homogenates (100 µL aliquots) were plated on solid LB medium containing 100 µg.mL^{-1} cycloheximide and 100 µg.mL^{-1} of kanamycin. Then, bacterial colonies were counted under UV light, and confirmed under fluorescence stereomicroscope, 3 days after incubation at 30°C.

Microscopy of epiphytic and endophytic colonization by PsJN

The epiphytic and endophytic colonizations of roots, stalks, and reproductive organs such as pre-flower buds and/or flowers or young berries by PsJN::*gfp*2x were monitored at different time intervals (as presented in Table 2) after soil inoculation with 5.10^8 CFU g^{-1} soil. The different parts were sampled separately and ten longitudinal sections were made on independents samples of different plant parts taken from 20-60 plants (roots or inflorescences) before examination under an epifluorescence and/or a confocal microscope (MRC 1024; Biorad, Hercules, CA,. USA) equipped with a x63 PlanApo objective.

Statistical analysis

Microscopic observations were made on samples taken from independent experiments. Population density data (CFU) from experiments on fruiting cuttings were pooled over 3 years. These data were submitted to logarithmic transformation (Loper *et al.*, 1984) and statistically analysed using Student's *t* test.

Results

Density populations on the rhizoplane, in the endorhiza and inside inflorescences following soil inoculations

The rhizoplane of fruiting cuttings was colonized by PsJN::*gfp*2x cells immediately after soil inoculation, reaching 6.99 +/- 0.43 $\log_{10}$ CFU g^{-1} FW of root tissue within 0-15 h after inoculation with $5x10^7$ CFU g^{-1} of soil (Table 1) and 7.41 $\log_{10}$ CFU g^{-1} FW with $5x10^8$ CFU g^{-1} of soil (Table 2). The bacterial population subsequently stabilized at 5.57 +/- 0.33 $\log_{10}$ CFU g^{-1} FW and at 5.72 +/- 0.37 $\log_{10}$ CFU g^{-1} FW 1 week after soil inoculation with $5x10^7$ CFU g^{-1} of soil (Table 1) or $5x10^8$ CFU g^{-1} of soil (Table 2)

Table 1: Colonization of grapevine fruiting cuttings by *Burkholderia phytofirmans* strain PsJN after inoculation with 5.10^7 CFU of PsJN::*gfp*2x g^{-1} of soil. Log numbers of plant populations of PsJN were determined on the rhizoplane, in the endorhiza as well as inside grape inflorescence stalk and reproductive organs. n.d.: not detectable. Base limit of detection was between 0 and –1 $\log_{10}$ CFU g^{-1} FW.

Weeks post inoculation	Rhizoplane	Endorhiza	Stalk	Reproductive organs
0	6.99 +/- 0.43	n.d.	n.d.	n.d. (preflower buds)
1	5.57 +/- 0.33	2.15 +/- 0.93	n.d.	n.d. (flowers)
4	5.45 +/- 0.36	2.26 +/- 0.54	n.d.	n.d. (young berries)
5	5.42 +/- 0.28	2.18 +/- 0.61	1.08 +/- 0.35	0.85 +/- 0.65 (young berries)
6	5.54 +/- 0.42	2.23 +/- 0.45	0.99 +/- 0.54	1.40 +/- 0.60 (young berries)

Table 2: Colonization of grapevine fruiting cuttings by *Burkholderia phytofirmans* strain PsJN after inoculation with 5.10^8 CFU of PsJN::*gfp*2x g^{-1} of soil. Log numbers of plant populations of PsJN were determined on the rhizoplane, in the endorhiza and inside grape stalks and reproductive organs. n.d.: not detectable. Base limit of detection was between 0 and –1 $\log_{10}$ CFU g^{-1} FW.

Weeks post inoculation	Rhizoplane	Endorhiza	Stalk	Reproductive organs
0	7.41 +/- 0.01	n.d.	n.d.	n.d. (preflower buds)
1	5.72 +/- 0.37	2.3 +/- 1.0	n.d.	n.d. (flowers)
4	5.62 +/- 0.52	2.34 +/- 0.74	n.d.	n.d. (young berries)
5	5.63 +/- 0.45	2.35 +/- 0.65	1.56 +/- 0.41	1.36 +/- 0.32 (young berries)
6	5.77 +/- 0.51	2.4 +/- 0.62	1.66 +/- 0.22	1.89 +/- 0.31 (young berries)

respectively. Then, these bacterial titer numbers remained almost unchanged over the 6 week duration of this study (Tables 1 and 2).

Following rhizoplane colonization, PsJN::*gfp*2x cells appeared in the endorhiza of all tested plants 2-3 after soil inoculation and reached their highest level 1 week after soil inoculation (2.15 +/- 0.93 $\log_{10}$ CFU g^{-1} FW after inoculation $5x10^7$ CFU g^{-1} of soil and 2.3 +/- 1.00 $\log_{10}$ CFU g^{-1} FW with $5x10^8$ CFU g^{-1} of soil; Tables 1 and 2). Bacterial titer numbers stabilized at 2.23 +/- 0.45 $\log_{10}$ CFU g^{-1} FW after inoculation with $5x10^7$ CFU g^{-1} of soil and 2.4 +/- 0.62 $\log_{10}$ CFU g^{-1} FW with $5x10^8$ CFU g^{-1} of soil 2-6 weeks post inoculation (Tables 1 and 2).

In addition to these root analyses, endophytic subpopulation densities of strain PsJN in inflorescences were determined using plate counting. After soil inoculation with $5x10^7$ CFU g^{-1} of soil, PsJN::*gfp*2x were not found in grape stalk, pre-flower buds and flowers during the first 2 weeks after inoculation (Table 1). However, they were detected in grape inflorescence stalks and in young berries after 5 and 6 weeks post inoculation. Nevertheless, bacterial concentrations in these organs were low with 1.08 +/-0.35 $\log_{10}$ CFU g^{-1} FW in grape stalks and 0.85 +/- 0.65 $\log_{10}$ CFU g^{-1} FW in young berries following inoculation with $5x10^7$ CFU g^{-1} of soil, and with 1.56 +/- 0.41 $\log_{10}$ CFU g^{-1} FW and 1.36 +/- 0.32 $\log_{10}$ CFU g^{-1} FW in stalks and berries respectively 5 weeks after $5x10^8$ CFU g^{-1} of soil (Tables 1 and 2). Six weeks post-inoculation, the densities of strain PsJN remained constant in inflorescence organs: 0.99 +/- 0.54 $\log_{10}$ CFU g^{-1} FW in grape stalks and 1.40 +/- 0.60 $\log_{10}$ CFU g^{-1} FW in young berries following soil inoculation with $5x10^7$ CFU g^{-1} of soil (Table 1) and 1.66 +/- 0.22 $\log_{10}$ CFU g^{-1} FW in grape stalks and 1.89 +/- 0.31 $\log_{10}$ CFU g^{-1} FW in berries after inoculation with $5x10^8$ CFU g^{-1} of soil (Table 2). However, even if these data represent the average colonization, a significant population (2 $\log_{10}$ CFU g^{-1} FW) in stalk and berries of individual cutting was only detected after inoculation with $5x10^8$ CFU g^{-1} of soil but not with $5x10^7$ CFU g^{-1} of soil. Consequently, some cuttings yielded up to 2.12 $\log_{10}$ CFU g^{-1} FW and 1.60 $\log_{10}$ CFU g^{-1} FW in grape stalks and 1.90 $\log_{10}$ CFU g^{-1} FW and 2.15 $\log_{10}$ CFU g^{-1} FW in berries, 5 and 6 weeks post inoculation, respectively, after soil inoculation with $5x10^8$ CFU g^{-1} of soil. However, some plants did not contain *gfp*-tagged bacteria in grape inflorescence stalk and young berries taken from bacterized plants: strain PsJN tagged with the *gfp* gene was only detected in 13% until 60% of grape stalk and berries, respectively.

Additionally, not all grape inflorescence stalks and berries from bacterized plants were colonized by strain PsJN: only 20 to 60% of grape stalks and berries , respectively, hosted the inoculated strain after $5x10^7$ CFU g^{-1} of soil and 13 to 60% of grape stalks and berries yielded strain PsJN tagged with the *gfp* gene after inoculation with $5x10^8$ CFU g^{-1} of soil.

Microscopic observations of rhizoplane population of *B. phytofirmans* strain PsJN

During the first 4 weeks post inoculation, microscopic observations of the rhizoplane demonstrated that strain PsJN tagged with the *gfp* gene colonized the root surface after inoculation with $5x10^8$ CFU g^{-1} of soil. Strain PsJN was found mainly in the root hair zone, lateral root emergence sites and root tips. However, differences in the bacterial densities of *gfp*-

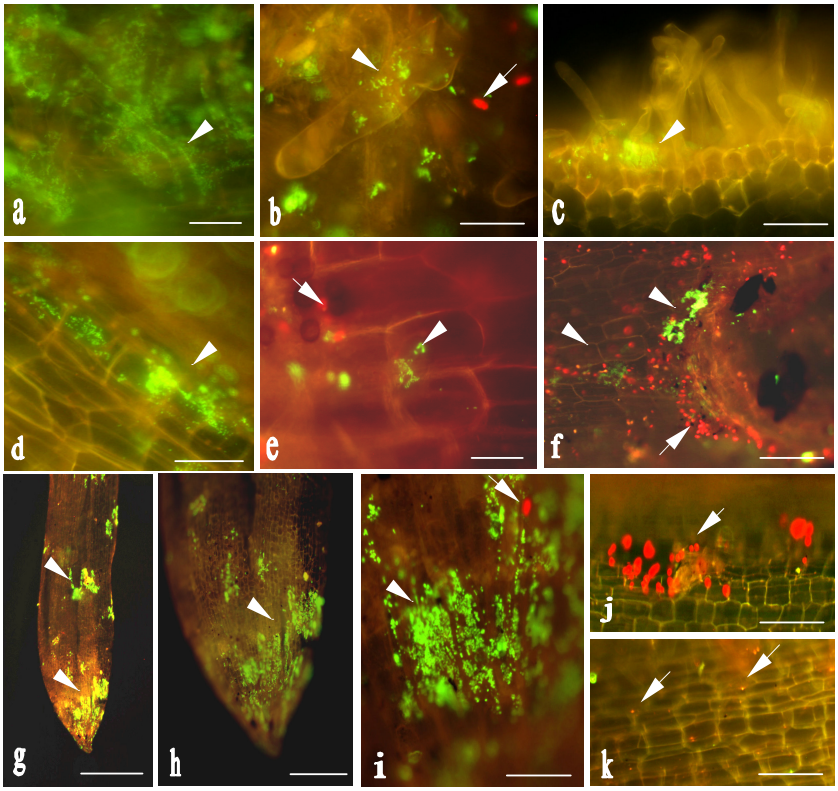

Figure 1: Images under an epifluorescence microscope of roots of grapevine fruiting cuttings inoculated with PsJN::*gfp*2x 1-4 weeks after soil inoculation with 5.10^8 CFU g^{-1} soil. *Gfp*-tagged bacteria (arrows) were visualized at the root hair zone (a-c) colonized root hairs (a-c), on other rhizodermal cells (d and e), at lateral root emergence sites (f) and at the root tip (g-i). A natural epiphytic microflora was also detected on the root surface of inoculated plants (arrows in b, e, f and i) as well as on roots of control plants (arrows in j and k). Similar rhizoplane colonizations by strain PsJN were found from 1 to 4 weeks post inoculation. Scale bars: (a) 100 µm, (b) 30 µm, (c) 75 µm, (d and e) 30 µm, (f) 100 µm, (g) 1 mm, (h) 500 µm, (i) 250 µm, and (j and k) 100µm.

tagged cells were observed in these different areas. The root hair zone was more highly colonized than other root zones with PsJN::*gfp*2x (Fig. 1a-c). Other rhizodermal cells were colonized differentially: some rhizoplane cells were filled with bacteria (Fig. 1d) whereas *gfp*-tagged cells were found closely attached to cell-walls in other cells (Fig. 1d-e). In addition, gfp-tagged cells were visualized in lateral root emergence sites and root tips (Fig. 1f-i). The root surface colonization pattern by strain PsJN was also observed with native microflora. This included yellow, green (Fig. 1e and 1f) and/or blue fluorescing bacteria that formed slight halos (data not shown) that were detected in control plants (Fig. 1k). Red fluorescing algae were also detected under blue light in the rhizosphere of bacterized (Fig. 1b, 1f and 1i) or control plants (Fig. 1j) due to the use of non-sterile conditions and tap water. Some fungi (yeasts and mold), and even worms were detected on the root surface of these plants, in both control and inoculated plants (data not shown), indicating the diverse array of microorganisms in the rhizosphere.

Visualization of endorhiza colonization by *B. phytofirmans* strain PsJN

Following rhizoplane colonization, PsJN::*gfp*2x was detected inside the root cortex as well as in the central cylinder, both 1 week and 4 weeks after soil inoculation (Fig. 2). *Gfp*-tagged bacteria were subsequently visualized in the exodermis, other cortical cell layers (Fig. 2a-b) and passing through the endodermis *via* an inter- and/ or intracellular pathway (Fig. 2c). Although the pericycle is expected to be the first cellular layer within the central cylinder, it was not clearly defined under epifluorescence, making visualization of the bacterium in this tissue difficult. However, in the central cylinder, *gfp*-tagged bacteria were detected inside xylem vessels 4 weeks after inoculation (Fig. 2d-e). As with the root surface, other autofluorescent microorganisms (mainly orange fluorescing bacteria) were observed in the endorhiza. These microorganisms were detected inside all root internal tissues as well as in xylem vessels of both control and bacterized plants (Fig. 2f and 2g).

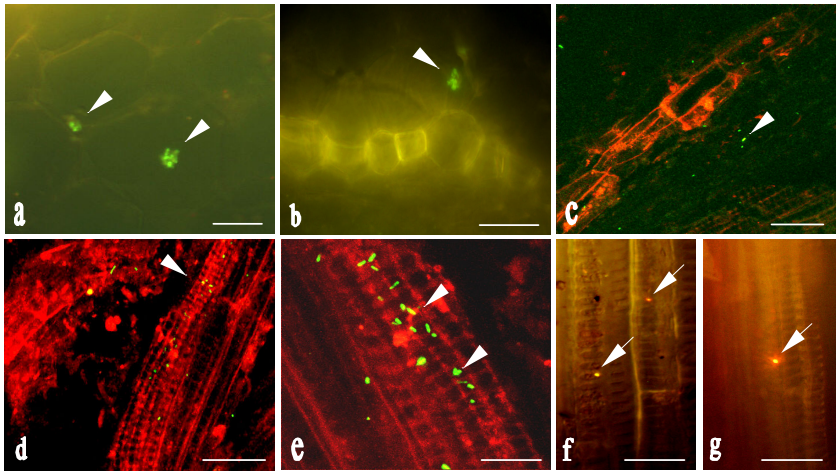

Figure 2: Images from epifluorescence and/or confocal microscope of roots of grapevine fruiting cuttings inoculated with PsJN::*gfp*2x 1-4 weeks after soil inoculation with 5.10^8 CFU g^{-1} soil showing strain PsJN in the endorhiza. *Gfp*-tagged bacteria (arrow heads) were visualized in the cortex (a) near the endodermis barrier (b), passing this barrier (c) and colonizing xylem vessels (d-e). Other fluorescent microorganims were visualized inside xylem vessels of inoculated cuttings (arrows in f) as well as of non-treated plants (arrows in g). Similar endorhiza colonizations by strain PsJN were visualized at 1 until 4 weeks post inoculation Scale bars: (a and b) 30 µm, (c) 50 µm, (d) 30 µm, (e) 15 µm, and (f and g) 20 µm

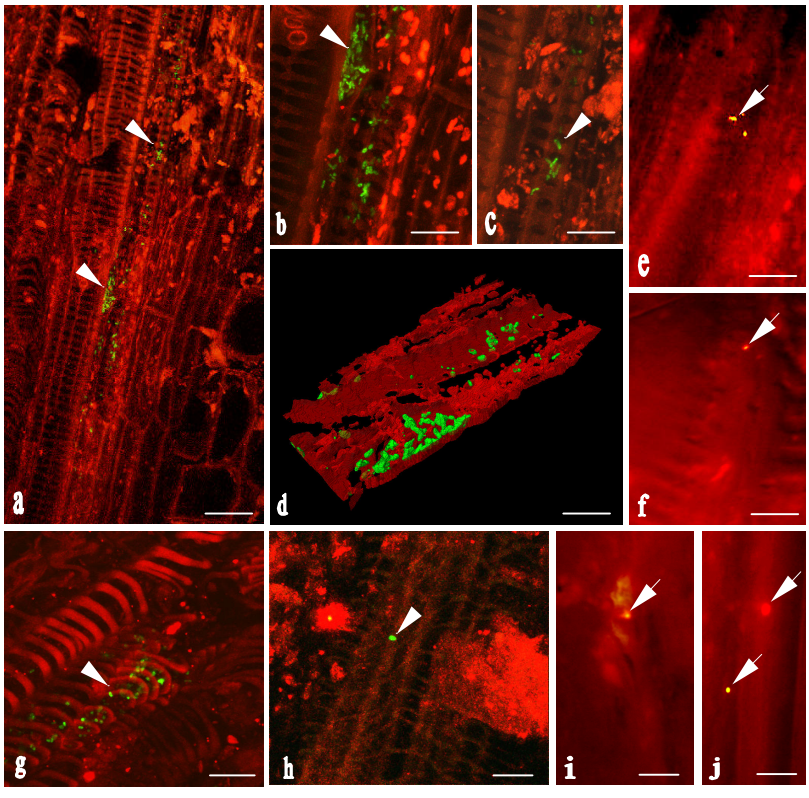

Figure 3: Images under epifluorescence and/or confocal microscope of grape inflorescence organs of fruiting cuttings inoculated with PsJN::*gfp*2x, 5 weeks after inoculation with 5.10^8 CFU g^{-1} soil. PsJN cells tagged with the *gfp* gene (arrow heads) were detected inside xylem vessels of grape stalk (a and c). (d) 3-D reconstruction of *gfp*-tagged cells inside a xylem vessel of grape stalk showing PsJN::*gfp*2x in the lumen of this vascular bundle. Inflorescence stalks can contain other fluorescent microorganisms (e) as in control plants (f). PsJN::*gfp*2x (arrow heads) was also detected inside xylem vessels of pedicel (g) and young berries (h) together with other fluorescent microorganisms (arrows in i) similar to control plants (arrows in j). Scale bars: (a) 30 µm, (b-e) 15 µm, (f) 10 µm, (g) 15 µm, (h) 5 µm, and (i and j) 20 µm.

Endophytic subpopulations of *B. phytofirmans* strain PsJN in grape inflorescence stalk

Following endorhizal colonization, PsJN::*gfp*2x was detected inside xylem vessels of grape inflorescence stalks through epifluorescence or confocal microscopy 5 and 6 weeks post inoculation with $5x10^8$ CFU g^{-1} of soil. However, in some fruiting cuttings, *gfp*-tagged cells of PsJN were not present in the grape inflorescence stalk although many longitudinal sections of stalk were examined. Inflorescence stalk colonization by strain PsJN was visualized in only 10-60% of bacterized fruiting cuttings. In these plants harbouring *gfp*-tagged cells, PsJN::*gfp*2x was visualized within xylem vessels, exclusively in the lumen of the vascular bundles (Fig. 3a-d). However, xylem vessels were not replete with PsJN::*gfp*2x; only single or few bacteria were detected inside the vascular bundles (Fig. 3a-d), mainly packaged within xylem elements (Fig. 3b and 3c). Again, a natural microflora was observed in the xylem vessels of stalks of bacterized plants (Fig. 3e) as well as control plants (Fig. 3f). This included orange (Fig. 3e and f) and also blue autofluorescent microorganisms (data not shown).

Endophytic colonization of grape berries

Strain PsJN has been detected as an endophyte within young berries following soil inoculation. This endophytic colonization of the fruit by *B. phytofirmans* strain PsJN followed the same pattern shown in grape stalks. Strain PsJN was thus detected inside grape berries in 10 to 60% of fruiting cuttings in each of the six independent repetitions of this study after inoculation with $5x10^8$ CFU g^{-1} of soil. As described for grape stalk colonization, *gfp*-tagged cells were found exclusively inside xylem vessels of pedicel and/or berries 5 and 6 weeks post inoculation. However, PsJN::*gfp*2x was not found inside all berries although four to five longitudinal sections were examined for pedicel and berries. Some plants that demonstrated PsJN colonization in grape stalks did not host the inoculant strain in pedicels or in young berries. In addition, some plants hosted *gfp*-tagged cells in pedicels or in young berries, despite their abscence in grape stalks. Even in infructescence, only some of the young berries and their pedicels were colonized by strain PsJN. Some PsJN::*gfp*2x bacteria were thus detected in the pedicel and were visualized as singlets, doublets or clusters of a few cells inside some xylem elements (Fig. 3g). In some young berries, *gfp*-tagged cells were also visualized within xylem vessels (peripheral or central vascular elements), either as singlets (Fig. 3h) or doublet s(data not shown). These *gfp*-tagged bacteria were visualized in the presence of a natural microflora, including mainly orange fluorescent microorganisms (Fig. 3i) as well as yellow and blue microorganisms (data not shown) that were also detected in control plants (Fig. 3j). This natural microflora was abundant inside young berries of some cuttings where *gfp*-tagged cells were not visualized. Strong defense reactions as well as necrotic tissues have been even associated with some of these microorganisms (data not shown).

Discussion

The present study clearly demonstrates the intimate association between *B. phytofirmans* strain PsJN and *Vitis vinifera* L. using fruiting cuttings, grown in non-sterile soil, with emphasis on colonization of inflorescence organs.

Following soil inoculation, grapevine fruiting cutting colonization by *B. phytofirmans* strain PsJN proceeded in distinct steps. Firstly, the root surface was rapidly colonized in a nonuniform manner. The highest concentrations occurred on the root hair zone compared to the emerging lateral roots and root tips. This colonization pattern was comparable to that one observed under a gnotobiotic system (Compant *et al.*, 2005b). However, *in vitro* plantlets do not develop root hairs when grown on agar (unpublished results). Similar rhizoplane colonization have been described for other plant-bacterial interactions (Hansen *et al.*, 1997; Gamalero *et al.*, 2004) where rhizosphere bacteria colonize exudate-rich zones such as root hairs, emerging lateral roots or root tips that provide them with nutrients (Walker *et al.*, 2003; Bais *et al.*, 2006). However, rhizoplane colonization by strain PsJN has been monitored in the present study with plants grown under non-sterile soil and thus in the presence of a natural microflora that may have competed for nutrients with the inoculated strain. Accordingly, this epiphytic population of strain PsJN declined 1 week after inoculation. However, strain PsJN survived in the presence of these other microorganisms and continued to colonize the root surface, demonstrating its rhizosphere competence.

Following root surface colonization, strain PsJN colonizes the endorhiza environment. To enter the root system, endophytic PGPR may secrete cell wall-degrading enzymes (James *et al.*, 2001; James *et al.*, 2002; Lodewyckx *et al.*, 2002; Compant *et al.*, 2005a; Rosenblueth and Martínez-Romero, 2006). This has been demonstrated for *B. phytofirmans* strain PsJN that secretes endoglucanase and endopolygalacturonase (Compant *et al.*, 2005b), as well as endo-β-D-cellobiosidase and exo-β-1,4-glucanase (unpublished results), which may falicitate its entrance to the endorhiza. Following its entry inside roots, strain PsJN progresses from the rhizodermis to the exodermis and then to the cortical cell layers intercellularly. Then, strain PsJN was detected passing the barrier of endodermis. Using a gnotobiotic biosystem we have previously reported the colonization of the peripheral cylinder by strain PsJN and that the endodermis barrier can be broken following the progression of strain PsJN in the endorhiza (Compant *et al.*, 2005b). However, in the present study, performed under non-sterile conditions, passage of strain PsJN through the endodermis could also be attributed to breaches opened by other microorganisms. Alternatively, the fact that the endodermis was broken can be also attributed to emergence of secondary roots (Hallmann, 2001). However, passage of strain PsJN through the endodermis was detected even without the presence of secondary root growth.

Following this progression, strain PsJN has been detected inside the central cylinder mainly inside xylem vessels and even in the presence of other microorganisms. This colonization of xylem vessels by strain PsJN was similar to our previous study conducted under gnotobiotic conditions (Compant *et al.,* 2005b), demonstrating the possibility to colonize these vascular bundles even under non sterile conditions.

Xylem vessels colonization at the root level by strain PsJN allowed it to spread inside plants. Following colonization of the endorhiza, strain PsJN was subsequently found in the vascular bundles of grape inflorescence stalks, pedicels and young berries. However, strain PsJN was not found in xylem elements of pre-flower buds and of flowers of the same plants. This can be attributed to the slow systemic spread of strain PsJN, requiring 5 weeks to reach the inflorescence following soil inoculation. Endophytic colonization of aerial plant parts by strain PsJN required more time compared to the *in vitro* system in our previous study, in which only 72h were required for the bacterium to spread systemically inside plants. This difference could be attributed to the difference of the physiology between the two systems. The xylem network of an *in vitro* plantlet is considerably smaller, and only a primarly xylem must occur in *in vitro* plantlets in comparison to cuttings as suggested by Thorne *et al.* (2006). This can explain the differences in colonization between the two models, together with the fact that cell-wall degrading enzymes activities of strain PsJN are probably required to allow passage from one vessel element to another and thus bacterial progression inside small plantlets and cuttings are different. However, Thorne *et al.* (2006) and Chatelet *et al.* (2006) have recently demonstrated that xylem structures and connectivity of grapevine provide passive mechanisms for a bacterial spread inside plants without the need for cell-wall degrading enzyme secretion. This also explains why the non-pathogenic endophyte *B. phytofirmans* strain PsJN could colonize aerial plant parts even under *in vivo* conditions without being harmful to its host. This does not explain, however, all the differences of colonization processes between the two models. In fruiting cuttings, some xylem elements may be occluded, restricting passage between xylem elements through pit membrane degradation, thereby lenghtening the time required for strain PsJN to reach the aerial plant parts. However, the present study demonstrated that some cuttings did not yield strain PsJN in the inflorescences. Thus problems with xylem connectivity can be envisaged. Some microorganisms from the natural microflora detected inside xylem vessels may be neutral, beneficial or even pathogenic strains that impact the physiology of xylem in cuttings. Alternatively, competition between strain PsJN and other microorganisms in the root, in the endorhiza or during xylem colonization, may have delayed systemic spread inside plants and contributed to differences between the two models.

In the present study, we demonstrated that the endophytic populations of strain PsJN were low (<2 $\log_{10}$ CFU g^{-1} FW) in inflorescence tissues after soil inoculation with 5.10^7 CFU g^{-1}, practically nonexistent from a biological viewpoint. Similar results were obtained after soil inoculation with 5.10^8 CFU g^{-1}. However, some cuttings contained more than 2 $\log_{10}$ CFU g^{-1} FW and berries of fruiting cuttings yielded up to 2.15 $\log_{10}$ CFU g^{-1} FW after soil inoculation with 5.10^8 CFU g^{-1}. This demonstrates that the berries of grapevine can be colonized by the bacterium after inoculation at a higher density. However, inconsistency of colonized grape inflorescences and the low density of endophytic populations support previous hypotheses about plant colonization by endophytic bacteria: endophytes are low or absent in generative organs like flowers and fruits (Hallmann, 2001). With *gfp* molecular markers, the colonization of inflorescence parts by endophytes can be monitored, even when a low endophytic colonization occurs. Strain PsJN tagged with the *gfp* gene was visualized inside inflorescences, in particular inside the lumen of xylem vessels of grape stalks, pedicels and young berries. This indicates bacterial progression from xylem vessels of the root system to the grape inflorescence stalks, pedicels and then to the berries. Consequently, stem stalks may serve only as a pathway for bacteria to reach the berries. However, this bacterial progression may be blocked sometimes inside plants. This may be related to the xylem structure of the plant or the natural microflora as discussed before. However, this microflora that may derived from the rhizosphere, is unlikely to have a strong impact on strain PsJN colonization, as in parallel to this study, the same average numbers of endophytic subpopulations were detected using sterilized soil and surfaces of fruiting cuttings (unpublished results). Plant defense responses can influence and regulate endophytic colonization (Iniguez *et al.,* 2005; Miché *et al.,* 2006) in addition to their potential to protect the plant against phytopathogen invasion and diseases (Maurhofer *et al.,* 1994; Wang *et al.,* 2005). Although this assumption has to be tested in grapevine-strain PsJN association, at both root and inflorescence levels, and against invasion of inflorescences by a pathogen (Compant *et al.,* in preparation), we demonstrated in the present study that strain PsJN can colonize roots epiphytically and thrive as an endophyte in the endorhiza, inside grape inflorescence stalks and subsquently inside young berries. Further works will be needed to determine if strain PsJN persists as an endophyte during berry ripening and at maturity.

Although endophytic subpopulations of strain PsJN are low in inflorescences of grapevine plants, they can be monitored by using *gfp* molecular marker. This technique and others have been used to monitor epi- and/or endophytic colonization of beneficial bacteria inside different plant hosts (Gamalero *et al.,* 2004; Chi *et al.,* 2005; Götz *et al.,* 2006). However, studies on colonization by rhizosphere-derived bacteria have mainly focussed on vegetative plant parts such as roots, stems and leaves. In this study, additional characteristics of plant colonization by PGPR have been elucidated through colonization of grapevine inflorescences by strain PsJN. Such knowledge will lead to a better understanding of plant-endophytic bacteria interactions, in particular in cultivated plants

with which some beneficial strains are currently or will be used for agricultural improvement.

Data from the present study relate to an old debate on plant colonization by microorganisms. It has been strongly argued by some plant anatomists that only pathogens can be transported *via* xylem vessels lumens and that endophytes colonize only nonfunctional vessels or move through the apoplaste to reach aerial plant parts (McCully, 2001). Although this has been argued for monocotyledons, it should also apply to dicotyledons. According to this hypothesis, the migration of strain PsJN from the endorhiza to inflorescence organs of grapevine would use nonfunctional vessels or strain PsJN should in fact be considered a phytopathogen. However, as strain PsJN can promote plant growth, protect its host again biotic and abiotic stress, it is a PGPR and unlikely to be a pathogen. Furthermore, xylem vessels are opened conduits that allow bacterial progression within plant (Thorne *et al.*, 2006; Chatelet *et al.*, 2006) and strain PsJN was detected in the lumen of xylem vessels. The present study is a substantial contribution to this debate with particular relevance to inflorescence colonization by an endophytic PGPR, on a woody plant for which the most valuable part used is the infructescence.

Acknowledgements

This work was supported by a Ph.D. grant from Europôl-Agro (Reims, France).
We are grateful to Dr. Richard E. Veilleux (Department of Horticulture, Virginia Polytechnic Institute and State University, Blacksburg) and Dr. Guy Abell (Department of Bioresources, Austrian Research Centers GmbH, Seibersdorf, Austria) for their critical review of the manuscript.

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Ces travaux ont permis de démontrer que *B. phytofirmans* souche PsJN peut former des populations épi- et endophytiques dans des boutures fructifères de vigne, en particulier au niveau de leurs inflorescences. En outre, cette étude a été réalisée en présence d'une microflore naturelle, ce qui montre les phénomènes de compétences rhizosphèrique et endophytique de *B. phytofirmans* souche PsJN. Pour cela, nous avons utilisé la souche transformée avec le gène *gfp* et caractérisée dans le chapitre 2 (Compant *et al.,* 2005b) afin de différencier la souche PsJN des autres micro-organismes naturellement présents au niveau des boutures fructifères.

Après inoculation du sol des boutures fructifères, il est apparu que *B. phytofirmans* souche PsJN colonise tout d'abord les surfaces racinaires, puis pénètre dans les racines. Cette souche bactérienne atteint ensuite les vaisseaux conducteurs du xylème et se propage jusqu'à la rafle et les jeunes baies. Ces résultats concordent avec nos travaux précédents sur la colonisation épi- et endophytique de la vigne par *B. phytofirmans* souche PsJN étudiée lors de conditions gnotobiotiques (Compant *et al.,* 2005b ; Chapitre 2). Néanmoins, la propagation bactérienne, du système racinaire jusqu'aux parties aériennes, a nécessité entre 5 et 6 semaines après inoculation du sol dans le cas des boutures fructifères, alors que 72 heures suffisaient avec les plantules *in vitro*. Ceci peut s'expliquer par les différences physiologiques entre les deux modèles utilisés ainsi que par la présence ou non de micro-organismes. En effet, les boutures fructifères sont beaucoup plus grandes que les plantules *in vitro* et présentent, ainsi, des physiologies différentes. De plus, nos expériences ont été réalisées en présence d'une microflore naturelle, bien qu'il soit possible que cette dernière ne présente pas d'impact sur la colonisation de la vigne par *B. phytofirmans* souche PsJN.

Lors de ces travaux, une colonisation des organes de l'inflorescence par la souche PsJN a été démontrée. Néanmoins, en utilisant tout d'abord des boutures présentant des bourgeons préfloraux, puis des fleurs et des baies, nous avons montré que la souche PsJN colonise les inflorescences seulement à partir du stade de jeunes baies (stade 71 selon l'échelle BBCH correspondant au stade de nouaison). Il est possible que la colonisation des inflorescences par la souche PsJN coïncide avec l'augmentation de la force puit des inflorescences lors de leur transformation en fruits (Lebon, 2005). En effet, cette force est plus importante à partir du stade 71 en comparaison aux stades de bourgeons préfloraux et de fleurs. Elle augmente ensuite progressivement lors du développement de la baie (Lebon, 2005). Il est de ce fait possible que la bactérie soit transportée jusqu'aux inflorescences par le

biais de ce phénomène. En parallèle et comme dans le cas de l'étude en conditions gnotobiotiques, la migration bactérienne dans les vaisseaux du xylème *via* l'utilisation de flagelles peut également être envisagée et devra faire l'objet d'une étude supplémentaire.

Lors de cette étude, nous avons montré que les populations endophytiques de la rafle et des jeunes baies sont peu nombreuses, c'est à dire inférieures à 2 log UFC/g MF après inoculation avec $5x10^7$ log UFC/g de sol. Une telle concentration est considérée comme inexistante d'un point de vue biologique puisque 2 log UFC/g MF est une limite reconnue dans le domaine de la microbiologie. D'autres analyses, après une inoculation avec $5x10^8$ UFC/g de sol, ont également permis d'observer une moyenne également inférieure à 2 log UFC/g MF. Néanmoins, des boutures ont présenté des populations maximales supérieures à 2 log UFC/g MF (2.12 $\log_{10}$ UFC.g^{-1} MF pour la rafle et 2.15 $\log_{10}$ UFC.g^{-1} MF pour les baies), ce qui démontre, quand même, la possibilité pour la souche PsJN d'établir des populations endophytiques significatives dans la rafle et dans les baies de la vigne.

Suite à ces travaux, nous nous sommes demandés si des mécanismes de défense étaient déclenchés dans la plante lors de la colonisation bactérienne. De plus, il semblait intéressant de voir si l'inoculation des boutures fructifères avec la souche PsJN pouvait entraîner une protection des inflorescences contre l'infection causée par *B. cinerea*. En effet, une ISR induite par la souche PsJN pouvait être envisagée au niveau des inflorescences. Ait Barka *et al.* (2000 ; 2002) avaient déjà montré une protection foliaire de la vigne contre *B. cinerea*, suite à l'inoculation de plantules *in vitro* avec la souche PsJN. Néanmoins, lors de cette étude, la souche PsJN était présente dans les feuilles et il était, par conséquent, possible que la protection contre *B. cinerea* résulte également d'une activité antagoniste de la souche PsJN envers le champignon pathogène.

L'ISR induite par les PGPR ne peut être étudiée que si il y a séparation dans l'espace entre les rhizobactéries et l'agent infectieux (van Loon *et al.*, 1998). Afin d'étudier une possible ISR envers *B. cinerea* au niveau des inflorescences, la bactérie PsJN ne doit pas être présente, par conséquent, dans ces parties de la plante. Il fallait, ainsi, déterminer une ISR avant la progression de la bactérie dans les inflorescences, c'est à dire avant le stade de jeunes baies (stade 71 selon l'échelle BBCH). De plus, il fallait débarrasser, le plus possible, les boutures fructifères de leur microflore naturelle afin d'éviter que l'ISR et ses mécanismes proviennent de l'interaction de la plante avec d'autres micro-organismes. Dans cette optique, nous avons stérilisé les surfaces externes des boutures fructifères et leur sol, puis inoculé la

bactérie PsJN. Ensuite, nous avons testé si l'inoculation des plants avec la souche PsJN induisait des mécanismes de défense et une protection de la vigne contre *B. cinerea*. Le laps de temps nécessaire avant que les PGPR initient une ISR et instaurent une protection de la plante contre les infections causées par les agents pathogènes est approximativement de 10 jours (van Loon et Bakker, 2005). De ce fait, nous nous sommes attachés à démontrer les mécanismes de défense mis en place suite à l'inoculation de plante avec la souche PsJN avant cette protection, c'est à dire au stade de bourgeons préfloraux. Ensuite, une possible résistance contre *B. cinerea* a été étudiée au niveau des fleurs, un lieu privilégié d'infection de ce champignon nécrotrophe (Pezet *et al.*, 2004).

Chapitre 4

Induced defense responses in *Vitis vinifera* L. by the endophyte *Burkholderia phytofirmans* strain PsJN and its related systemic resistance towards early flower infection by *Botrytis cinerea* Pers.

Stéphane Compant[1], Sandra Paquis[1], Sophie Bordiec[1], Fanjaniriana Rabenoelina[1], Angela Sessitsch[2], Jerzy Nowak[3], Fabienne Baillieul[1], Christophe Clément[1]and Essaïd Ait Barka[1]

Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cédex 2, France[1]*;*

Department of Bioresources, ARC Seibersdorf research GmbH, A-2444 Seibersdorf, Austria[2]*;*

Department of Horticulture, Virginia Polytechnic Institute and State University, 0327-301 Saunders Hall, Blacksburg, VA 24060, USA[3]

En préparation

Induced defense responses in *Vitis vinifera* L. by the endophyte *Burkholderia phytofirmans* strain PsJN and its related systemic resistance towards early flower infection by *Botrytis cinerea* Pers.

Stéphane Compant[1], Sandra Paquis[1], Sophie Bordiec[1], Fanjaniriana Rabenoelina[1], Angela Sessitsch[2], Jerzy Nowak[3], Fabienne Baillieul[1], Christophe Clément[1] and Essaïd Ait Barka[1] *

[1]*Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cédex 2, France;* [2]*Department of Bioresources, ARC Seibersdorf research GmbH, A-2444 Seibersdorf, Austria;* [3]*Department of Horticulture, Virginia Polytechnic Institute and State University, 0327-301 Saunders Hall, Blacksburg, VA 24060, USA*

Induced defense responses were evaluated during the interaction between *Vitis vinifera* L. and the endophyte *Burkholderia phytofirmans* strain PsJN. In addition, systemic resistance induced by strain PsJN was determined in grapevine flowers against early infection caused by *Botrytis cinerea* Pers. Fruiting cuttings of *V. vinifera* L. cv. 'Chardonnay' with emerging inflorescences were thus root inoculated with strain PsJN and early events, as well as phenolic compound accumulation, and PR encoding genes expression, were monitored in roots and/or preflowers before to check for an enhanced flower defense capacity towards *B. cinerea* infection. Results of this study demonstrated that following its epiphytic and/or colonization, strain PsJN induces visual local defense reactions such as phenolic deposition as well as oxydatif and nitric bursts at the root level. Some defense-related genes expressions such as for *VvGluC, VvChi4C, VvPR6, VvPR10.1, VvPR27* (encoding respectively PR-2, PR-3, PR-6, PR-10, and PR-17 proteins) and *Vv102* (a putative PR-encoding gene) were also detected, but with differences, in roots and preflowers. As some of these PR-encoding genes have known signaling, PR-encoding genes analyses suggest an SA dependent pathway in roots but a JA signaling in preflowers. Following these induced defense responses, the beneficial association between grapevine and strain PsJN leads to flowers protection against early infection caused by *B. cinerea.* As some typical SAR and ISR mechanisms are involved during grapevine-strain PsJN interaction and its associated systemic resistance, SAR and ISR mechanisms triggered by strain PsJN are discussed.

In both managed and natural ecosystems, plants can form intimate association with beneficial microorganisms. This can lead to profound effects on host growth, development, yield as well as soil quality (Raaijmakers *et al.*, 2002; van Loon and Bakker, 2004; Welbaum *et al.*, 2004; Compant *et al.*, 2005a; Haas and Défago, 2005). The associative bacteria designated as PGPR (Plant Growth-Promoting Rhizobacteria; Kloepper and Schroth, 1978) or PGPB (Plant Growth-Promoting Rhizobacteria; Bashan and Holguin, 1998) can also induced a systemic resistance (ISR) protecting their plant host to various environmental stress conditions (Nowak and Shulaev, 2003; van Loon and Bakker, 2005). This induced state of resistance is generally described as jasmonate (JA) and ethylene (ET) dependent and PR-encoding gene expression seems to not occur during this phenomenon of resistance (van Wees *et al.*, 1997; Pieterse *et al.*, 1996; 1998; van Loon and Bakker, 2005; Verhagen *et al.*, 2006). This differes from the well known systemic acquired resistance (SAR), induced by phytopathogens, which is associated with PR-encoding genes expression and a systemic signalling *via* the salicylate (SA) pathway (Metraux *et al.*, 2002; Ton *et al.*, 2006). Although these two types of resistances have been declared to be synonymous by Hammerschmidt *et al.* (2001), and more recently by Tuzun (2006), they are however phenotypically different and ISR mechanisms induced by PGPR seem to be more complex than those involved during SAR. Moreover, in some plant-PGPR associations, the bacterial partner induces typical SAR responses whereas others do not (Maurhofer *et al.*, 1994; van Loon, 2007). Recent studies have thus reported that some PGPR (or their microbial-associated molecular patterns; MAMPs) induce early signalling events such as production of H_2O_2 and nitric oxide (NO) as well as SA dependent PR gene expression and the induction of other defense responses (reviewed by van Loon, 2007). As some differences can occur during various plant-PGPR interactions, it thus seems important to study these plant responses to other PGPR and with other plant hosts. One of these is correlated to *Vitis vinifera* L.-*Burkholderia phytofirmans* strain PsJN interaction (Compant *et al.*, 2005b). *B. phytofirmans* strain PsJN (Sessitsch *et al.*,

* Corresponding author : Essaïd Ait Barka; E-mail : ea.barka@univ-reims.fr Phone/Fax : +33-3-26-91-34-41.

2005) can efficiently colonize various plants and can induce beneficial effects on plant growth and development as well as trigger resistance to abiotic and biotic stresses (Frommel *et al.*, 1991; Ait Barka *et al.*, 2000; 2002; Nowak and Shulaev, 2003). In an *in vitro* system this bacterium can also induce cell-wall reinforcement during internal colonization *via* accumulation of polyphenolic compounds (Compant *et al.*, 2005b; 2007), although this needs to be demonstrated using an *in vivo* system. Under gnotobiotic conditions, inoculation of grapevine with strain PsJN also enhanced plantlet tolerance to *Botrytis cinerea*, the causing agent of gray mould disease (Ait Barka *et al.*, 2002). The mechanism of this resistance however has not been yet investigated and it is actually unclear if this is associated to ISR or SAR mechanisms. However, a space between the bio-control bacterium and the phytopathogen is required to monitor and study these phenomenons in a fundamental view point (van Loon *et al.*, 1998). Mechanisms of plantlets resistance can not be determined as strain PsJN can thrive as endophyte inside plants and spread to aerial plant parts in few days post rhizosphere inoculation under gnotobiotic conditions (Compant *et al.*, 2005b). However, by using an other model involving fruitng cuttings, systemic colonization inside plants has been demonstrated on berries but not on flowers nor on preflowers (flower buds) in presence of a natural microflora (Compant *et al.*, submitted) or not (unpublished). Nowadays, it is well known that grapevine inflorescences are prone to severe infections and *B. cinerea* infects primarly flowers before enter a latency phase and then expresses disease symptoms in berries (Keller *et al.*, 2003; Viret *et al.*, 2004; Pezet *et al.*, 2004). Thus, flower infection can enabling us to study ISR or SAR mechanisms triggered by strain PsJN before its systemic spreading inside plants.

In this study we investigated grapevine plant defense reactions following root inoculation with strain PsJN at both local and systemic level as well as the resistance of flowers to early infection by *B. cinerea* before that the bacterium systemically spreads inside plants. Early events leading to the induction of the systemic resistance, such as nitric oxide and hydrogen peroxide (H_2O_2) formation, accumulation of phenolics were thus monitored in roots followed by the analysis of the local (in roots) and systemic (in flower buds) expression of some PR-encoding genes. As some of these genes expressions are dependant of SA or JA signaling pathway, we further discuss the signaling pathway triggered by strain PsJN and SAR and ISR mechanisms. Finally, we demonstrated that the beneficial association between grapevine and strain PsJN leads to flower protection against early infection by *B. cinerea* that could be correlated to the previous plant defense reactions investigated.

RESULTS

Accumulation of autofluorescent phenolics in roots.

Autofluorescent phenolics have been detected on the root surface of both control and bacterized plants at 12 and 96 h after inoculation with no significant differences between the two treatments (Figure 1a-d). A noticeable difference occurred, however, in root internal tissues 96 h post inoculation. Bacterized plants accumulated thus more phenolic compounds in exodermis and in the first layers of the cortex than non-bacterized controls (Figure 1e-f).

Hydrogen peroxide production in roots.

H_2O_2 production progressively increased from the basal parts to the root tips independently of the time after inoculation, as demonstrated by a red/pink colour in both control and bacterized plants by using the DAB method (Figure 2). No difference was found on the root surfaces between the bacterized and control treatments 12 h post inoculation (Figures 2a and 2b). However, a slight difference occurred between the two treatments at 96 h

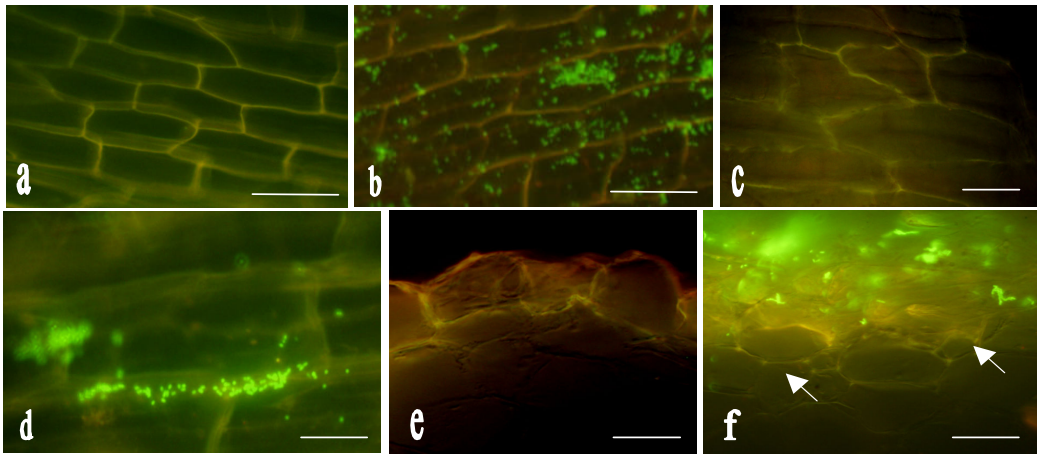

Fig. 1: Microphotographs of autofluorescent phenolic compounds on the root surfaces (a-e) and inside root internal tissues (e and f) after 12 h (a and b), and 96 h (c-f) post inoculation with control (a,c and e) or PsJN::*gfp*2x (b, d and f) showing no differences on the root surfaces but a more pronunced accumulation of autofluorescent phenols in exodermis and in the first cell layers of bacterized roots (arrows). PsJN::*gfp*2x was used to visualize bacterial cells on and inside the root system. Similar results were obtained with root inoculations with PsJN wild-type strain (data not shown). Scale bars : (a and b) 50 µm, (c) 20 µm, (d) 15µm, and (e and f) 50 µm.

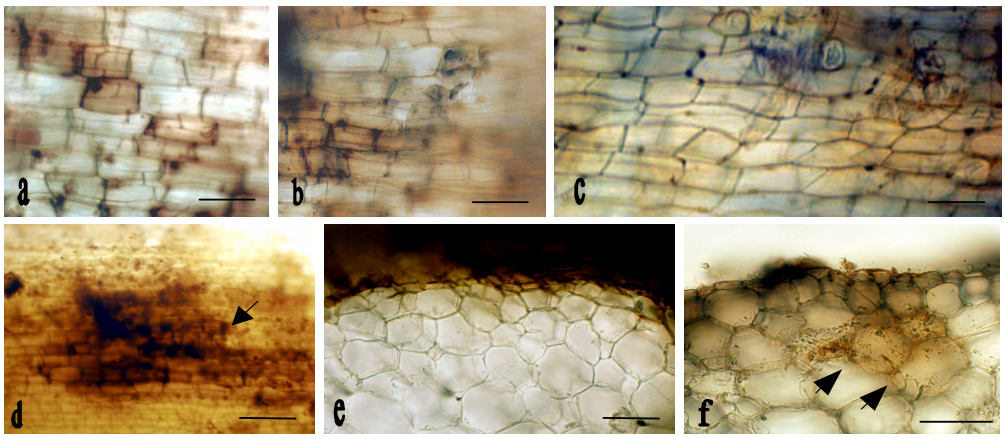

Fig. 2: Microphotographs of H_2O_2 accumulation, using DAB staining method, on the root surfaces (a-d) and inside root internal tissues (e and f) after 12 h (a and b), and 96 h (c-f) post inoculation with control (a, c and e) or PsJN wild type strain (b, d, and f) showing no difference in H_2O_2 accumulation at 12 h post inoculation but a slight more accumulation of H_2O_2, 96 h post inoculation in bacterized roots, as detected on the root surfaces (arrows) as well as inside root internal tissues (arrow heads). Scale bars : (a) 50 µm, (b and c) 100 µm, (d) 200µm, (e and f) 100 µm.

after inoculation. H_2O_2 accumulation was thus found more pronounced on the root surfaces (Figure 2c and 2d) and inside root internal tissues colonized by strain PsJN (Figure 2e and 2f).

Nitric oxide production in roots.

Similarly to H_2O_2, nitric oxide accumulated in roots of control plants as demonstrated using DAF-2DA fluorescent probe (Figure 3). NO production was thus detected on the root surfaces of control plants as visualized on the cell-wall of rhizodermal cells and increased from the proximal parts of the roots to the tips. On the root surfaces of bacterized plants, NO accumulation was comparable to that found in the control treatment 12 h post inoculation (Figures 3a and b). However, the two treatments showed slight differences at 96 h post inoculation. DAF-2DA/NO associated fluorescence was thus visualized at the subcellular level of roots colonized by strain PsJN in comparison to the control treatment in which no such accumulation was observed (Figures 3c-f).

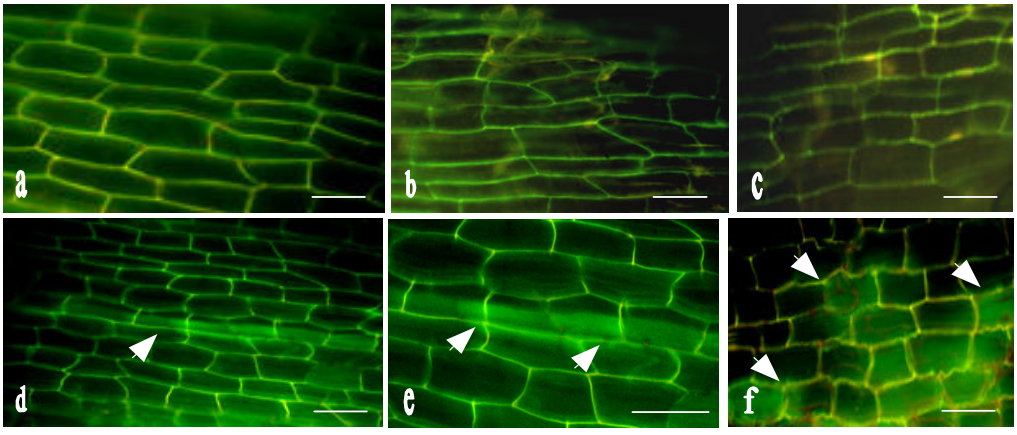

Fig. 3: Microphotographs of nitric oxide accumulation on the root surfaces after 12 h (a and b), and 96 h (c-f) post inoculation with control (a and c) or PsJN wild type strain (b, d, e and f) showing no difference at 12 h post inoculation but a slight more accumulation (arrow heads) of NO at subcellular level of some rhizodermal cells in bacterized roots 96 h post inoculation (d-f) using DAF-2DA fluorescent probe. Scale bars : (a-c) 50 µm, (c) 75 µm, (eand f) 50 µm.

Table 1: Sequences of defense gene primers used for real time quantitative polymerase chain reaction[a].

Names	Forward primers	Reverse primers
VvChi4C	5'-TCGAATGCGATGGTGGAAA-3'	5'-TCCCCTGTCGAAACACCAAG-3'
VvGluC	5'- TCAATGGCTGCAATGGTGC-3'	5'-CGGTCGATGTTGCGAGATTTA-3'
VvPR6	5'-AFTTCAGGGAGAGGTTGCTC-3'	5'-CGTCGACCCAAACACGGACCCTAGTGC-3'
VvPR10.1	5'CGTTAAGGGCGGCAAAGAG-3'	5'GCATCAGGGTGTGCCAAGA-3'
VvPR27	5'-CAGACTTCTTCGTGGAGCTTCTG-3'	5'-TGGACTGCTAGTTAATTTCCATATTT-3'
Vv102	5'-CATGGATTGGAGCCTTGATCA-3'	5'-TGGCCTTGACTTGCATATGC-3'
VvEF1α	5'-GAACTGGGTGCTTGATAGGC-3'	5'-AACCAAAATATCCGGAGTAAAAGA-3'

VvChi4C, *VvGluC* and *VvPR6* also named *CHI4C*, *GLUI* and *PIN* (Aziz *et al.*, 2003), *VvPR10.1* (Robert *et al.*, 2001). Others sequences from F. Baillieul, personal communication.

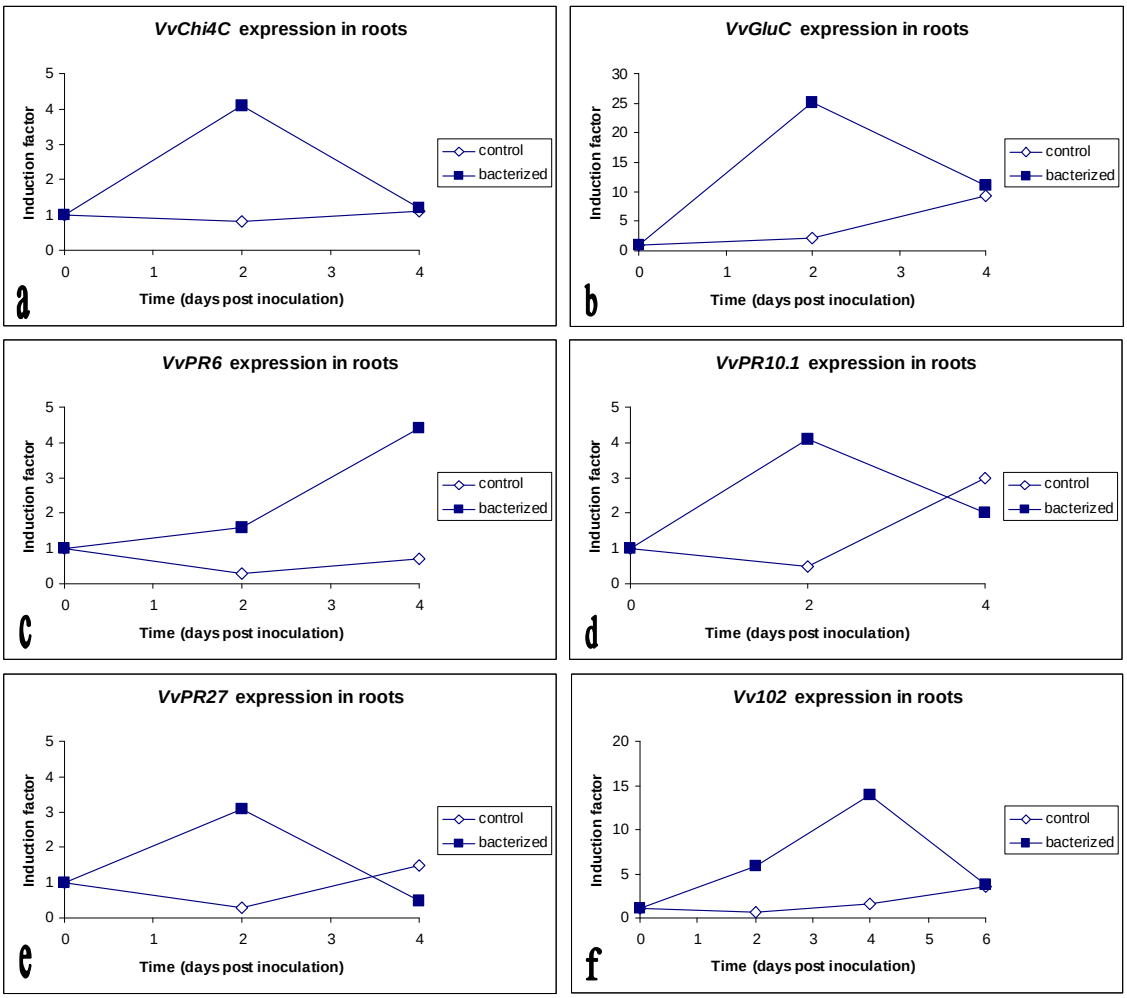

Fig. 4: Kinetics of the expression of some PR-encoding genes in grapevine roots following root colonization by *B. phytofirmans* strain PsJN. (a) *VvChi4C*, (b) *VvGluC*, (c) *VvPR6*, (d) *VvPR10.1*, (e) *VvPR27* and (f) *Vv102*.

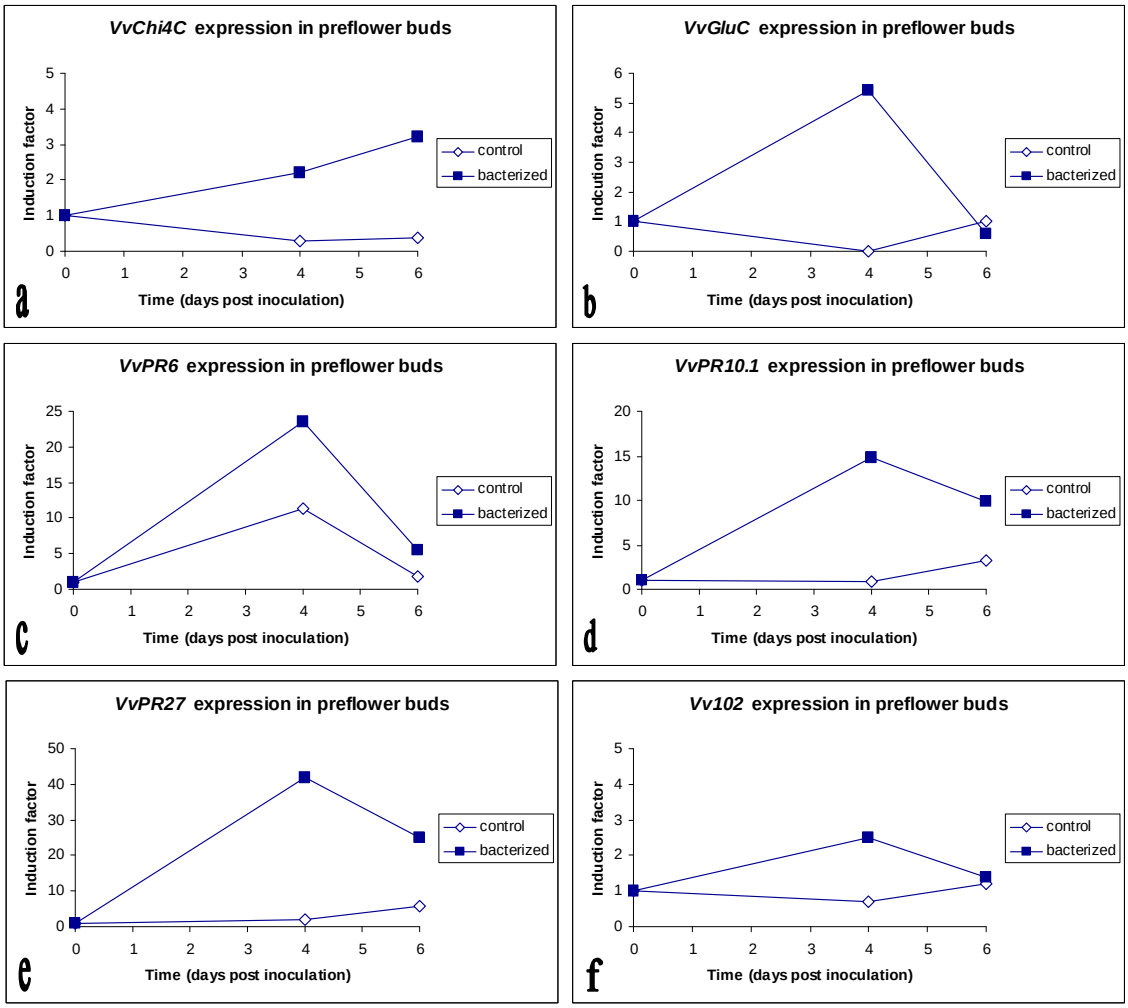

Fig. 5: Kinetics of the expression of some PR-encoding genes in grapevine preflower buds following root colonization by *B. phytofirmans* strain PsJN. (a) *VvChi4C*, (b) *VvGluC*, (c) *VvPR6*, (d) *VvPR10.1*, (e) *VvPR27*, and (f) *Vv102*.

Defense-related gene expression in roots.
The expression of the all tested PR encoding genes have been induced locally in roots following soil inoculation with PsJN (Figure 4). *VvChi4C*, *VvGluC*, *VvPR6*, *VvPR10.1* and *VvPR27*, encoding PR-2, PR-3, PR-6, PR-10, and PR-17, respectively, showed 4, 25, 4, 4, and 3 fold higher expression in roots of bacterized plants at two days after inoculation compared to control plants (Figures 4a-e). Their level of expression then decreased to reach the level of non-inoculated plants at four days after inoculation (Figures 4a-e). Similarly, *Vv102* which encodes a putative PR protein with unknown function was expressed 15 fold higher in roots of bacterized plants than in controls at two days after inoculation (Figure 4f). *Vv102* expression then decreased in bacterized roots until to reach the level of control plants (Figure 4f).

Defense-related gene expression in flower buds.
Defense-related gene expression was also detected in inflorescences (*i.e.* flower buds) following soil inoculation with strain PsJN (Figure 5). *VvChi4C* expression peaked at six days post inoculation with a 3-4 fold higher expression in the bacterized treatment than in control plants(Figure 5a). Similarly, the expression of *VvGluC*, *VvPR6*, *VvPR10.1*, *VvPR27*, *and Vv102* was 6, 25, 15, 40 and 3 times, respectively, higher in flower buds of bacterized plants, four days post inoculation (Figure 5b-f). The expression of all the tested genes then decreased to the level of non-treated plants (Figure 5a-f).

Induced flower protection towards early *Botrytis cinerea* infection and colonization in bacterized fruiting cuttings.
Grapevine inflorescences at the stage of flower buds were not protected against *B. cinerea* infection by the PsJN inoculation of soil (data not shown). However, at the flowering stage, when 70% of flowers were opened, protection against *B. cinerea* infection was observed in bacterized plants in comparison to control plants following soil inoculation. Three days post-inoculation with a conidial suspension of a twenty days old *B. cinerea* culture, applied ten days post soil inoculation with strain PsJN, no grey mould disease symptoms appeared on

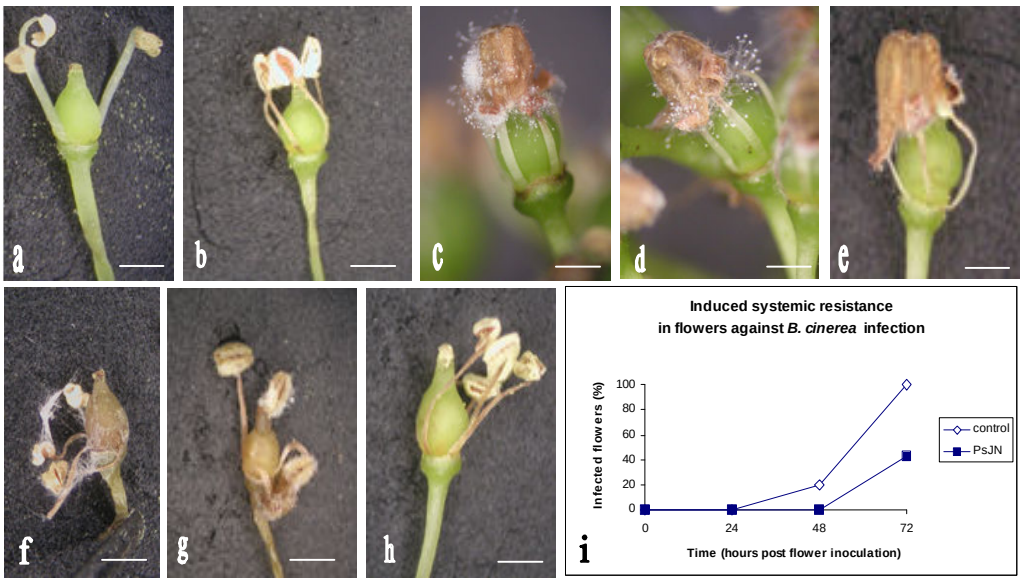

Fig. 6: Systemic resistance induced in grapevine flowers by strain PsJN against *Botrytis cinerea* infection. (a-h) Photographs of flower infection by *Botrytis cinerea*. Two methods have been used: inoculation were realized with (a and b) control treatment, (c-e) conidia from twenty or (f-h) ten days old-growing fungus on flowers from (a, c and f) control or (b, d, e, g and h) bacterized plants with strain PsJN showing that some flowers of bacterized plants (d and g) were infected whereas some others (e and h) were not colonized by the pathogenic fungus. (i) Percentages of protection against *B. cinerea* using conidia from ten days old growing fungus. Similar percentages of protection were obtained using the two differents methods. Scale bars: (a and b) 1 mm, (c-e) 1.5 mm, and (f-h) 1 mm.

flowers. Nevertheless, a white mycelium covered the calyptra of all controls and of some bacterized plants (Figure 6) indicating a pathogen's latency phase (Figures 6a-d). Sixty percent of flowers on the bacterized plants did not develop, however, this white mycelium (Figures 6e), demonstrating a – at least partly - protection of flowers of bacterized plants against *B. cinerea* infection. When a fresh conidial inoculum from 10 days old *B. cinerea* was applied, some differences occurred. Flowers were necrotic and aborted in comparison to non-inoculated flowers (Figures 6f and 6g). Similar symptoms take place on flowers from both control and bacterized plants (Figures 6h). However, only 40% of flowers were infected in bacterized plants in comparison to control plants, where all flowers were infected by the pathogen (Figure 6i).

Similar results of infection and protection were obtained with conidial suspension aged from ten or twenty days old conidia taken from BC3-GFP isolate transformed with the *gfp* gene (data not shown). The *gfp*-transformed *B. cinerea* has allowed us to visualize fungal infection and to monitor its colonization pattern of the grapevine flowers (Figure 7). No B. *cinerea* was detected on non-inoculated flowers from control and bacterized plants (Figures 7a and 7b). By using conidia from twenty day old growing fungus, *B. cinerea* tagged with the *gfp* gene (Figures 7c and 7d) was not also not detected microscopically in flowers of bacterized plants but was found on the calyptra of others, similarly than in the case of flowers on non-bacterized controls (Figures 7e and 7f). Using conidia from ten days old growing fungus, *B. cinerea* was also not detected in some flowers of bacterized plants but found as infecting others similarly than in control plants (Figure 7g-t).

B. cinerea was visible on the calyptra (Figures 7g and 7h), pistil (mainly on the ovary and style but never on the stigma; Figures 7i and 7j), stamen filaments (Figures 7k and 7l), anthers (Figures 7m and 7n), inside a very few (1 - 5 %) pollen grains (Figures 7o and 7p), on sepal and receptacle area (Figures 7q and 7r), and on pedicell (Figures 7s and 7t) of both control and bacterized plants. However, it should be taken into consideration that all these flower parts were not colonized by *B. cinerea* at the same intensity and the phytopathogen was thus detected colonizing more the calyptra, the ovary, the receptacle area as well as the pedicell than others flower parts in both control and bacterized plants. In some case however, no fungus was detected in some flowers of bacterized plants (Figure 7u).

DISCUSSION

The present study clearly demonstrates that *Burkholderia phytofirmans* strain PsJN induced grapevine plant defense responses in roots and flower buds and then enhanced resistance of flowers to *B. cinerea* infection.

Grapevine defense responses triggered by strain PsJN involved firstly early events such as H_2O_2 and NO accumulations. These substances were detected both on the root surfaces of control and bacterized plants independently of time after inoculation. This is in accordance with previous studies showing that H_2O_2 and NO are usually produced during root elongation and plant development (Torres and Dangl, 2005; Stöhr and Stremlau, 2006). At an early stage, bacterized and control plants did not behave differently in terms of H_2O_2 and NO production, but differences were detected 96 h post inoculation.

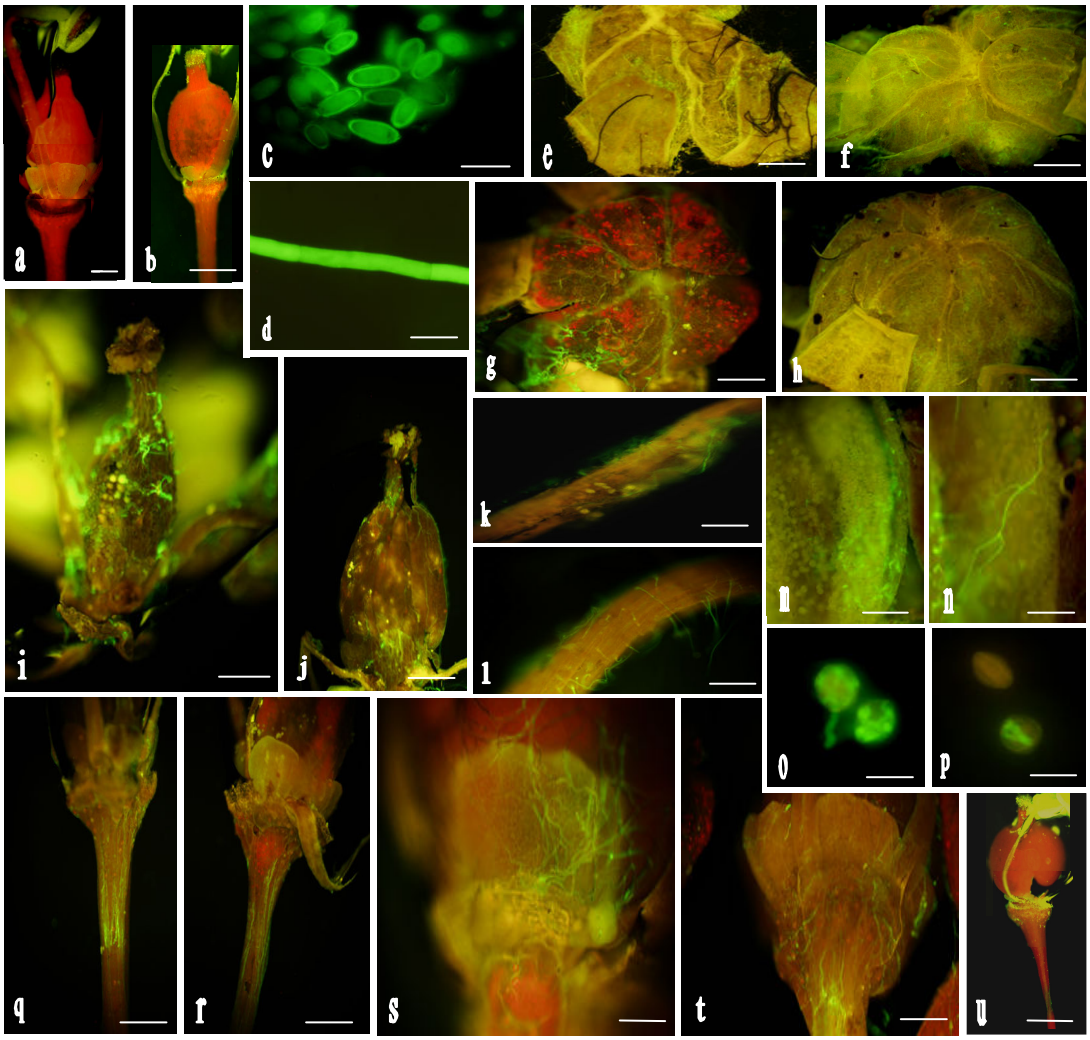

Fig. 7: Epifluorescence microphotographs of *B. cinerea* colonization in infected flowers using the *gfp* derivative BC3-GFP. (a and b) non infected flowers from (a) control plants or (b) bacterized plants. (c and) conidia and mycelium of BC3-GFP. (e and f) *B. cinerea* infection on the calyptra from (e) control plants or (f) bacterized plants after inoculation of twenty days old growing fungus. (g-t) *B. cinerea* infection on flowers was detected on both control and bacterized plants after inoculation with ten days old growing fungus. *B. cinerea* was thus visualized on (g and h) the calyptra, (i and j) on the pistil (mainly on the ovary and style but neither on the stigma;), (k and l) on stamen filaments, (m and n) anthers, (o and p) inside pollen, (q and r) on pedicell and (s and t) on sepal and receptacle area. (u) In some flowers of bacterized plants no *B. cinerea* was detected after inoculation with conidia from ten days old growing fungus. Scale bars: (a and b) 1.5 mm, (c) 10 µm, (d) 30 µm, (e and f) 100 µm, (g and h) 75 µm, (i and j) 1.5 mm, (k and l) 100 µm, (m and n) 50 µm, (o and p) 10 µm, (q and r) 1 mm, (s and t) 0.5 mm, and (u) 1.5 mm.

Interestingly, more pronounced H_2O_2 and NO production were visualized on and/or inside root tissues of bacterized roots in comparison to control plants, demonstrating that these early events are triggered in grapevine plants by strain PsJN. It might be that rhizoplane is required for inducing H_2O_2 and NO production. However, it has been reported that these so-called early events are triggered in a few hours post inoculation (Garcia-Brugger *et al.*, 2006; Mur *et al.*, 2006). As we detected them on and/or inside root internal tissues 96 h post inoculation and as strain PsJN can enter inside root internal tissues within the first 2-3 days post inoculation (data not shown), the hypothesis that H_2O_2 and NO production could be correlated to the bacterial penetration inside root internal tissues can not be excluded. According to this, strain PsJN secretes various cell-wall degrading enzymes that allow it to enter inside roots (Compant *et al.*, 2005b). These enzymes can be potential MAMPs that induce plant defense responses including H_2O_2 and NO accumulations (Garcia-Brugger *et al.*, 2006). As H_2O_2 and NO are typically produced when a pathogen infects and invades plant tissues or by their MAMPs (Apel and Kirt, 2004; Wendehenne *et al.*, 2004; Garcia-Brugger *et al.*, 2006; Mur *et al.*, 2006; Vandelle *et al.*, 2006), strain

PsJN seems to be recognized first as an invader by grapevine and then, as a beneficial partner.

During grapevine-strain PsJN interaction, autofluorescent phenolic compounds are produced. However, strain PsJN did not induce polyphenol accumulation on the root surfaces but these defense compounds were detected inside grapevine roots, mainly in the exodermis and in the first cell layers of cortical cells. This suggests that the production of phenolic compounds is linked to the endophytic colonization of strain PsJN and is in accordance to our previous study under gnotobiotic conditions (Compant *et al.,* 2005b). The accumulation of autofluorescent phenolic compounds has been often linked to hypersensitive responses (HR; Heath, 2000). However, PGPR generally do not induce HR as they do not trigger strong plant defense reactions (van Loon and Bakker, 2005). Rather, only a slight polyphenolic compound accumulation is induced during plant-PGPR interaction. This have been often described during various plant-endophytic PGPR interactions (Hallmann, 2001; Rosenblueth and Martinez-Romero, 2006) and this is not surprising for endophytes as endophytic PGPR create little stress when they enter inside plant tissues, without being harmful for their host (Hallmann, 2001; Rosenblueth and Martinez-Romero, 2006).

In the present study, grapevine inoculation with strain PsJN leads to PR-encoding gene expression in roots and preflowers of grapevine plants. The PR-encoding genes tested, *VvGluc*, *VvChi4C*, *VvPR6*, *VvPR10.1*, *VvPR27* and *Vv102*, were thus induced locally and systemically after soil inoculation of grapevine plants with strain PsJN. It has been postulated that PGPR do not induce PR gene expression (Pieterse *et al.*, 1996; van Wees *et al*., 1997). However, various publications have demonstrated that certains PGPR induce the expression of PR-encoding genes during their interaction with a plant host, locally and/or sytemically. For examples, *PR1*, *PR2* and *PR3* were induced in roots and leaves during *P. fluorescens* CHAO-tobacco interaction (Maurhofer *et al*., 1994). Similarly, in *A. thaliana, PR5* was induced in the root xylem in response to *P. fluorescens* WCS417r (Leon-Kloosterzield *et al.,* 2005). Furthermore, Timmusk and Wagner (1999) showed PR1 accumulation in roots after *Paenibacillus polymyxa* inoculation, and *PR2*, *PR3*, *PR4*, *PR5* and/or *PR12* were induced in response to *P. fluorescens* PFT9601-T5 (Wang *et al.,* 2005), *P. thivarlensis* MLG45 (Cartieaux *et al.,* 2003), or *Serratia marcescens* 90-166 (Ryu *et al.,* 2004). In some of these studies, high levels of PGPR were applied, however, that can be toxic for the plant host and thus induce PR-encoding genes (described by van Loon and Bakker, 2005). Nevertheless, for others, even at low density levels of PGPR, some PR-encoding genes are induced in the plant hosts although their expression are very low. In our study, the induction levels of defense-related genes tested were also low but significant, demonstrating that grapevine-strain PsJN interaction leads to PR-encoding gene expression.

With regard to their level of induction, some differences of the PR-encoding genes tested occur in roots and preflowers of grapevine plants interacting with strain PsJN. *VvChi4C* is the only gene that does not present differences. However, *VvGluC* and *Vv102* are more induced at the root level than in preflowers. Interestingly, in case of *VvPR6*, *VvPR10.1* and *VvPR27*, this seems to totally change. Thus, these genes have been detected as more induced in preflowers than in roots. This suggests that a difference of signaling occurs locally and systemically in fruiting cutting plants inoculated with strain PsJN. In *A. thaliana*, signaling of PR-encoding genes is well known and *PR1*, *PR2*, *PR3* and *PR5* acids depend of the SA pathway that caracterized them furthermore as SAR markers (van Loon *et al.,* 2006). However, for other plants, this is not so clear. In grapevine, PR-1a depends of the SA pathway, whereas PR-4 depends on JA (Hamiduzzaman *et al.,* 2005). PR-1a and PR-4 encoding genes were not studied in the present paper but others, which also represent different signaling pathways. The signaling pathways of *VvChi4C*, *VvGluc*, *VvPR10.1 and VvPR27* is correlated with SA and JA. However, *Vv102* expression depends only of SA signaling, whereas *VvPR6*, depend only of JA (unpublished results). This allow to indicate that SA-dependent PR-encoding genes were more induced at the root level, whereas JA-dependent genes were more induced in preflowers during grapevine-strain PsJN interaction.

It is well known that some PGPR strains can produce SA under iron limitation that is then incorporated into the iron-chelating compound siderophore (Bakker *et al.,* 2007). Strain PsJN is a siderophore producer (Sessitsch *et al.,* unpublished results) and thus signaling through SA could derive from the bacterium. However, bacterial SA production is expected mostly under Fe limiting conditions, that did not encounter in the present study and rather SA must derive from the plant response.

It have been reported that PGPR trigger JA dependent responses systemically (van Loon, 2007) and JA (together with ET) as well as non induction of PR-encoding gene have thus been described to be characteristic for ISR induced by PGPR (van Loon *et al*., 1998). SAR associated with a systemic SA signaling and PR-encoding gene expression (Nawrath *et al*., 2006; Ton *et al*., 2006) has been associated mostly with pathogen attack. If we place our results in the context of these definitions, the interaction between grapevine and strain PsJN seems to overlap both common mechanisms of ISR and SAR as strain PsJN induces lower PR-encoding genes expression and signalling *via* JA seems to be involved systemically. This strongly resembles to the interaction between *A. thaliana* and the endophyte *P. fluorescens* PFT9601-T5 as recently described by Wang *et al.* (2005), for which low PR-encoding gene expression and JA/ET signaling were involved systemically. However, this has been determined on leaves but not on flower buds. With the present study, the mechanisms seems to be the same for a flowering plant with another endophytic PGPR.

In the present study, strain PsJN induces flowers protection against *B. cinerea* infection as a follow of plant defense establishment at the preflowering stage. This has been proved by using two different procedures, *i.e.* young or old conidia and two different *B. cinerea* isolates. By using old conidia (twenty days old growing fungus), *B. cinerea* develops mainly on the calyptra and strain PsJN seems to protect calyptra with a 60% rate of protection before that the fungus enter in a latency phase. Interestingly, when young conidia were used, some flowers were aborted. This has been previously reported by Keller *et al.* (2003) using fruiting cuttings and could be related to the fact that senescing flowers can be sources for *B. cinerea* infection which uses them in order to colonize berries at maturity (Pezet *et al.,* 2004). However, if studies reported

that *B. cinerea* can grow on senescing flowers, they do not report a flower abortion due to the fungus, excepted during lab experiments using Chardonnay cultivar, very sensitive to *B. cinerea* infection (Keller *et al.*, 2003). It is thus possible that this is far in natural conditions. However, this allow us to visualize again systemic resistance induced by strain PsJN in a fundamental viewpoint. It appears, therefore, that the bacterium protects grapevine flowers toward fungal infection with a 60% rate of protection. This induced state of resistance of flowers from bacterized plants toward *B. cinerea* infection can be correlated to the PR-encoding gene expressions together with others mechanisms demonstrated in the present study. However, it have been postulated that PR-encoding gene expressions and others defense-related compounds that can take place sometimes during plant-PGPR interactions are not required for ISR. Rather, plant defense responses are primed and respond better after pathogen challenge (van Loon and Bakker, 2005). Although this needs to be determined in the system grapevine-strain PsJN-*B. cinerea*, we have demonstrated in the present study that grapevine-strain PsJN interaction seems to overlap both common mechanisms of ISR and SAR, as well as induces a protection of flowers against *B. cinerea* infection. This greatly contributes to our understanding of the grapevine–strain PsJN interaction, and also more largely on SAR or ISR mechanisms induced by PGPR.

MATERIAL AND METHODS

Bacterial strains, growth conditions and inoculum preparation.

Strain PsJN and the *gfp*-marked strain PsJN::*gfp*2x (Compant *et al.*, 2005b) were grown in 100 ml Luria-Bertani liquid medium in 250 ml Erlenmeyer flask and incubated at 25°C, on a shaker (150 rpm) for 48 h as described by Compant *et al*. (2005b). Bacteria were collected by centrifugation (4500 *g*; 10 min) and washed twice with sterile phosphate buffer, pH 6.5 (PBS). The bacterial concentration of the inoculum was then adjusted with PBS, based on optical density (600 nm) confirmed with plate counting (Pillay and Nowak, 1997).

Plant material and growth conditions.

Three-node cuttings (20 cm long) of *Vitis vinifera* L. cv. Chardonnay were cane pruned from 6-year-old plants from the 'Moët et Chandon' vineyard (Epernay, France). Fruiting cuttings were then prepared according to Lebon *et al*. (2005) with some modifications. Briefly, cuttings were treated with 0.05 % Cryptonol®, and the distal node was covered with grafting wax (Agrochemie®, Germany) containing a fungicide (0.1% Oxiquinoleine) and a plant growth regulator (0.00175% 2,5-Dichlorobenzoïc acid). Cuttings were then stored in the dark at 4°C for at least 2 weeks. After 15 h of hydratation at 28°C, fruiting cuttings were surface sterilized during 30 min in an ethanol 70% bath, then they were placed in a 2.5% bleach solution for 10 min to decontaminate cuttings. They were subsequently rinsed three times in sterilized tap water baths. The two proximal nodes were removed and cuttings were immersed for 30 s in indole-3-butyric acid (2 g/L) to promote rhizogenesis and then stored in containers with sterilized tap water until roots were developped. They were then placed after in plastic pots (9x9x10 cm) filled with sterilized 1/5 (v/v) clay balls and 4/5 (v/v) potting soil, irrigated daily with sterilized tap water and incubated in a growth chamber at 25/20°C day/night temperature, 16 h photoperiod, under 500 $\mu mol.s^{-1}.m^{-2}$ (Lumilux cool white L30W/840 and Fluora L30W/77, Osram, Germany) and 70% relative humidity. To facilitate development of inflorescence leaves were removed daily as described by Lebon *et al.* (2005).

Plant inoculation and growth conditions.

Soils of fruiting cuttings with fully developed inflorescences, corresponding to stage 57 (preflowers) according to the BBCH scale (Meyer, 2001), were inoculated with *Burkholderia phytofirmans* strain PsJN at a density of $5x10^7$ CFU colonizing per g of roots, *e.g.* after inoculation with $5x10^8$ CFU/g of soil.

Autofluorescence detection of phenolic compounds.

Phenolic autofluorescent compounds were detected according to Compant *et al.* (2005b). Roots were placed on glass slide at various time after inoculation and examined under an epifluorescence microscope under blue light.

Cytological detection of hydrogen peroxide.

For histochemical detection of H_2O_2, the 3,3-benzidine-HCl (DAB, Sigma-Aldrich) method was used according to Thordal-Christensen *et al.* (1997). Entire roots were immersed at various time intervals after inoculation in the DAB solution (1 mg/mL for 8 h at 37°C) before observation under an optical microscope.

Nitric oxide detection.

Nitric oxide localisation was determined by using the fluorophore 4,5-diamino-fluorescein diacetate (DAF-2DA, Sigma-Aldrich) according to Desikan *et al*. (2002) with some modifications. Roots were immersed at various time points after inoculation in DAF-2DA solution (10 µM in a KCl buffer) for 10 min, followed by a wash step (in a KCl buffer) for 20 min before that samples were examined under epifluorescence microscope.

Plant parts sampling and mRNA extraction.

Roots and inflorescences tissues (pedicell and preflowers) were sampled at 0, 2, 4, and 6 days after root inoculation to monitor gene expression. Messenger RNA was then extracted from roots and inflorescences with magnetic beads (Dynal, France) according to the manufacturer's instructions with changes in extraction buffer (Tris-HCl 600mM pH 7.5, LiCl 500 mM, EDTA10mM pH8, LiDS 1.5%, sodium deoxycholate 1.5%, NP-40 1.5%, DTT 10mM, β-mercaptoethanol 1%).

Real-time RT-PCR analysis.

RT and RT-PCR analysis of gene expression of roots and inflorescences were determined according to Bézier *et al*. (2002). Briefly, RT of RNA from roots and inflorescences was performed with 0.5–1 µg of total RNA reverse-transcribed with 5 µM Oligo(dT) (Life Technologies/Gibco-BRL) following the manufacturer's instructions. RT-PCR reactions were carried out in 96-well plates (25 µL per well) in a reaction buffer containing 1xSYBR Green I mix (PE Biosystems; including Taq polymerase, dNTPs, SYBR Green dye), 300 nM primers (forward and reverse) and 3.2–1600 pg of reverse transcribed RNA depending of experiments on roots or

preflowers. PCR conditions were 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension) for 40 cycles on a GeneAmp 5700 sequence Detection System (PE Biosystems). Each time point was determined as an average from data obtained from triplicate samples. The grapevine gene EF1α was used as internal control and the other gene specific primers used in this study are listed in Table 1.

Determination of the state of resistance in grapevine inflorescences induced by strain PsJN.

Potential protection of flowers against early colonization and infection by *Botrytis cinerea* was monitored by spraying, ten days after inoculation with strain PsJN, 200 µL of a conidial suspension of the pathogen (10^5 conidies per mL from 10 or 20 days-old growing B630 isolate or BC3-GFP in ¼ potatodestrose broth) per inflorescence at full bloom (*i.e.* 70% opened flowers). Three days after pathogen inoculation, flower infections were analysed between control and bacterized plants and flower colonization was particularly examined under epifluorescence microscope using BC3-GFP isolate.

Statistical analysis

For phenolic compound determination, H_2O_2 and NO accumulation, analyses were realized on samples taken from eight independent experiments. PR-encoding gene expressions were analysed on two independent batches, each taken from five control or bacterized plants. Flowers protection and microscopic analyses were determined on seven independent experiments and data were statistically analysed using Student's *t* test for flower infections with $P<0.05$.

ACKNOWLEDGEMENTS

This study was supported by a Ph.D. grant from Europôl-Agro (Reims, France). We are grateful to Dr. W. Mahafee and T. Neill (Horticultural Crop Research, Corvallis, Oregon, USA) for providing BC3-GFP isolate and Dr. Y. Barbier-Brygoo (Versailles, France) for B630 isolate.

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Lors de ce chapitre, nous avons montré que *B. phytofirmans* souche PsJN induit des réactions de défense dans les boutures fructifères de vigne ainsi qu'une résistance systémique contre l'infection florale causée par *B. cinerea.*

Au niveau racinaire, *B. phytofirmans* souche PsJN n'induit pas de réponse d'hypersensibilité contrairement aux agents pathogènes (Watanabe et Lam, 2006). Cependant, cette PGPR induit une accumulation de composés phénoliques, un stress oxydatif ainsi qu'un stress nitrique au niveau du rhizoplan et / ou à l'intérieur des racines. Ceci démontre que des défenses mises en évidence dans le cas d'agents pathogènes ou lors de l'application d'éliciteurs (Appel et Kirk, 2004 ; Garcia-Bruger *et al.,* 2006 ; Mur *et al.,* 2006) peuvent également se mettre en place, chez la vigne, suite à l'inoculation par une PGPR, notamment la souche PsJN. Néanmoins, ces défenses ont été détectées à des niveaux très faibles, ce qui suggère que les endophytes induisent un stress léger lors de leur colonisation.

Lors de cette étude, nous avons également mis en évidence, d'après des résultats préliminaires, une induction locale et systémique des gènes *VvGluC, VvChi4C, VvPR6, VvPR10.1* et *VvPR27* codants respectivement des protéines PR-2, PR-3, PR-6, PR-10 et PR-17, ainsi que *Vv102,* un gène à fonction inconnue. Il semble que la régulation de l'expression de ces gènes est différente dans les racines et les inflorescences des boutures bactérisées, tant au niveau des gènes induits que de leurs niveaux d'expression. En effet, des différences de profils d'expressions spatio-temporelles et de niveaux d'induction de ces gènes ont pu être constatées dans ces deux parties de la plante, à l'exception du gène *VvChi4C*. *Vv102* et *VvGluC* sont ainsi plus induits au niveau racinaire que dans les inflorescences et à l'inverse, *VvPR6, VvPR10.1,* et *VvPR27* sont plus induits dans les inflorescences que dans les racines. Bien que *VvGluC, VvChi4C, VvPR10.1* et *VvPR27* dépendent du SA et du JA, la signalisation de *Vv102* dépend majoritairement de la voie du SA et *VvPR6*, seulement du JA (Névians, 2007). Il semble par conséquent que la réponse de la plante à la souche PsJN soit dépendante du SA, au niveau racinaire, et du JA, au niveau systémique.

Les mécanismes de la SAR comprennent l'induction de gènes codants des protéines PR et une signalisation *via* le SA au niveau systémique. Par ailleurs, l'ISR est indépendante de l'induction de gènes codants des protéines PR et de l'accumulation du SA au niveau systémique (van Loon *et al.,* 2006b ; Ton *et al.,* 2007). Il semble donc que la souche PsJN induit à la fois des mécanismes communs de la SAR et de l'ISR. Néanmoins, ces premiers résultats ne permettaient d'étudier que les mécanismes associés à ces phénomènes de

résistances. Ces résultats doivent être de plus confirmés puisque seulement deux répétitions ont été analysées.

Lors de ces travaux, nous avons pu mettre en évidence que l'inoculation de *B. phytofirmans* souche PsJN réduit l'infection florale par *B. cinerea*. Comme nous avons pu le décrire précedemment, les expériences ont été réalisées avant que la souche PsJN colonise les inflorescences. Par conséquent, il apparaît que la souche PsJN induit une résistance systémique au niveau des inflorescences. Divers travaux avaient montré que l'utilisation de micro-organismes bénéfiques pouvait résulter en une protection des fleurs de vigne contre *B. cinerea*. Ces microorganismes tels que des souches du genre *Trichordema*, *Bacillus* ou *Pseudomonas* étaient cependant directement appliquées au niveau floral et la protection était liée à l'activité antagoiniste de ces microorganismes (pour revue Elmer et Reglinski, 2006). Par conséquent, aucune donnée ne concernait, auparavant, l'application de PGPR chez la vigne à l'origine d'une ISR permettant de contrer le développement de *B. cinerea* et de plus rien n'avait été montré au niveau floral. Il est possible que cette résistance diminue les symptômes de pourriture grise à maturité puisque l'infection des fleurs par *B. cinerea* est une étape précoce à l'origine de symptômes au niveau des fruits (Nair *et al.*, 1995 ; Pezet *et al.*, 2004). Néanmoins, ceci n'est pour l'instant qu'une hypothèse et devra, de ce fait, faire l'objet d'une étude complémentaire.

Chapitre 5 :

Conclusions et Perspectives

L'étude des interactions entre les PGPR endophytiques et leurs plantes hôtes constitue à l'heure actuelle un enjeu majeur. Dans cette thèse, une de ces interactions a été étudiée avec le modèle *Vitis vinifera* L.-*Burkholderia phytofirmans* souche PsJN. Une partie de cette interaction a été caractérisée, notamment la colonisation de la plante par la bactérie, l'élicitation des défenses de la plante, ainsi que l'induction d'une ISR conduisant à un certain niveau de protection contre *B. cinerea*.

Colonisation des plantules *in vitro* de vigne par *Burkholderia phytofirmans* souche PsJN

La colonisation de la vigne par *B. phytofirmans* souche PsN a tout d'abord été caractérisée sur des plantules *in vitro* (Figure 20).

Après inoculation artificielle de la rhizosphère des plantules *in vitro*, *B. phytofirman*s souche PsJN colonise les surfaces racinaires puis pénètre dans les racines. Cette propagation bactérienne déclenche des réactions de défense de la vigne, principalement au niveau de l'exoderme et des cellules corticales, qui se manifestent par des épaississements pariétaux et une accumulation de composés phénoliques.

La pénétration intra-racinaire de la souche PsJN et les défenses de la vigne associées peuvent être corrélées à la sécrétion d'enzymes dégradant les parois cellulaires par la bactérie PsJN. En effet, nous avons démontré que *B. phytofirmans* souche PsJN synthétise des polygalacturonases et des cellulases. Ces enzymes ou leurs produits d'hydrolyse (oligosaccharides, oligogalacturonides) peuvent être à l'origine de réactions de défense chez la vigne. Nous avons également montré que *B. phytofirmans* souche PsJN colonise de façon intracellulaire tous les tissus du cylindre périphérique pour atteindre, ensuite, la barrière de l'endoderme qu'elle va dégrader à certains endroits.

Suite à cette première étape de pénétration, *B. phytofirmans* souche PsJN entre dans le cylindre central. Cette étape de colonisation est également à l'origine de réactions de défense de la plante telles qu'au niveau des cellules du parenchyme du xylème où des épaississements pariétaux prennent place suite à la colonisation bactérienne. Ensuite, *B. phytofirmans* souche PsJN atteint les vaisseaux du xylème et les utilise pour se propager de façon systémique jusqu'aux feuilles, où elle colonise également les chambres sous-stomatiques, en 72-96 heures après inoculation. Ceci suggère une colonisation bactérienne *via* le phénomène

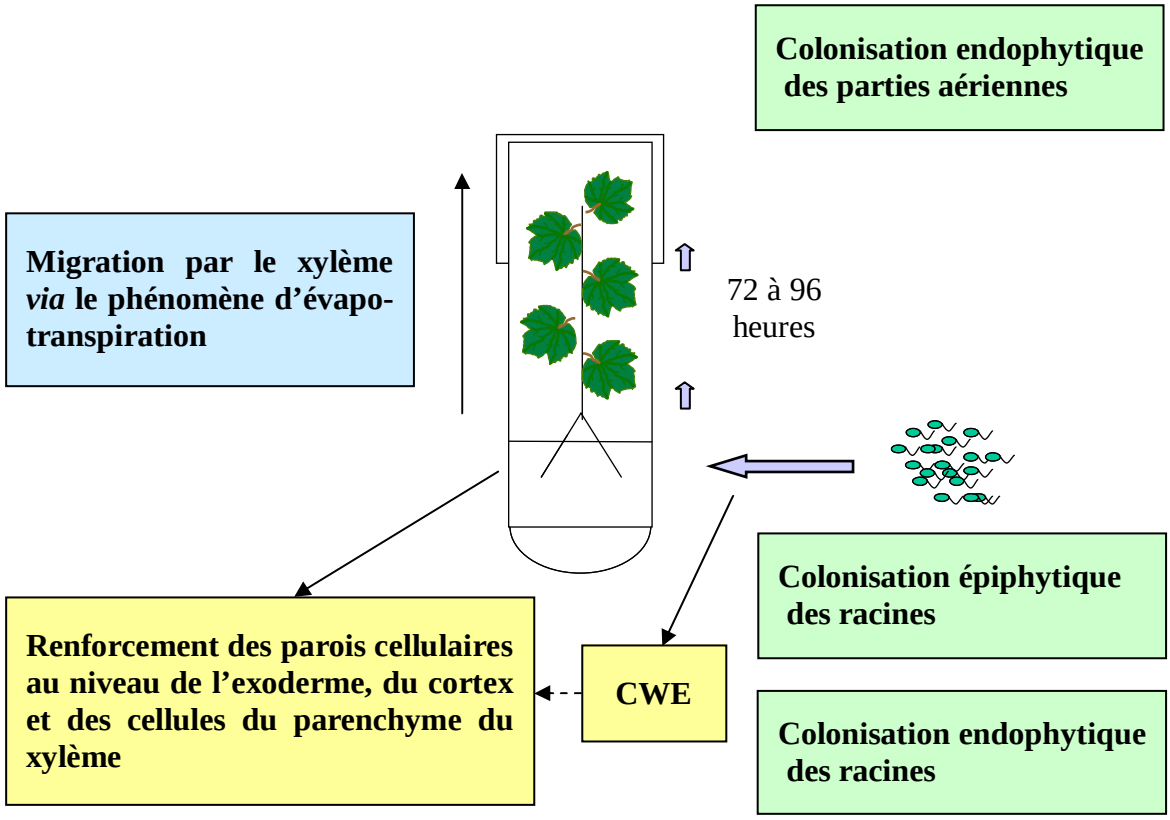

Figure 20 : Illustration des résultats sur la colonisation des plantules *in vitro* de vigne par *B. phytofirmans* souche PsJN. CWE : Enzymes dégradant les parois cellulaires.

d'évapotranspiration de la plante par le biais des vaisseaux du xylème, bien qu'une migration active de la bactérie par le biais de ses flagelles peut également être envisagée.

Colonisation des boutures fructifères de vigne par *Burkholderia phytofirmans* souche PsJN

Suite à l'étude de la colonisation des plantules *in vitro*, la colonisation de boutures fructifères par *B. phytofirmans* souche PsJN a été caractérisée (Figure 21). Ceci a permis d'évaluer si cette souche bactérienne pouvait migrer dans un tronçon de tige aouté et coloniser les differents organes de l'inflorescence.

Comme sur les plantules *in vitro*, *B. phytofirmans* souche PsJN colonise en premier les surfaces racinaires des boutures fructifères avant de pénétrer dans les tissus de la plante jusqu'aux vaisseaux du xylème. Ensuite, la bactérie PsJN migre jusqu'aux inflorescences par le biais de ces vaisseaux conducteurs.

Lors de ces travaux, des boutures fructifères qui présentaient à l'origine des bourgeons préfloraux, puis des fleurs et des baies, ont été utlisées. Il est apparu que la souche PsJN ne colonise les inflorescences qu'à partir du stade de jeunes fruits. En effet, la colonisation systémique de la plante n'est apparue qu'après 5 à 6 semaines après inoculation. Dans ce délai, les inflorescences se sont développées et ont atteint le stade 71 selon l'échelle BBCH correspondant à la nouaison, puis le stade 73 correspondant au stade de plomb de chasse. Ainsi, la rafle et les jeunes fruits n'ont pu présenter des sous-populations endophytiques de la souche PsJN qu'après un certain temps de développement des inflorescences. Cette colonisation endophytique des organes de l'inflorescence est différente des résultats obtenus avec le système *in vitro* où la bactérie est apparue au niveau systémique à partir de 72 heures après inoculation. Ceci peut s'expliquer par les différences de taille des plantes utilisées, par la différence de physiologie des plantes ainsi que par la présence ou non d'une microflore naturelle comme cela a pu être suggéré dans le chapitre 3.

Diverses études avaient préalablement démontré la présence de bactéries endophytiques dans les fruits (pour revue Hallmann, 2001). Néanmoins, aucune étude n'avait corrélé jusqu'à présent la présence des bactéries endophytiques dans ces organes avec une origine rhizosphèrique, même si cela avait été suggéré (Hallmann, 2001). Par le biais de cette thèse, nous avons montré qu'une PGPR, la souche PsJN, migre jusque dans les fruits après inoculation du sol. Les sous-populations endophytiques de la souche PsJN sont, cependant,

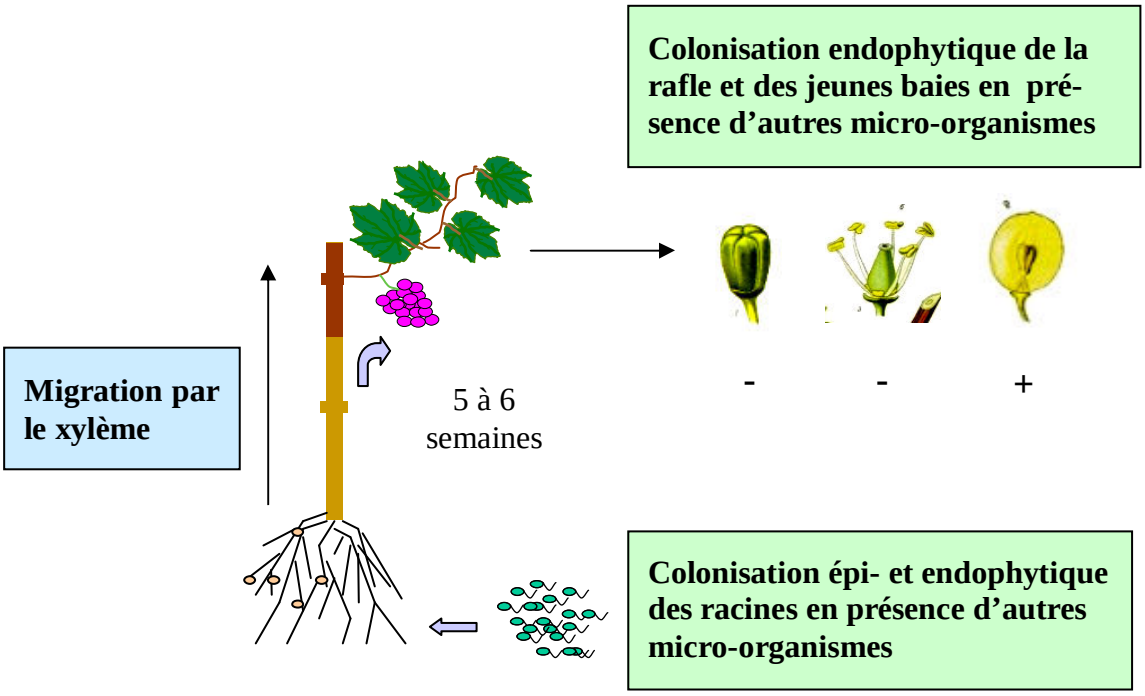

Figure 21 : Illustration des résultats sur la colonisation des boutures fructifères par *B. phytofirmans* souche PsJN. (-) Absence ou (+) présence de la souche PsJN.

peu nombreuses dans ces organes avec une moyenne détectée comme inférieure à 2 log UFC/g MF. Il est, de ce fait, possible qu'en application au vignoble, la souche PsJN ne colonise pas les inflorescences, compte tenu de cette faible densité bactérienne détectée en utilisant un modèle de boutures fructifères, même si cela reste à être vérifier.

Induction de défenses de la vigne par *Burkholderia phytofirmans* souche PsJN

Lors de cette thèse, l'induction de défenses par la souche PsJN a été étudiée sur des boutures fructifères présentant des bourgeons préfloraux, c'est à dire avant sa migration dans les organes de l'inflorescence. Ceci a permis de caractériser une réponse locale et systémique induite chez la plante suite à l'inoculation bactérienne (Figure 22).

Dans un premier temps, une accumulation de composés phénoliques, un stress oxydatif ainsi qu'un stress nitrique ont été montré au niveau racinaire suite à la colonisation épi- et / ou endophytique des racines par *B. phytofirmans* souche PsJN. Par ailleurs, des inductions de gènes codants des protéines PR ont été mis en évidence tels que pour les gènes *VvGluC*, *VvChi4C*, *VvPR6*, *VvPR10.1*, *VvPR27* et *Vv102*. Il est apparu que ces gènes sont d'abord induits au niveau racinaire et ensuite au niveau systémique dans les bourgeons préfloraux. Néanmoins, des différences de niveaux d'induction de ces gènes aux niveaux local et systémique ont pu être mis en évidence, par le biais de résultats préliminaires. Bien que la signalisation de certains gènes correspond à la fois au SA et au JA, les travaux de cette thèse ont permis de montrer une signalisation *via* le SA au niveau racinaire et impliquant le JA au niveau systémique. En effet, le gène *Vv102*, dépendant majoritairement de la voie de signalisation du SA, est plus induit au niveau local qu'au niveau systémique et à l'inverse, le gène *VvPR6* dépendant seulement de la voie du JA, est, quant à lui, plus induit dans les inflorescences que dans les racines.

Par le biais de cette étude, nous avons montré que le modèle *V. vinifera* L.- *B. phytofirmans* souche PsJN englobe les mécanismes communs des phénomènes de SAR et d'ISR. En effet, la souche induit un stress oxydatif et un stress nitrique ainsi que des gènes codants des protéines PR, comme dans le cas de la SAR (van Loon *et al.*, 2006b). Néanmoins, il n'y a pas de HR. De plus, la réponse de la plante semble dépendre du JA au niveau systémique comme dans le cas de l'ISR (van Loon et Bakker, 2005). En outre, l'induction de gènes par la souche PsJN est faible en comparaison à ce qu'il se passe chez les plantes en réponse aux agents pathogènes, même si celà nécessite d'être confirmé.

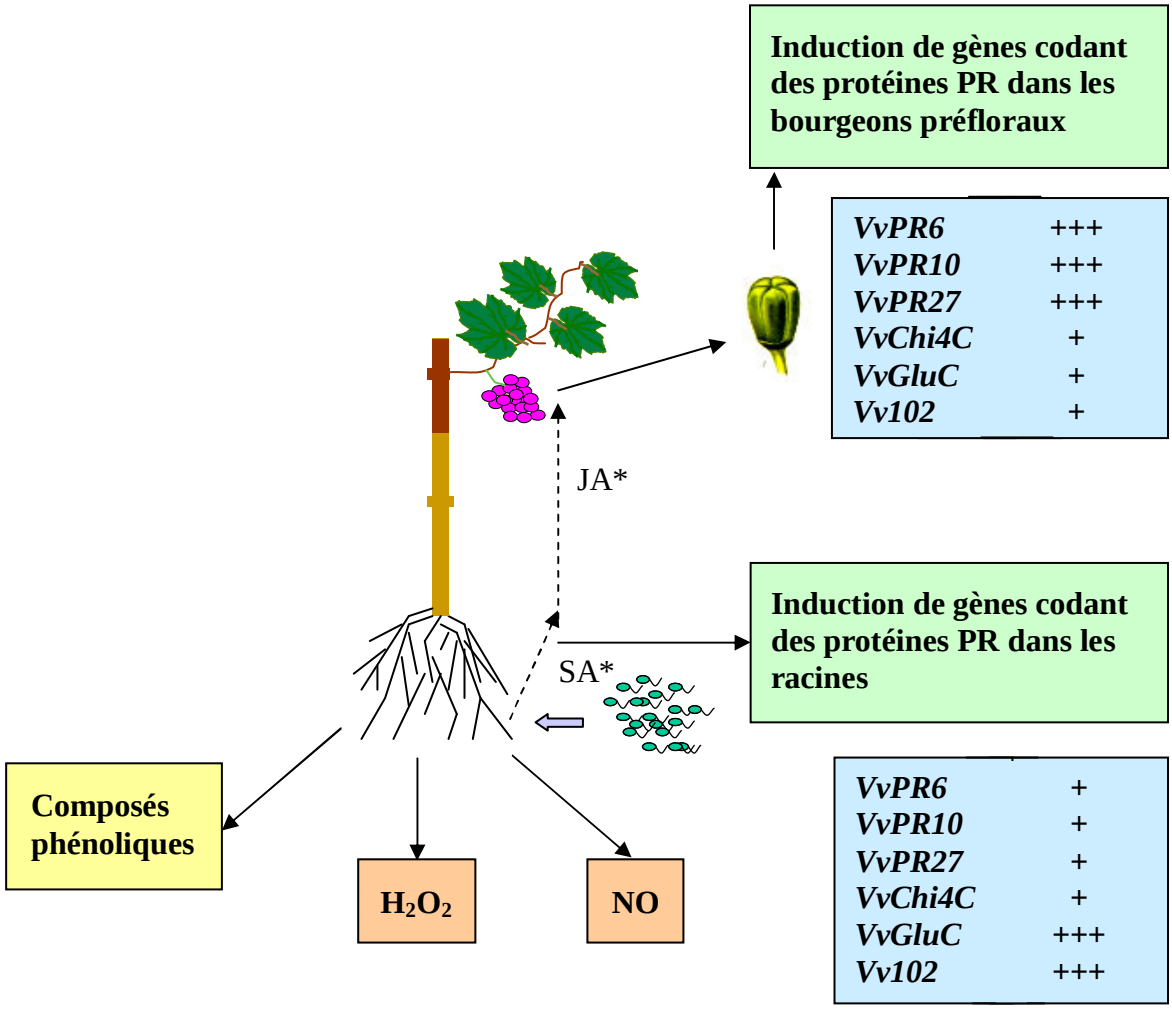

Figure 22 : Illustration des résultats sur l'étude de l'induction de défenses chez des boutures fructifères par *B. phytofirmans* souche PsJN. H_2O_2 : peroxyde d'hydrogène, JA : acide jasmonique, NO : oxyde nitrique et SA : acide salicylique. *VvChi4C, VvGluC, VvPR6, VvPR10.1, VvPR27* : gènes codants respectivement une PR-2, PR-3, PR-6, PR-10 et *PR-17*. *Vv102* : gène à fonction inconnue. * signalisation intercellulaire probable.

Des travaux similaires ont été publiés récemment par Wang *et al.,* (2005) concernant les effets de l'endophyte *P. fluorescens* PFT9601-T5 sur *A. thaliana.* Néanmoins, dans cette étude, rien n'a été démontré au niveau local mais plutôt au niveau systémique. Nous avons ainsi démontré une partie des réponses locales et systémiques des plantes suite à l'inoculation des PGPR endophytiques avec *B. phytofirmans* souche PsJN. Ceci a été réalisé, en particulier, chez une plante ligneuse, la vigne, sur laquelle peu d'études ont porté sur son interaction avec des PGPR. De plus, notre étude a porté sur les inflorescences, des organes peu étudiés suite à l'inoculation de PGPR.

Cette étude n'a permis, cependant, d'analyser que les mécanismes de défense induits lors de l'interaction entre la vigne et la souche PsJN. Il a fallu ensuite déterminer si ces défenses étaient accompagnées d'une résistance de l'inflorescence contre *B. cinerea*. Ceci ne pouvait qu'être déterminé au stade floral puisque la mise en place d'une résistance systémique induite nécessite environ une dizaine de jours après inoculation d'un micro-organisme (van Loon *et al.*, 1998). De plus, nous ne pouvions pas déterminer l'ISR d'un point de vue fondamental dans les fruits puisqu'afin d'étudier l'ISR, le micro-organisme bénéfique doit être absent de lieu d'infection de l'agent pathogène (van Loon *et al.,* 1998).

Résistance systémique induite par *Burkholderia phytofirmans* souche PsJN chez la vigne contre l'infection florale causée par *Botrytis cinerea*

Une partie de cette thèse a ainsi permis d'analyser l'incidence de l'inoculation de *B. phytofirmans* souche PsJN chez la vigne envers l'infection florale causée par *B. cinerea* (Figure 23). Nous avons montré que les inflorescences de boutures bactérisées sont moins infectées (60% de protection) que les boutures non traitées. Ceci démontre le potentiel de l'interaction entre la vigne et la souche PsJN pour contrer le développement de *B. cinerea*. De plus, cette étude nous a permis de caractériser l'ISR induite par *B. phytofirmans* souche PsJN. En effet, la protection observée ne pouvait résulter que d'une résistance systémique induite au niveau racinaire, la bactérie PsJN ne s'étant pas encore propagée jusque dans les inflorescences.

Il est probable que cette protection résulte des réactions de défense de la vigne mises en place lors de son interaction avec *B. phytofirmans* souche PsJN. Néanmoins, l'ISR induite par les PGPR a le plus souvent été corrélée à un phénomène de potentialisation des défenses existantes (pour revue van Loon, 2007). Il est de ce fait possible qu'une potentialisation se

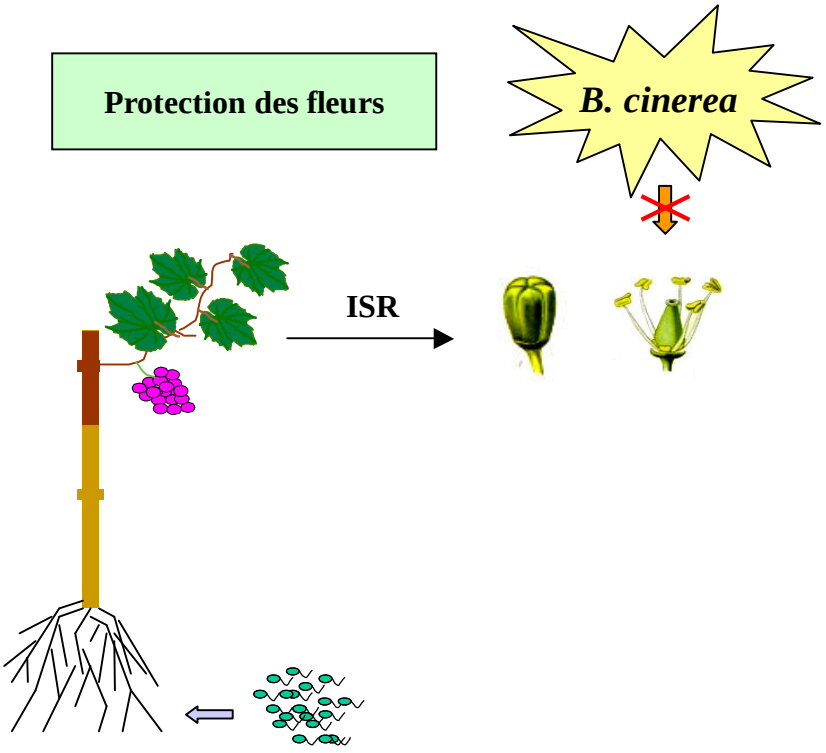

Figure 23 : Illustration des résultats sur l'ISR au niveau floral induite par *B. phytofirmans* souche PsJN envers l'infection précoce causée par *B. cinerea.*

mette en place lors de l'infection des plants bactérisés par *B. cinerea* et que les défenses mises en évidence lors de cette thèse ne permettent pas d'expliquer le phénomène d'ISR. Néanmoins, à l'heure actuelle, nous ne savons encore rien sur ce possible phénomène de potentialisation et il serait, par conséquent, intéressant de l'envisager comme perspective.

Perspectives

En perspective de ces travaux de thèse, différents points peuvent être envisagés. Tout d'abord d'un point de vue fondamental et ensuite d'un point de vue appliqué.

Perspectives fondamentales

Des changements de flux d'ions ainsi que des MAP kinases interviennent-ils lors de l'interaction *Vitis vinifera* L.-*B. phytofirmans* souche PsJN ?

Une étude approfondie de la signalisation intracellulaire peut tout d'abord être envisagée. En effet, lors de ces travaux, nous avons décrit une partie de la signalisation intracellulaire qui se met en place lors de l'interaction entre la vigne et *B. phytofirmans* souche PsJN et qui se traduit par une accumulation d'H_2O_2 et de NO au niveau racinaire. Or, il existe d'autres événements précoces lors de l'interaction entre les plantes et les micro-organismes ou leurs composés éliciteurs. Ainsi, des changements de flux d'ions (influx de Ca^{2+}, efflux de K^+, Cl^-) et des MAP kinases peuvent intervenir dans cette cascade de signalisation (pour revue Garcia-Brugger *et al.*, 2006). Il serait, de ce fait, intéressant d'analyser si ces phénomènes moléculaires interviennent lors de la reconnaissance entre la vigne et la souche PsJN. Ceci pourrait être déterminé sur des suspensions cellulaires, des plantules *in vitro* voire même des boutures fructifères. Une telle étude permettrait de mieux comprendre les mécanismes impliqués lors de l'interaction *Vitis vinifera* L.-*B. phytofirmans* souche PsJN.

Signalisation intercellulaire à approfondir ?

Une étude approfondie de la signalisation intercellulaire peut être également envisagée.

Nos résultats suggèrent que la voie de signalisation du SA se met en place au niveau racinaire. En revanche, le JA semble être impliqué au niveau systémique. Il serait, cependant, intéressant de quantifier ces phytohormones par chromatographie phase gazeuse puisque les

seules indications concernant l'implication de ces phytohormones dans notre modèle d'étude proviennent de l'étude de l'expression de gènes codants des protéines PR.

Lors de l'interaction entre les plantes et les PGPR, il a été montré que la signalisation *via* le JA au niveau systémique se fait en synergie avec l'ET (pour revues van Loon et Bakker, 2005 ; Pieterse *et al.*, 2007 ; Pieterse et van Loon, 2007). Il est donc possible que l'ET soit impliqué au niveau systémique dans l'interaction que nous étudions. Néanmoins, nous ne savons pas à l'heure actuelle si cette phytohormone joue un rôle de signalisation dans notre modèle. Il serait, par conséquent, intéressant de déterminer son rôle lors de l'interaction entre la vigne et la souche PsJN.

Etude du suivi d'autres gènes de défense ?

Une étude plus approfondie des gènes de défenses induits chez la vigne en réponse à *B. phytofirmans* souche PsJN peut également être envisagée.

Au cours de cette thèse, nous avons pu montrer l'expression de gènes de défense, en particulier ceux codants des protéines PR. Néanmoins, nous n'avons pas étudié tous les gènes codants des protéines PR connus chez la vigne (Bézier *et al.*, 2007). Certains d'entre eux ont bien été étudiés lors de l'interaction entre la vigne et des micro-organismes ou suite à l'application d'éliciteurs (Bézier, 2003 ; Hamiduzzaman *et al.*, 2005 ; Aziz *et al.*, 2007 ; Bézier *et al.*, 2007). C'est le cas par exemple de *VvChi1b*, *VvChi4D*, *VvPR10.2*, *VvCH3*, *VvPR1* et *VvPR4*. Ces gènes peuvent être notamment induits en réponse à *B. cinerea* (Bézier *et al.*, 2007), *Pseudomonas syrinage pv. pisi* (Robert et al., 2001), au BABA (Hamiduzzaman *et al.*, 2005), ou à d'autres éliciteurs (Aziz *et al.*, 2003 ; 2007). Néanmoins, leurs expressions n'ont pas été étudiées dans le cas de l'interaction *Vitis vinifera* L.-*B. phytofirmans* souche PsJN. Il serait, de ce fait, intéressant de vérifier si ces gènes sont induits dans notre modèle d'étude. De plus, les voies de signalisation qui régulent l'expression de certains de ces gènes sont connues. L'expression de *VvPR1* dépend ainsi de la voie du SA et *VvPR4* de la voie du JA (Hamiduzzaman *et al.*, 2005). Le suivi de l'expression de ces gènes permettrait de confirmer l'implication de la voie du SA au niveau racinaire et du JA au niveau systémique.

D'autres gènes de défense tels que *VvPAL* et *VvLox* codants respectivement la PAL et la LOX pourraient également être étudiés. En effet, il serait intéressant d'analyser leur expression puisque *VvPAL* dépend de la voie du SA et *VvLox*, de celle du JA (Hamiduzzaman *et al.*, 2005). Une telle démarche permettrait de compléter l'étude précédente sur les gènes

VvPR1 et *VvPR4* et ainsi de confirmer la signalisation qui se met en place lors de l'interaction entre la vigne et *B. phytofirmans* souche PsJN.

Enfin, l'étude du transcriptome de la vigne en réponse à *B. cinerea* (Bézier *et al.*, 2007) a permis de mettre en évidence plusieurs gènes impliqués dans les réponses de défense de la plante. Tous ces gènes, *Big* ou *Brg*, pourraient être analysés lors de l'interaction entre la vigne et la souche PsJN. Le suivi de ces gènes pourrait nous apporter des informations considérables sur les défenses de la plante qui se mettent en place en réponse à la PGPR. Une étude plus globale pourrait même être réalisée en utilisant des puces à ADN. Ceci contribuerait à accroître nos connaissances actuelles sur l'interaction *V. vinifera* L.-*B. phytofirmans* souche PsJN.

Détermination de composés phénoliques ?

La détermination des composés phénoliques impliqués lors de l'interaction entre la vigne et *B. phytofirmans* souche PsJN serait également une perspective intéressante. En effet, lors de cette thèse, nous avons décrit une accumulation de composés phénoliques au niveau racinaire. Néanmoins, nous ne connaissons pas encore à l'heure actuelle leur composition chimique exacte. De ce fait, il serait intéressant de les caractériser. Ceci permettrait de savoir quels types de phénols s'accumulent chez la vigne en réponse à la souche PsJN.

En outre, les stilbènes tels que le resvératrol n'ont pas été analysés lors de ces travaux de thèse. Compte tenu du fait que ces métabolites secondaires ont bien été caractérisés chez la vigne (Jeandet *et al.*, 2007), il serait de ce fait intéressant d'analyser si ces composés sont produits lors de l'interaction entre la vigne et *B. phytofirmans* souche PsJN. Cette analyse permettrait de mieux comprendre leur rôle dans les défenses des plantes et leur possible implication dans notre modèle d'étude.

Potentialisation ?

Pour la suite de ces travaux, nous pourrions également étudier si un phénomène de potentialisation se met en place lors de l'interaction *Vitis vinifera* L.-*B. phytofirmans* souche PsJN-*B. cinerea*. Ce phénomène a bien été caractérisé chez diverses plantes en réponse à des PGPR, en particulier chez *A. thaliana* (Ton *et al.*, 2007 ; van Loon, 2007). Néanmoins, nous ne savons pas encore si des défenses sont potentialisées dans notre modèle d'étude. Un suivi des gènes de défenses ou de leurs produits sur des plants témoins et bactérisés, en présence ou

non de *B. cinerea*, permettrait de répondre à cette question. Cette démarche permettrait en outre de déterminer si les défenses constatées lors de notre thèse sont indispensables à la protection de la vigne contre *B. cinerea*. Il est possible que ces défenses ne présentent pas de rôle dans l'ISR comme cela a pu être décrit par van Loon et Bakker (2005). En revanche, il est possible qu'elles soient potentialisées lors d'une infection ultérieure par un agent pathogène, bien que celà reste à être démontrer dans le cas de l'interaction que nous venons d'étudier.

Perspectives d'un point de vue appliqué

Identification de l'espèce *Burkholderia phytofirmans* dans le vignoble champenois ?

Tout d'abord, il serait intéressant d'étudier si *B. phytofirmans* est une PGPR naturellement présente chez la vigne. A l'heure actuelle, nous ne savons pas si cette espèce fait partie de la microflore rhizosphèrique et / ou endophytique des vignes champenoises. Or, la réglementation française est très restrictive concernant l'application d'un micro-organisme (directive 2001/36/CE). Jusqu'à 10 ans d'attente peuvent être nécessaires avant son utilisation, en particulier quand celui ci n'est pas d'origine française. Il serait, de ce fait, intéressant de rechercher si cette espèce bactérienne est naturellement présente dans nos régions septentrionales. Ceci permettrait de l'appliquer plus rapidement au vignoble et de vérifier si elle protège réellement la vigne contre l'infection causée par *B. cinerea*.

Impact de *B. phytofirmans* souche PsJN sur la vinification ?

Un suivi de la colonisation de la vigne par la souche PsJN jusqu'à la maturité du raisin ainsi que son impact sur la vinification peuvent également faire l'objet d'un projet intéressant. Lors de ces travaux de thèse, nous avons décrit la colonisation de la vigne par la souche PsJN, en particulier au niveau des inflorescences. Néanmoins, nous n'avons pas été jusqu'à la fermeture de la grappe ni jusqu'à la maturation du fruit. Il serait, de ce fait, intéressant d'étudier si la souche PsJN persiste dans les baies de raisin. Cette étude pourrait être effectuée sur des boutures fructifères mais également sur des plantes en pots, voire même au vignoble si l'espèce *B. phytofirmans* y a été auparavant détectée. Les résultats de cette démarche permettraient d'éclairer les viticulteurs sur une éventuelle persistence de cette souche

bactérienne dans les fruits à maturité et d'envisager ensuite l'étude de son impact sur la vinification des vins de Champagne.

Résistance des plants bactérisés avec *B. phytofirmans* souche PsJN envers *B. cinerea* lors de la maturation du raisin ?

Un autre projet peut correspondre à l'analyse de la résistance des plants bactérisés contre l'infection des baies matures par *B. cinerea*. Nous n'avons pas été jusqu'à maturité du fruit lors de cette thèse. Etant donné que *B. cinerea* provoque des dégâts importants au niveau des baies matûres, il serait intéressant d'analyser si les plants bactérisés sont résistants envers *B. cinerea* au cours de la maturation. Dans le cas où une réduction de la pourriture grise serait constatée, l'utilisation de *B. phytofirmans* souche PsJN permettrait de diminuer l'utilisation de produits phytosanitaires, produits dont certains sont néfastes pour l'environnement.

Résistance contre d'autres agents pathogènes ?

Enfin, un autre projet peut correspondre à l'étude du suivi de la protection des plants bactérisés envers divers agents pathogènes. En effet, la vigne de nos régions septentrionales est infectée par divers agents pathogènes comme par exemple *Plasmopara viticola, Erysiphe nector,* ou les champignons responsables de la maladie de l'Esca (Huglin et Schneider, 2003). D'un point de vue appliqué, il serait par conséquent intéressant d'étudier si *B. phytofirmans* souche PsJN réduit le développement de ces champignons. Si cette alternative était mise en place, elle permettrait de réduire l'utilisation massive de produits phytosanitaires sur la vigne et serait par conséquent une approche plus respectueuse de l'environnement.

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Annexes

Interaction between plants and *Burkholderia* spp.

Stéphane Compant[1], Jerzy Nowak[2], Tom Coenye[3], Christophe Clément[1], and Essaïd Ait Barka[1]

Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cédex 2, France[1];

Department of Horticulture, Virginia Polytechnic Institute and State University, 0327-301 Saunders Hall, Blacksburg, VA 24060, USA[2]

Laboratorium voor Farmaceutische Microbiologie, Universiteit Gent, Harelbekestraat 72, B-9000 Gent, Belgium[3]

Soumis à FEMS Microbiology Reviews après invitation.

Interactions between plants and *Burkholderia* spp.

Stéphane Compant [1], Jerzy Nowak [2], Tom Coenye [3], Christophe Clément [1] & Essaïd Ait Barka [1*]

[1]*Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UFR Sciences – EA 2069, Université de Reims Champagne-Ardenne, 51687 Reims Cedex 2, France;* [2]*Department of Horticulture, Virginia Polytechnic Institute and State University, 0327-301 Saunders Hall, Blacksburg, VA 24060, USA;* [3]*Laboratorium voor Farmaceutische Microbiologie, Universiteit Gent, Harelbekestraat 72, B-9000 Gent, Belgium*

Correspondence: Essaïd Ait Barka, Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cédex 2, France. Tél./Fax : +33-326913441; e-mail: ea.barka@univ-reims.fr

Received 18 June 2007

Editor : Eva Top

Keywords
Burkholderia; pathogen; phytosphere; rhizosphere; endophytic bacteria; symbiotic bacteria.

Abstract

Both in natural and managed ecosystems, bacteria are common inhabitants of the phytosphere and the internal tissues of plants. Some of these bacteria can be hazardous to human, animal or plant health, but the majority is either neutral or beneficial. Probably the most diverse and environmentally adaptable bacteria belong to the genus *Burkholderia.* This genus is best known for its human and plant pathogenic members. However, this genus also contains plant pathogens as well as beneficial strains which have a great potential to be exploited in biotechnological processes. Here we present an overview of plant associated-*Burkholderia* sp. with special emphasis on beneficial plant-*Burkholderia* interactions and discuss the potential for utilization of stable plant-*Burkholderia* spp. associations in the development of low input cropping systems.

Introduction

Over the past two decades, research on *Burkholderia* species has been steadily expanding. Members of the genus *Burkholderia* are very abundant, occupying diverse ecological niches (Estrada de los Santos *et al*., 2001; Coenye and Vandamme, 2003), including soil (van Elsas *et al*., 2002; Salles *et al*., 2002; 2004; Janssen, 2006), and can cause infections in humans, animals (Coenye and Vandamme, 2003; Valvano *et al*., 2005; 2006). Nevertheless, during the recent years, a growing number of *Burkholderia* strains and species with interactions with plants have been reported. Indeed, plant-associated *Burkholderia* can be free-living in the rhizosphere or endophytic, including obligate endosymbionts as well as phytopathogens (Coenye and Vandamme, 2003; Janssen, 2006). Several of them are known to enhance disease resistance in plants, contribute to better water management, improve nitrogen fixation and overall host adaptation to environmental stresses (Coenye and Vandamme, 2003; Compant *et al.*, 2005a; Nowak and Shulaev, 2003; Sessitsch *et al.*, 2005; Ait barka *et al*., 2006; Balandreau and Mavingui, 2006; Barrett and Parker, 2006; Janssen, 2006). However, several members of this genus are opportunistic or obligate pathogens causing human, animal and/or plant diseases (Coenye and Vandamme, 2003). Thus any development of biotechnological applications using *Burkholderia* germplasm needs to include a stringent assessment of the potential risks. The review summarizes the current status of knowledge of interactions between *Burkholderia* species and plants with special emphasis on beneficial associations, and discuss of their potential for biotechnological applications.

Brief overview of the genus *Burkholderia*

Walter H. Burkholder described one of the first *Burkholderia* sp., *Phytomonas caryophylli* (Burkholder, 1942), later known as *Pseudomonas caryophylli*. In the mid 1940's, vegetable growers in New York State discovered infections by a bacterium previously known to cause rot in onion bulbs. Following this outbreak, in 1949 Walter H. Burkholder described this phytopathogen and gave it the species name "cepacia", meaning derived from "onion" (Burkholder, 1950); it was later known as *Pseudomonas cepacia*. Since then, however, the taxonomy of the genus *Pseudomonas* has changed dramatically (reviewed by Kersters *et al*., 1996). Due to its broad and vague phenotypic definition, many incompletely characterized, polarly flagellated, rod-shaped, aerobic, Gram-negative bacteria were initially placed in the genus *Pseudomonas*. However, rRNA-DNA hybridization analyses during the early 1970s demonstrated considerable genetic diversity among members of this genus, which was thus divided into five so-called rRNA homology groups (Palleroni *et al.*, 1973). Subsequent genotypic analyses confirmed that these five groups were actually only distantly related to each other. Consequently, the genuine genus *Pseudomonas* was restricted solely to homology group I, containing the type species, *P. aeruginosa* (De Vos *et al.*, 1985). In 1992, the seven species belonging to rRNA homology group II (*P. solanacearum*, *P. pickettii*, *P. cepacia*, *P. gladioli*, *P.*

mallei, *P. pseudomallei* and *P. caryophylli*) were transferred to the novel genus *Burkholderia* (Yabuuchi *et al*., 1992) which resides in rRNA superfamily III *sensu* (De Ley, 1987) or subgroup β-3 of the β-*Proteobacteria sensu* (Woese, 1987). In recent years, the number of species included in this genus has increased dramatically (Coenye and Vandamme, 2003).

Many of these changes involve the species *B. cepacia* as during the past few years, several polyphasic taxonomic studies (Vandamme *et al.*, 1997; 2000; 2002; 2003; Coenye *et al.* 2001a; 2001b; 2001; Vermis *et al.* 2004) have indicated that strains identified as *B. cepacia* based primarily on phenotypic analysis actually represent a complex of several closely related genomic species or genomovars. This group, collectively referred to as the *B. cepacia* complex, currently consists of nine species including, *B. cepacia* (genomovar I), *Burkholderia multivorans* (genomovar II), *B. cenocepacia* (genomovar III), *B. stabilis* (genomovar IV), *B. vietnamiensis* (genomovar V), *B. dolosa* (genomovar VI), *B. ambifaria* (genomovar VII), *B. anthina* (genomovar VIII) and *B. pyrrocinia* (genomovar IX).
Others species of *Burkholderia* have been also discovered and nowadays, there are currently over 40 validly described *Burkholderia* species (see http://www.bacterio.cict.fr/b/burkholderia.html for an up-to-date overview).
Most members of the genus *Burkholderia* are soil commensals that rarely cause human infection, although several *Burkholderia* species are obligate (*B. mallei*, *B. pseudomallei*) or opportunistic (*B. gladioli*, *B. cepacia* complex) human pathogens (for a recent review see Vandamme *et al.*, 2007). The genus *Burkholderia* also contains some species which can be endosymbionts in plant-associated fungi or insects (*e.g. Burkholderia sordidicola*, *B. rhizoxina*) as well as plant-pathogens (*e.g. B. cepacia*, *B. caryophylli*, *B. plantarii*, *B. gladioli*, *B. glumae* and *B. andropogonis*). However, certain *Burkholderia* species can be free-living or live in close associations with some plants and even can form obligate symbiosis with their host (e.g. *B. mimosarum*, *B. phymatum*).

Phytopathogens in the genus *Burkholderia*

Several species of the genus *Burkholderia* can thus induce diseases in plants as described for *B. cepacia*, *B. caryophylli***,** *B. plantarii***,** *B. gladioli*, *B. glumae* and *B. andropogonis* (Burkholder, 1942; Coenye and Vandamme, 2003). As noted above, *B. cepacia* can cause onion rot by infecting onion leaves and bulbs (Burkholder, 1950). The pathogen produces an endopolygalacturonase at low pH that is responsible for the maceration of onion tissues. The other phytopathogenic *Burkholderia*, *B. caryophylli*, induced formation of bacterial wilt in various plant species as identified in Russel prairie gentian in Japan (Furuya *et al*., 2000). *B. plantarii,* , first isolated in Japan in 1982, provokes in its host, seedling blight on rice and also infects koyawarabi (*Onoclea sensibilis* L.) (Azegami *et al*., 1987; Tanaka and Katoh, 1999). *B. gladioli,* is also a potent phytopathogen of various plant host. It thus induces bacterial soft rot in onions, leaf-sheath browning and grain rot in rice, and leaf and corm diseases in gladiolus and iris species (Palleroni, 1984; Lee *et al*., 2005; Ura *et al*., 2006). The two last known phytopathogenic *Burkholderia* sp., *B. glumae* and *B. androponis*, have also a wide range hosts. *B. glumae* can thus cause seedling and grain rot in rice and wilting symptoms in tomato, sesame (*Sesamum indicum* L.), perilla (*Perilla frutescens*), eggplant and hot pepper (Jeong *et al*., 2003). *B. andropogonis* infects more than 52 species of 15 families of unrelated monocot and dicotyledonous plants. This later phytopathogenic bacterium is thus an important causal agent for example of stripe disease of sorghum (*Andropogon* sp.), leaf spot of clover (*Trifolium* sp.), *Odontioda* orchids, velvet bean (*Stizolobium deeringianum*), jojoba (*Simmondsia chinensis*), *Amaranthus cruentus* L., and of *Bougainvillea* sp. in addition to cause considerable economic lost of carnation (*Dianthus caryophyllus*) (Smith, 1911; Murai and Goto, 1996; Scortichini *et al*., 2001; Cother *et al*., 2004; Takahashi *et al*., 2004; Li and De Boer, 2005). Although phytopathogenesis was described for these several *Burkholderia* sp., the ecological role of some *Burkholderia* spp. such as *B. glathei*, *B. graminis*, *B. phenazinium*, *B. caribensis*, *B. caledonica*, *B. hospita*, *B. terricola* and *B. saccharii* remains largely unknown at this time (Coenye and Vandamme, 2003; Vandamme *et al.*, 2007). It is thus possible that some plant diseases will be futher correlated to some Burkholderiales members.

Endosymbiotic *Burkholderia* in phytopathogenic fungi

Nowadays, it is clear that some phytopathogenic fungi can contain endosymbionts from the genus *Burkholderia.* This is the case for *Burkholderia sordidicola*, isolated from the white-rot fungus, *Phanerochate sordida*, which inhabits fallen branches of hardwood tress (Lim *et al*., 2003). These two species have distantly related 16S rDNA sequences, suggesting that associations between fungi and *Burkholderia* have arisen on multiple occasions.
Burkholderia rhizoxina also identified as an endosymbiont of the pathogenic fungus *Rhizopus microsporus* is responsible for the synthesis of the phytotoxin rhizoxin causing rice seedling blight (Partida-Martinez and Hertweck, 2005; Scherlach *et al*., 2006). Rhizoxin inhibits mitosis in rice plant cells and effectively weakens or even kills the plant, with both the host and the symbiont benefiting from nutrients derived from the decaying plant material (Partida-Martinez and Hertweck, 2005). It is obvious that the fungus profits from the biosynthetic capabilities of the endosymbiont in order to access nutrients. However, the direct benefits to the bacterial symbiont are not clear, as it may grow, albeit slowly, outside the host and produce the toxin which induces disease on plants (Partida-Martinez and Hertweck, 2005).

Endosymbiotic *Burkholderia* in plant associated insects

Nowadays, *Burkholderia* species have been also associated with insect feeding on plants. For instance, the plant-associated Japanese common broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* contain *Burkholderia* sp. as endosymbionts. These insects possess a number of crypts in the posterior region of the midgut, whose lumen contains a copious amount of bacterial cells. The predominant 16S rRNA gene sequences obtained from different individuals and species of the bugs were very similar but not identical to each other. Homology searches in the DNA databases revealed that the sequences showed the highest levels of similarity (96% to 99%) to the sequences of *Burkholderia* spp and *in situ* hybridization with specific oligonucleotide probes confirmed the localization of the *Burkholderia* symbiont in the lumen of the midgut crypts. Molecular phylogenetic analysis recently demonstrated that the *Burkholderia* symbionts of the bugs formed a well-defined monophyletic group, although the group also contained several environmental *Burkholderia* strains (Kikuchi and Graf, 2007). The important physiological roles of the symbionts for their hosts come from the discover that symbiont-free insects exhibit retarded growth, nymphal mortality, and/or sterility. Because of the dense and specific colonization in the midgut crypts and the prevalence in natural populations of *R. clavatus* and *L. chinensis*, it appears likely that the *Burkholderia* symbionts play important roles in the host bugs (Kikuchi and Graf, 2007).

Non phytopathogenic and beneficial *Burkholderia* sp. associated with the phytosphere

Nowadays, the majority of *Burkholderia* species are non pathogenic and can be neutral or beneficial to their hosts, plants in particular (De Costa and Erabadupitiya, 2005). Some species can establish themselves as epiphytes and/or endophytes colonizing interior of plant organs and stimulate plant growth, fix nitrogen and/or enhance host's resistance to abiotic and biotic stresses (Sharma and Nowak, 1998; Estrada de los Santos *et al.*, 2001; Nowak and Shulaev, 2003; Compant *et al.*, 2005a; Sessitsch *et al.*, 2005; Ait Barka *et al.*, 2006). Members of the genus *Burkholderia* are also able to form beneficial symbiotic associations responsible of plant nodulation (Van Oevelen *et al.*, 2002; Elliott *et al.*, 2007). Agricultural industry has been attempting to explore properties of these bacteria for use as biopesticides (Li *et al.*, 2002; De Costa et Erabadupitiya, 2005).

Burkholderia sp. associated with the rhizosphere

Some members of the genus *Burkholderia* species are cuurently known as common inhabitants of plant rhizosphere (Table 1), *i.e.* soil immobilized on root surfaces and adjacent to roots, forming neutral or beneficial associations although the nature of these interactions cannot always be defined. For example, *B. cepacia* complex can form associations with various plant species under different geographical zones (Chiarini *et al.*, 2000; Dalmastri *et al.*, 2003). The bacteria were thus isolated from the rhizosphere of maize in the U.S. (Hebbar *et al.*, 1992; Ramette *et al.*, 2005), rice (Tran Van *et al.*, 1994), pea (Parke, 1990), cotton (Parke and Gurian-Sherman, 2001), as well as of oak trees, common groundsel, horsetail, arum lily and wheat in the U.K. (Richardson *et al.*, 2002). However, *B. cepacia* was not the only *Burkholderia* species detected in the rhizosphere. Thus, *B. graminis* is a common rhizobacteria of corn, pasture and wheat in Australia and France (Viallard *et al.*, 1998), *B. unamae* of maize, sugarcane and coffee (Caballero-Mellado *et al.*, 2004), *B. ambifaria* from pea in U.S.A. (Richardson *et al.*, 2002), *B. silvatlantica* from maize in Brazil (Perin *et al.*, 2006) and *B. caledonica* from diverse plant rhizospheres in Scotland (Coenye *et al.*, 2001).

Plant hosts can be colonized by more than one species of the genus *Burkholderia*. *B. vietnamiensis*, which was first described in the rice rhizosphere in Vietnam (Tran Van *et al.*, 1994; Gillis *et al.*, 1995), was thus isolated along with other *Burkholderia* species from the rhizosphere of maize and coffee in Mexico (Estrada de los Santos *et al.*, 2001). Furthermore, a recent study on *Sphagnum* rhizophere of peat bogs of the boreal and tundra zones of Russia, Canada and Estonia demonstrated also that this plant forms associations with several *Burkholderia* spp. (Belova *et al.*, 2006). It has also been documented that different soil/crop management practices and land use history affect diversity of *Burkholderia* spp. in the rhizosphere of plant crops (Salles *et al.*, 2006), indicating that the formation of plant-microbial associations is under both plant genetic and environmental control.

The endophytic *Burkholderia* sp. and their plant hosts

Isolation of bacteria from surface-sterilized plant roots or shoots has often been reported during plant-bacteria studies. It is now recognized that some rhizosphere bacteria can enter internal tissues of plants and thrive as endophytes without causing harm to the host (Ait Barka *et al.*, 2000; Sturz *et al.*, 2000; Hallmann, 2001; Gray and Smith, 2005). Recently, the analysis of N_2-fixing bacteria associated with maize and coffee plants grown under field conditions revealed the presence of *B. vietnamiensis*, as well as the richness of novel diazotrophic bacterial species belonging to the genus *Burkholderia* (Estrada-de los Santos *et al.*, 2001; Estrada *et al.*, 2002), identified recently as *B. tropica* (Reis *et al.*, 2004). Such bacteria are able to enter the root tissue, transcend the endodermis barrier, cross from the root cortex to the vascular system and subsequently establish endophytic populations in various organs. This has been described in a wide range of plants including both monocotyledonous and dicotyledonous (Balandreau *et*

Table 1: Classified rhizospheric and endophytic *Burkholderia* spp. isolated from plant hosts.

Species	Plants	Refs
Rhizospheric isolates		
B. ambifaria	pea, maize	Richardson *et al*., 2002
B. caledonica	grapevine	Coenye *et al*., 2001
B. cepacia	maize,	Ramette *et al*., 2005
	pea, coton,	Parke and Gurian-Sherman, 2001
	birch trees, oak trees, common groundsel, horsetail, arum lily, wheat	Richardson *et al*., 2002
B. graminis	corn, pasture, wheat	Viallard *et al*., 1998
B. silvatlantica	maize	Perin *et al*., 2006
B. unamae	maize, sugarcane coffee	Caballero-Mellado *et al*., 2004
B. vietnamiensis	rice,	Gillis *et al.*, 1995
	maize, coffee	Estrada de los Santos *et al*., 2001
Endophytic isolates		
B. cepacia	wheat, lupine, maize,	Balandreau *et al*., 2001
	citrus,	Araujo *et al.* 2002
	rice	Singh *et al*., 2006
B. gladioli	coffee	Vega *et al*., 1995
B. phytofirmans	onion	Sessitsch *et al*., 2005
B. pyrrocinia	lodgepole pine	Bal and Chanway, 2000
B. silvatlantica	sugar cane	Perin *et al*., 2006
B. tropica	pineapple	Cruz *et al*., 2001

al., 2001; Araujo *et al.*, 2002; Compant *et al*., 2005a; Sessitsch *et al*., 2005). Some of these *Burkholderia* sp. have been identified (Table 1). For instance, *B. phytofirmans* (Sessitsch *et al*., 2005) was thus isolated as an endophyte from onion roots in Canada (Nowak and Shulaev, 2003), and the well known *B. cepacia*, from branches of *Citrus* cultivated in Brazil (Araujo *et al.*, 2002) and from roots of rice in India (Singh *et al*., 2006). Others *Burkholderia* sp. such as *B. cenocepacia and* the newly-described species *B. silvatlantica* were also determined as endophytes. *B. cenocepacia*, was thus isolated from the inner tissues of wheat, lupine and maize in a study conducted in France and Australia (Balandreau *et al*., 2001) and *B. silvatlantica*, from surface-desinfected leaves of sugar cane in Brazil (Perin *et al*., 2006). Endophytic *Burkholderia* sp. can be even found in gymnospermae as described by Bal and Chanway (2000) who isolated *B. pyrrocinia* from the stems of lodgepole pine and other *Burkholderia* spp. from stems of western red cedar grown in Canada, demonstrating the wide host range of *Burkholderia* spp.

Recent evidences suggested that some *Burkholderia* can also thrive as endophytes in both vegetative and reproductive organs of their plant hosts. This includes *B. tropica* originally designated as *B. tropicalis*, which was isolated from the stems and fruit of pineapple in Brazil (Cruz *et al.*, 2001), and *B. gladioli* which was isolated from roots, stems, seeds and berries of coffee, together with other *Burkholderia* strains inside the pulp and seeds of coffee berries (Vega *et al.*, 2005).

Burkholderia sp. as endosymbionts of beneficial endophytic fungi

Recently, evidences on niches speciation of some species of the genus *Burkholderia* have revealed that certains of these species are potent endosymbionts in beneficial endophytic fungi, especially in mycorrhiza (Nurmiaho-Lassila *et al.*, 1997; Timonen *et al*., 1998). The association of *Burkholderia* with ectomycorrhizal fungi forming has been thus demontrated with *Suillus variegatus* and *Tomentellopsis submollis* in two corsican *Pinus nigra* (Izumi *et al.*, 2007). Using the *Pinus radiata –Rhizopogon luteolus* symbiosis, Garbaye and Bowen (1989), further proof the role of Burkholderiales by showing that a number of bacteria isolated from inside the ectomycorrhizal mantle had stimulatory effects on the mycelial growth of *R. luteolus*, and in some cases, enhanced mycorrhiza formation. *Burkholderia* spp. have also been detected as endosymbionts in endomycorrhizal

Table 2: Classified *Burkholderia* spp. and plant nodulation

Species	Nodulated plant	Refs
Root endosymbiont	**Fabaceae**	
B. tuberum	*Aspalathus carnosa*	Vandamme *et al*., 2002
B. phymatum	*Machaerium lunatum, Mimosa invisa, Mimosa pudica*	Vandamme *et al*., 2002 Elliott *et al.*, 2007
B. caribensis	*Mimosa diplotricha* *M. pudica*	Chen *et al*., 2003a
B. mimosarum	*Mimosa* spp.	Chen *et al*., 2005a, b, 2006
B. nodosa	*Mimosa* spp.	Chen *et al*., 2005b, 2007
Leaf endosymbiont	**Rubiaceae**	
'*Candidatus* Burkholderia calva'	*Psychotria calva*	Van Oevelen *et al*., 2004
'*Candidatus* Burkholderia kirkii'	*Psychotria kirkii*	Van Oevelen *et al*., 2002
'*Candidatus* Burkholderia nigropunctata'	*Psychotria nigropunctata*	Van Oevelen *et al*., 2004

fungi, such as *Gigaspora margarita* (Bianciotto *et al.*, 2000; Ruiz-Lozano and Bonfante, 2000), forming beneficial associations with herbaceous plants. Furthermore, Andrade *et al*. (1997) found that *B. "cepacia"* was ubiquitous in the hyphosphere of three arbuscular fungi tested, while other bacterial species were not as widely distributed which demonstrate that some species of *Burkholderia* are common inhabitants of symbiotic fungi.

Burkholderia sp. and plant nodulation

Actually, there is ample evidence that the genus *Burkholderia* play a key role in plant nodulation.

Since the 19th century, research on plant-bacteria symbiotic associations has been focussed on the interaction between leguminous plants and gram-negative *α-Proteobacteria* within, or closely-related to, the genus *Rhizobium* (Hirsch *et al.*, 2001). Lately, bacteria in the *Burkholderiales* (*Cupriavidus* and *Burkholderia*) have also been isolated from plant nodules and recognized as effective symbionts associated with roots of *Fabaceae* and leaves of *Rubiaceae* (Table 2). Historically, it was demonstrated that some soil microorganisms, like *B. cepacia*, facilitate the infection of plant nodulating *Frankia* sp., probably through induction of root hair deformation (Knowlton *et al.*, 1980). Later on, Knowlton and Dawson (1983) reported that this strain helped nodulation, increasing up to four times the number of nodules, but was not an absolute prerequisite. Then, *B. tuberum* was isolated from root nodules of *Aspalathus carnosa* in South Africa, *B. phymatum* from *Machaerium lunatum* in French Guiana (Moulin *et al*., 2001; Vandamme *et al*., 2002), and *B. caribensis* from *Mimosa diplotricha* and *M. pudica* in Taiwan (Chen *et al*., 2003a). As a follow of this, several *Burkholderia* strains were then isolated from *Abarema macradenia* and *Pithecellobium hymenaefolium* in Panama and Costa Rica (Barrett and Parker, 2005, 2006) and from nodules of Mimosoid legumes, such as *B. phymatum* from *M. invisa* and *M. pudica* in Papua New Guinea (Elliott *et al*., 2007), *M. pigra*, *M. casta*, *M. pudica*, in Panama and Costa Rica (Barrett and Parker, 2005, 2006), *Mimosa pudica* in India (Pandey *et al*., 2005), and from *Mimosa* spp. in Taiwan, Venezuela, and Brazil (Chen *et al.*, 2005a, b). This research area has lead to the discoveries of newly-described species such as for example *B. mimosarum* (Chen *et al.*, 2006) and *B. nodosa* (Chen *et al*., 2007) and further identification of the others strains will surely lead to the discovery of new species of *Burkholderia*.

Actually, there is even more and more evidence that the genus *Burkholderia* exhibits affinity to Mimosoid legumes. *B. phymatum* for example can nodulate a large number of *Mimosa* species, in a wide range of geographic zones and taxa (Elliott *et al*., 2007). This demonstrates that the genus *Mimosa* forms symbiotic associations with *β-Proteobacteria* that differs from other legumes that are nodulated by *α-Proteobacteria.* Attempts to nodulate *Mimosa* with *α-Proteobacteria* have resulted in ineffective symbioses (Barrett and Parker, 2005), suggesting a close co-evolution of Mimosoid legumes and *Burkholderia* spp. The same observation was recently observed by studying nodulation of four *Papillonaceae* (*Vigna* sp., *Clitoria* sp., *Crotalaria* sp. *and Centrosema* sp.) and results of this study demonstrate that nodules contain equally rhizobial species and *Burkholderia* species (J. Balandreau, personnal communication).

However, although *Burkholderia* and β-rhizobial species had been isolated from leguminous root nodules, their direct involvement in the formation of root nodules and nitrogen fixation has been documented only recently. *Burkholderia* sp. and *Cupriavidus taiwanensis* (syn. *Ralstonia taiwanensis*) contain thus symbiosis-essential

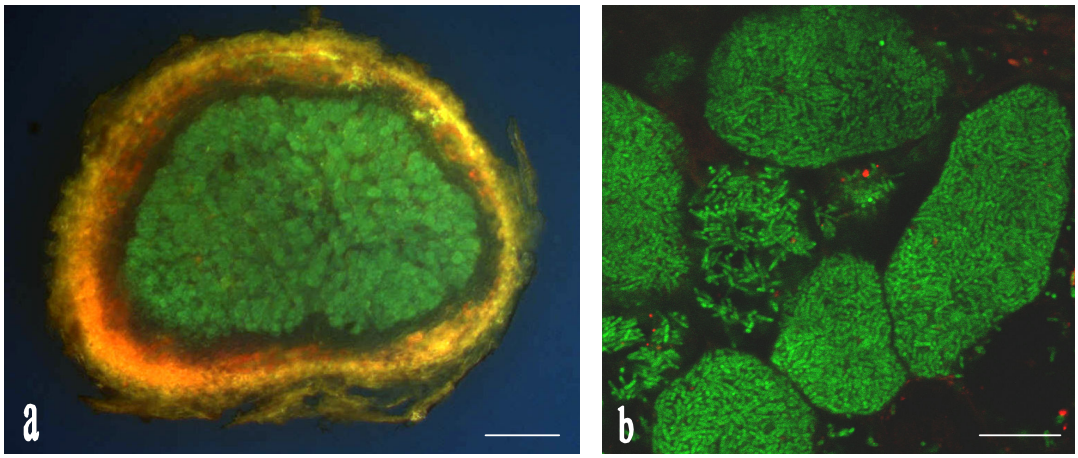

Fig. 1: Microphotographs under confocal microscope of section of *Mimosa pudica* nodules infected with *B. phymatum* STM815GFP (a and b). Scale bars: (a) 500 µm and (b) 5 µm. Pictures provided by Dr. Geoffrey N. Eliott (School of life Sciences, University of Dundee, UK).

genes such as *nod* and *nif* and exhibit significant acetylene reduction activity (Barrett and Parker, 2005, 2006; Chen *et al*., 2003a, b; Chen *et al.*, 2005a, b; Elliott *et al*., 2007). As a further proof of their role in nodule formation, similar to rhizobia, their nodulation activity has been confirmed through light and electron microscopy studies with green-fluorescent protein (*gfp*) marked strains (Chen *et al*., 2003b; Chen *et al.*, 2005a, b; Elliott *et al*., 2007; Figures 1a and 1b).

The occurrence of bacterial leaf nodules in some genera of angiosperms (tropical plants belonging to *Rubiaceae*, *Myrsinaceae* and *Dioscoreaceae*) has been known since 1902 (Zimmermann, 1902) and some of them have been recently attribued to the genus *Burkholderia*. Leaves of these tropical plants can develop nodules allowing for symbiotic interaction between bacteria and their host (Silver *et al.* 1963; Centifanto and Silver, 1964; Lersten and Horner, 1967; Whitmoyer and Horner, 1970; Miller *et al.*, 1983). This symbiosis is correlated to the fact that the bacterial endosymbionts form intimate associations with their host throughout the plant's entire life cycle as well as can be transmitted to the next generation *via* seed transmission without the need for an external infection event (von Faber, 1912, Miller, 1990). In *Rubiaceae*, known leaf endosymbionts of *Psychotria* spp. include actually the recently designated *Burkholderia* spp., 'Candidatus *Burkholderia calva'* (van Oevelen *et al.*, 2004), 'Candidatus *Burkholderia kirkii'* (van Oevelen *et al.*, 2002), and 'Candidatus *Burkholderia nigropunctata'* (van Oevelen *et al*., 2004). Further studies on this plant bacterial association have demonstrated that plant tissue cultures of *Psychotria* sp. without the bacteria had distorted leaves, stunted growth and eventually died, demonstrating an obligate association between *Burkholderia* spp. and *Psychotria* spp. (van Oevelen *et al.*, 2003).

Use of *Burkholderia* sp. for biocontrol of plant diseases and plant health stimulation

Biocontrol properties of some *Burkholderia* sp.

Biological control of soil-borne plant pathogens with bacteria has been studied as an alternative or complementary approach to physical and chemical disease control measures for the past three decades (Compant *et al.*, 2005a). In the last few years, attention has also been focused on the potential impact of plant pathogens on biocontrol agents and their disease-suppressive activity. Nowadays, the genus *Burkholderia* has become popular in the agricultural sector as a result of the biocontrol abilities of some species (Hebbar *et al.*, 1998; Vandamme *et al.*, 2007).

As described above, *Burkholderia* spp. can be free-living and endophytic forming mutualistic and symbiotic associations that benefit plant growth and health status. Several *Burkhoderia* species are thus, considered to be beneficial in the natural environment. These species are well-known for their biological and metabolic properties which can be exploited for biological control of fungal diseases in plants but also for bioremediation and plant growth promotion (Govan *et al.*, 1996; Holmes *et al.*, 1998; Parke and Gurian-Sherman, 2001). Many species belonging to the genus *Burkholderia* have thus the ability to produce compounds with antimicrobial activity (Hu and Young, 1998; Kang *et al.*, 1998) and can potentially be used as biocontrol agents of phytopathogenic fungi. This was well demonstrated with *B. cenopacia*, *B. cepacia*, *B. ambifaria*, *B. pyrrocinia*, *B. vietnamiensis* and *B. phytofirmans* strains towards *Pythium aphanidermatum*, *Pythium ultimun*, *Fusarium* sp., *Phytophthora capsici*, *Botrytis cinerea*, and/or *Rhizoctonia solani* (Parke, 1990; Parke *et al.*, 1991; McLoughlin *et al.*, 1992; Bowers and Parke, 1993; King and Parke, 1993; Hebbar *et al*., 1992; 1998; Heydari and

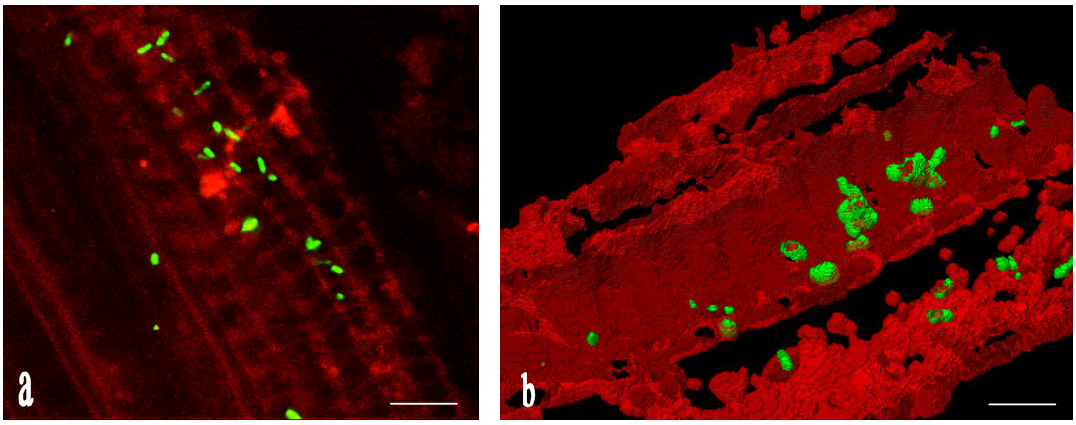

Fig. 2: Microphotographs under confocal microscope (a) and 3D reconstruction (b) of grapevine roots inoculated with *Burkholderia phytofirmans* strain PsJN tagged with *gfp* showing *gfp*-bacteria inside root xylem vessels. Scale bars: (a) 20 µm and (b) 15 µm. Pictures by Stephane Compant and Dr. Hervé Kaplan (University of Reims, France).

Misaghi, 1998; Heugens and Parke, 2000; Cain *et al*., 2000; Ait Barka *et al*., 2000; 2002; Parke and Gurian-Sherman, 2001; Singh *et al*., 2006). Many *Burkholderia* species have thus antifungal properties but are also able to inhibit growth of other bacteria and of protozoa (Cain *et al*., 2000). Their ability to suppress plant diseases was observed in many different crops, such as corn, sweet corn, cotton, pea, tomato, and pepper. Moreover, crop rotation and soil management practices affect population diversity and numbers of the antagonistic strains of *Burkholderia* (van Elsas *et al*., 2002; Salles *et al*., 2006).
Similar to other plant growth-promoting bacteria, beneficial effects of *Burkholderia* involve diverse mechanisms of action, including rhizosphere competence determining their population density on root surface, secretion of allelochemicals, including antibiotics and siderophores, competition for nutrients, induced systemic resistance (Baldani *et al*., 2000; Van loon *et al*., 1998; Welbaum *et al*., 2004; Compant *et al*., 2005b), and potentially others. The outlined beneficial properties of *Burkholderia* spp. indicate that they can be potentially utilized in agricultural production as a substitute, or a complementary agent to chemicals in management of plant diseases. However, there is more and more precaution taken using certains species of the genus *Burkholderia* in particular for *B. mallei*, *B. pseudomallei*, *B. gladioli*, and species of the *B. cepacia* complex which can have biocontrol properties, but as the same time can be opportunictic human pathogens although nothing is known actually whether if the bad and good strains are actually the same (Coenye and Vandamme, 2003). Searching others *Burkholderia* sp. with biocontrol properties has been thus an alternative and the potential of some new species are actually under investigation as for strain PsJN of *B. phytofirmans* which directly inhibits *Botrytis cinerea* growth (Ait Barka *et al*., 2000; 2002) or can induce defense plant responses (Compant *et al*., 2005b) which lead indirectly to protection of the host plant toward this phytopathogenic fungus (Compant *et al*., in preparation).

Use of *Burkholderia* spp. for direct stimulation of plant growth enhancement

Some members of the genus *Burkholderia* can also directly stimulate plant growth. As an example, *Burkholderia* spp. inoculation of crops such as maize and sorghum has thus resulted in increases in both root and shoot dry weights (Chiarini *et al*., 1998; Bevivino *et al*., 2000). It is likely that rhizosphere adapted bacteria simultaneously use several mechanisms to increase the growth of their host-plant (Govindarajan *et al*., 2006). As demonstrated for *B. vietnamiensis* (Tran Van *et al*., 2000), production of phytohormones and/or fixation of atmospheric nitrogen, are common causes of plant growth promotion induced by this genus. Estrada de los Santos *et al*. (2001) isolated also new diazotrophic *Burkholderia* species from coffee and maize plants which were phylogenetically unrelated to *B. vietnamiensis* which further proofs the wide range capacity of Burkholderiales to fix atmospheric nitrogen.
Research on mechanisms involved in plant growth promotion by *Burkholderia* sp. have also demonstrated that some members of this genus such as *B. phytofirmans* strain PsJN can express a quinolinate phosphoribosyl transferase (Wang *et al*., 2006) or a high level of 1-aminocyclopropane-1-carboxylate deaminase (Sessitsch *et al*., 2005). This later enzyme is well known for hydrolyze the precursor of the inhibitory hormone ethylene, 1-aminocyclopropane-1-carboxylate By lowering the production of this hormone *in planta*, the bacterium can thus decrease the inhibitory effect of ethylene on root elongation and stimulation of senescence which occurs upon plan exposure to stress (Sessitsch *et al*., 2005).
Recently, Ait Barka *et al*. (2007) also reported interestingly that *in vitro* inoculation of *Vitis vinifera* L. cv. Chardonnay explants with *Burkholderia phytofirmans*

strain PsJN, increased grapevine growth and physiological activity at a low temperature, improving thus plant ability to withstand cold stress. Because the nucleation temperature of plants increases with increasing population sizes of Ice^+ bacteria, Ait Barka *et al.* (2007) suggest thus that preemptive competitive exclusion of Ice^+ bacteria with naturally occurring non-ice nucleation-active bacteria such *B. phtofirmans* strain PsJN could be an effective and practical means of frost control, demonstrating thus the wide range properties of *Burkholderia* spp.

Endophytic establishment of some *Burkholderia* sp. in non natural hosts

As a follow on research on plant-Burkholderia sp. association, some studies have reported that certains *Burkholderia* spp. can thrive as epi- and endophytes in plants other than the original hosts. The *B. phytofirmans* strain PsJN, originally isolated from surface desinfected roots of onion, can thus readily establish rhizospheric and endophytic populations in grapevine (as demonstrated by *gfp* derivative, Figure 2a and 2b), potato, tomato, cucumber, watermelon and chickpea (Frommel *et al*., 1991; Nowak and Shulaev, 2003; Ait Barka *et al*., 2000; Compant *et al*., 2005b; Sessitsch *et al*., 2005; Compant *et al*., submitted). This demonstrates that *Burkholderia* strains may be potentially utilized as rhizospheric and endophytic inoculants, for stimulation of plant growth and management of plant health. Nowadays, the use of endophytic bacteria for plant disease management has great potentials as the bacteria can act as direct bio-protectants of specific micro-environmental niches as well as prime plant metabolism to respond faster to encountered stresses (reviewed in Nowak and Shulaev, 2003). Furthermore, endophytes are more effective than free-living bacteria colonizing root and leaf surfaces as they form closer associations with the plant hosts (Conn *et al*., 1997; Chanway *et al*., 2000) and such bacteria can translocate to the plant reproductive organs and thus can be used as delivery agents in the development of sustainable crop rotations (Sturz *et al*., 2000).

Use of *Burkholderia* species to protect plants: what we have to know before?

Searching for new *Burkholderia* species and strains of special interest needs however to address the issue whether the same genotypes can be pathogenic or non pathogenic depending upon the environment (Coenye and Vandamme, 2003). The origin of the differences in the level of human, animal and plant pathogenicity or non pathogen within *Burkholderia* species seems very likely determined by both the bacterial genome and its evolutionary adaptation to specific environments (Holden *et al.*, 2004), especially for a small set of genes (Kim *et al*., 2005). However, the present advances in sequencing of *Burkholderia* genomes such as for various strains of *B. ambifaria, B. cepacia*, *B. cenopacia*, *B. dolosa, B. glumae*, *B. mallei*, *B. multivorans, B. phymatum, B. phytofirmans, B. pseudomallei*, *B. thailandensis*, *B. vietnamiensis*, *B. xenovorans* (http://www.ncbi.nlm.nih.gov/) will undoubtely contribute to a better understanding of the adaptation phenomena of these common proteobacteria and development of new management practices to control their pathogenicity and/or to harness their benefits.

One of the regulatory criteria determining the use of *Burkholderia* species for plant protection, biofertilization, or phytostimulation could come however from their phylogenetic distance as between the known human and animal pathogens. The potential for horizontal gene transfer between non pathogenic and pathogenic strains, such as opportunistic acquisition of deleterious DNA sequences by some strains of *B. cepacia* from Human pathogens, may nevertheless still exist. Recently, insertion sequences within *B. pseudomallei* have even been identified in *B. cepacia,* including an isolate belonging to an epidemic strain (Jones *et al.,* 2001). This further proof that the genome sequencing of members of Burkholderiales or its analyses is required before that some *Burkholderia* sp. will be used in agriculture, although actually more and more species of *Burkholderia* are known as non pathogenic for both human and plant.

Conclusions and future prospects

Burkholderia spp. are one of the most abundant bacteria in the environment. Their plasticity of their genomes, capable of adapting to changing conditions can allow them to colonize diverse environmental niches. Several species of this genus are classified as human, animal or plant pathogens but others exhibit beneficial effects on their plant hosts, and can potentially be utilized as powerful pesticides in control of soil-borne diseases. Some *Burkholderia* can also be harnessed as biofertilizers either fixing nitrogen or releasing phosphorus from rock phosphates to the benefit of crops cultivated in low fertility soils, thus reducing inputs of costly fertilizers. The potential economic and ecological benefits can be considerable. A widespread use would however increase human exposure to these potentially hazardous bacteria. Sequencing of the *Burkholderia* species will undoubtedly generate new knowledge about their genetic makeup and ranges of molecular and functional adaptation within each species. We also would like to know how the host and/or co-colonizing microflora can modulate such adaptation? These and other gaps of knowledge still need to be filled before *Burkholderia* can be utilized to their full potential in the development of new crop management practices and/or in industrial processes. The conflict of interest between human health and agriculture needs to be resolved.

Acknowledgements

We would like to thank Jacques Balandreau for providing information on the genus *Burkholderia*. We are gratefull to Dr. Geoffrey N. Eliott (School of life Sciences, University of Dundee, Dundee, DD1 5EH,UK) for providing confocal pictures of *B. phymatum* STM

815 in *Mimosa pudica* nodules and to Dr. Hervé Kaplan (University of Reims, France) for helping in confocal scanning of roots of *Vitis vinifera* L. inoculated with *B. phytofirmans* strain PsJN and 3D reconstruction. We are also gratefull to Dr. Euan K. James (School of life Sciences, University of Dundee, Dundee, DD1 5EH,UK) for critical reading of the manuscript. This work was partly supported by a grant from Europôl-Agro (Reims, France).

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Polyphenolic compound accumulation as a response of grapevine (*Vitis vinifera* L.) to colonization by *Burkholderia phytofirmans* strain PsJN.

Stéphane Compant[1], Jerzy Nowak[2], Christophe Clément[1], and Essaïd Ait Barka[1]

Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UPRES EA 2069, UFR Sciences, Université de Reims Champagne-Ardenne, 51687 Reims Cédex 2, France[1];

Department of Horticulture, Virginia Polytechnic Institute and State University, 0327-301 Saunders Hall, Blacksburg, VA 24060, USA[2]

"Macromolecules from grape and vine"
(Jeandet P., Conreux A. and Clément C., eds),
Lavoisier, Paris, France. 2007, pp. 89-93.

Macromolecules and Secondary Metabolites from Grapevine and Wine

Polyphenolic Compound Accumulation as a Response of Grapevine (*Vitis vinifera* L.) to colonization by *Burkholderia phytofirmans* Strain PsJN

S. Compant[1], J. Nowak[2], C. Clément[1] and E. Ait Barka[1]

[1]Laboratoire de Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne, UFR Sciences – UPRES EA 2069, Université de Reims Champagne-Ardenne, 51687 Reims Cedex 2, France
[2]Department of Horticulture, Virginia Polytechnic Institute and State [3]University, 0327-301 Saunders Hall, Blacksburg, VA 24060, USA

Plants are natural habitats for a variety of microorganisms, some of which are deleterious causing diseases whereas others are beneficial to their host. Among the latters, plant growth-promoting bacteria (PGPB) are a proeminent group of beneficial microorganisms commonly associated with diverse plant species in various environments [4, 14]. These microorganisms can stimulate plant growth either directly, by producing phyto- hormones and/or allelochemical suppressing phytopathogens, or, indirectly, by inducing systemic resistance in the plant host to biotic and abiotic stresses [4, 14].
Grapevine is susceptible to infection by viruses, bacteria and fungi. To cope with this challenge, viticulturists use agrochemicals as a relatively reliable method of plant protection. However, these products can induce pathogen resistance to the active ingredients as well as they can have non-target environmental impacts. PGPBs are considered as alternative, or complementary bio- control agents that may significantly reduce the use of chemicals in agriculture [4].
PGPB-induced-plant host defenses against phytopathogens involve a large arsenal of responses, which include strengthening of the plant cell wall and synthesis of plant defense compounds such as PR proteins, peroxidases, phenylalanine ammonia lyase (PAL), polyphenol oxidases, chalcone synthase and/or various phenolics [4, 14].
Burkholderia phytofirmans strain PsJN, is an effective plant growth-promoting bacterium which can enhance plant growth and resistance to environmental stresses without harming their host [13]. This bacterium is able to establish epi- and endophytic subpopulations and thrive as endophyte in potato, tomato, cucumber, watermelon, chickpea and grapevine [1, 3, 5, 11, 13]. Our own previously published results demonstrated that this bacterium induces host defense responses following epi- and endophytic colonization [3]. The present work provides further evidence of the interaction between the grapevine and the colonizing bacterium.

Material and Methods

Bacterial growth and inoculum preparation: The wild type of *B. phytofirmans* strain PsJN was grown in King's B medium as described in Pillay and Nowak [12]. The bacterial inoculum was transferred to 100 mL of King's B liquid medium containing the appropriate antibiotic in a 250 ml erlenmeyer flask and incubated at

20°C on a shaker (150 rpm) for 48 h. Bacteria were collected by centrifugation (4,500 x g, 15 min) and washed twice with phosphate-buffered saline (pH 6.5) (PBS). The concentration of the inoculum was then adjusted to approximately 3 x 10^8 CFU/mL with PBS, based on absorbance at 600 nm, confirmed by plate counting as described by Pillay and Nowak [12].

Plant material, growth conditions and inoculation: Disease-free plantlets of *Vitis vinifera* L. cv. Chardonnay were propagated by nodal explants in 25 mm diameter test tubes containing 15 mL of Martin medium [10]. Cultures were grown in a growth chamber under white fluorescent light (200 µmol.m^{-2}.s^{-1}) with a 16 h photoperiod at 26°C (constant temperature).

Plant inoculation was done by spreading 250 µL aliquots of the bacterial suspension in PBS on the surface of Martin medium [10]. Five-week-old rooted plantlets with five developed leaves were then delicately transferred into the new test tubes which were previously inoculated with bacteria. Attention was paid that only the roots were in contact with the bacterial inoculum. Non-inoculated checks were treated in the same way using PBS (control). The plantlets were then incubated in the culture chamber as described above.

Microscopic observation of phenolic compound accumulation in grapevine roots inoculated with *B. phytofirmans* strain PsJN: Fresh roots removed from six plantlets inoculated with either strain PsJN or a control (PBS) were collected 96 h after inoculation. Samples were then prepared for microscopy analysis as described in Compant et al. [3], with some modifications. The roots were fixed for 24 h at room temperature in 2% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.24) with 2% (w/v) sucrose and 0.1% (v/v) Tween 20. After three rinses (5 min each) with the phosphate buffer containing 2% (w/v) sucrose, samples were fixed for 4 h in 1% (w/v) osmium tetroxide in phosphate buffer with 2% (w/v) sucrose. The samples were then dehydrated in an alcohol series, transferred to acetone, and finally embedded in araldite. Semi-thin sections (1 µm) from different treatments were performed with a microtome (Jung RM2055; Leica, Nussloch, Germany), collected on glass slides, stained with 0.1% toluidine blue, and examined with a microscope (BH2; Olympus).
In parallel, hand-cut transverse sections of roots of 6 plantlets inoculated with either PBS (control) or PsJN bacteria were collected to detect host defense reactions by visualizing autofluorescence using epifluorescence microscopy.

Results

Cell-wall strenghtening in exodermis and cortex: A strenghtening of the cell wall was detected in root internal tissues (**Figures 1a-b**) 96 hours post inoculation with PsJN. Compared to the non-inoculated control (**Figure 1a**) an intense cell-wall thickening was seen in the exodermis and some cortical cells (**Figure 1b**).

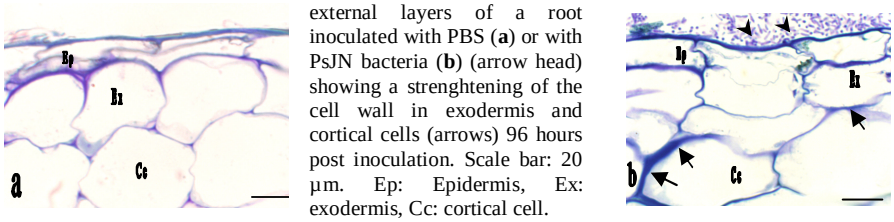

Figure 1: Microphotographs of external layers of a root inoculated with PBS (**a**) or with PsJN bacteria (**b**) (arrow head) showing a strenghtening of the cell wall in exodermis and cortical cells (arrows) 96 hours post inoculation. Scale bar: 20 µm. Ep: Epidermis, Ex: exodermis, Cc: cortical cell.

Phenolic compounds accumulation in cortical cell layers: In the roots of control plantlets, rhizodermis and endodermis layers, as well as xylem vessels, exhibited a yellow fluorescence under blue light within 96 hours post inoculation, indicating the accumulation of phenolic compounds in the cell wall (**Figure 2a**). A yellow auto-fluorescence was also observed in the roots of plantlets inoculated with PsJN, although, in comparison to control, several additional cortical cell layers exhibited fluorescence (**Figure 2b**).

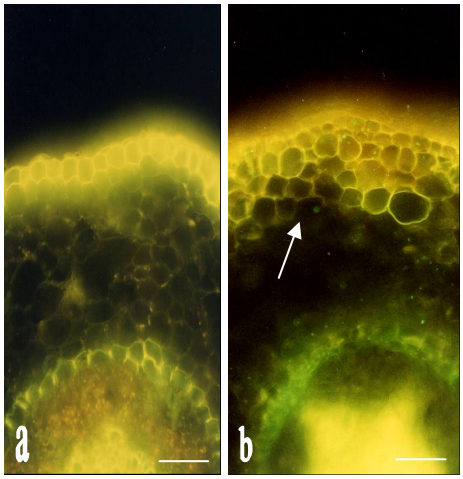

Figure 2: Epifluorescence microphotographs of root internal tissues of *V. vinifera* L. cv. Chardonnay plantlets 96 hours after inoculation with control (PBS) (**a**) or *B. phytofirmans* strain PsJN (**b**), showing accumulation of fluorescent phenolic compounds in the cortical cell layers (arrow). Scale bars: 100 µm.

Xylem defense responses: Grapevine defense reponses have also been visualised in xylem within 96 hours post inoculation with the PsJN (**Figure 3a**). A strenghtening of the xylem cell wall (**Figure 3b**), probably of the xylem parenchymal cell walls, was observed. Such responses were not observed in the control experiments (data not shown).

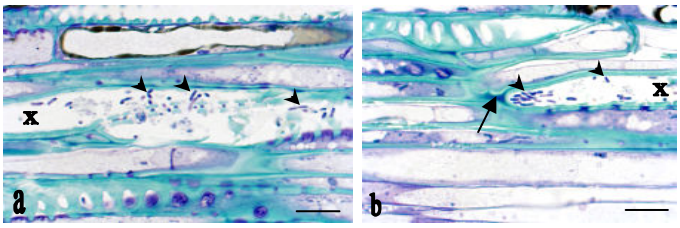

Figure 3: Microphotographs of grapevine root xylem colonization by *B. phytofirmans* strain PsJN showing PsJN bacteria in xylem vessels (**a, b**) (arrow heads) and xylem defense as strengthening of the xylem cell wall (b) (arrows). Scale bars: 10 µm. x: xylem.

Discussion

We have previously reported that grapevine defense responses are induced after inoculation with the plant growth-promoting bacterium *Burkholderia phytofirmans* strain PsJN [3]. This study provides further evidence of the induction of grapevine defenses following endophytic colonization by this plant- beneficial bacterium that includes accumulation of phenolics in several cortical cell layers, cell-wall strenghtening in exodermis, cortex and xylem vessels. Other studies on plant endophytic bacteria have linked these types of responses to the plant defense reactions usually observed upon infection of host plants by pathogens [4, 6, 8, 9]. Moreover, *B. phytofirmans* strain PsJN secretes cell-wall degrading enzymes enabling the bacterium to enter root internal tissues [3], the phenomenon usually linked to localized host infection by pathogens [7]. The data reported here demonstrate localized defense reactions in root tissues upon PsJN inoculation. Our most recent results indicate that similar responses occur in the plant when colonized by a PsJN that induces a systemic resistance associated with a decrease in the infection rate caused by a necrotrophic phytopathogen, *Botrytis cinerea* (Compant et al., unpublished results).

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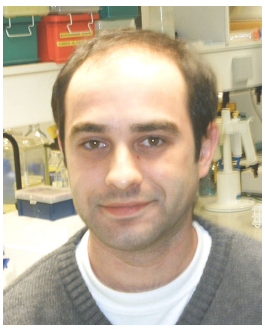
COMPANT Stéphane
Laboratoire SDRP
Unité de Recherche Vignes et Vins de Champagne,
UPRES EA 2069, UFR Sciences,
Université de Reims Champagne-Ardenne,
51687 Reims Cédex 2,
France
Phone +33 3 26 91 34 41
e-mail : stephane.compant@univ-reims.fr



Curriculum Vitae

Personnal Data

Date of Birth : 05/28/1977 (Wassy, France)

Marital statut: Single

French Nationality

Education

2007-2007	**Visiting Scientist**	Seibersdorf, Austria
2003-2007	**PhD** Plant Biology and Physiology	Reims, France
2001-2002	**MS degree in Sciences**	UTC Compiègne, France
2000-2001	**BS degree in Biology**	Reims, France

Academic appointments

2005-2007 **ATER (equivalent to Assistant Professor)** Reims, France

80-96 h teaching per year

Courses involving: Mechanisms of Biocontrol by Plant Growth Promoting Fungi and Plant Growth-Promoting Rhizobacteria

Microbial ecology of beneficial microorganisms
Interaction between rhizobia and leguminous plants
Plant Culture in Champagne-Ardenne
In vitro Cultures
How to read and write a scientific publication
The diverse Kingdoms of Nature

2004-2005 **Lecturer** Reims, France
10 h teaching
Courses involving: Interaction between rhizobia and leguminous plants
Plant Culture in Champagne-Ardenne

Research Interest

Microbial ecology, rhizosphere, endophyte, induced systemic resistance, allelochemical synthesis, elicitation, *Burkholderia* spp., *Vitis vinifera* L.

Student Training

2005-2007 **Mélanie Benard. Study on chemotaxis of *Burkholderia phytofirmans* strain PsJN towards root exudates from grapevine.** Bs and Ms degree.

2004-2005 **David Franka. Rhizospheric and endophytic competences of *Burkholderia phytofirmans* strain PsJN in *Vitis vinifera* L.** Bs degree.

Publications

Compant S., Duffy B., Nowak J., Clément C., and Ait Barka E. (2005a) **Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects.** *Applied and Environmental Microbiology* 71: 4951-4959.

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Posters

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Award

Finalist for oral communication: Biological control in grapevine: comprehension of mechanism of action of biocontrol bacteria in grapevine against *B. cinerea*. "Prix national millésime Europol-Agro" Maison des Agriculteurs, Reims, France. 2006.

1st price for oral communication: Microscopic study of endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN. "Concours Jeunes chercheurs de la Société de Biologie de Reims" Reims, France. 2005.

Grant

PhD grant from Europôl-Agro and Vineal associations (Reims, France).

PhD thesis

09/2003-11/2007 **Interaction between grapevine and the endophytic bacterium *Burkholderia phytofirmans* strain PsJN: colonization, induced defense responses and systemic resistance towards *Botrytis cinerea*.** SDRP, UFR Sciences, Reims, France. Directors: Dr. Ait Barka E. and Pr. Clément C.

Internships before PhD

2002-2003 **Scientist.** Confidential industrial projects involving microorganisms based bioproducts for controlling grapevine gray mold caused by *Botrytis cinerea*. Laboratory SDRP, UFR Sciences, Reims, France.

09/2001-09/2002 **Master of Sciences: Caracterization of the efficacity of systemic biofungicides containing chitosan for controlling the phytopathogenic fungus *Botrytis cinerea* in grapevine *Vitis vinifera* L.** Laboratory SDRP, UFR Sciences, Reims, France. Directors: Dr. Ait Barka E. and Pr. Clément C.

06/2001-08/2001 **Application of R.A.P.D. technique for research of polymorphism between various strains of *Botrytis cinerea.*** Laboratory SDRP, UFR Sciences, Reims, France. Director: Dr. Mazeyrat-Gourbeyre F.

2000-2001 **Characterization of sequences and expressions of *Abdominal-B* gene in uro-genital development of both vertebrates and invertebrates.** Laboratory of Cellular and Molecular Biology, UFR Sciences, Reims, France. Directors: Pr. Tarpin M. and Pr. Bierne J.

Internships after PhD

08/2007-09/2007 **Incidence of girdling and soil fertilization on bacterial communities in bulk soil and rhizosphere of forest trees of Vienna.** Austrian Research Centers, Seibersdorf, Austria. Directors: Dr. Sessitsch A. and Dr. Rasche F.

Private Professional Activities

09/08/1999-10/09/1999	Laboratory assistant	Malteurop, France
10/08/1998-28/08/1998	Laboratory assistant	Malteurop, France
23/06/1997-11/07/1997	Laboratory assistant	Malteurop, France

Languages

French: read, spoken, written

English: read, spoken, written

Résumé

Lors de ces travaux, l'interaction entre la vigne, *Vitis vinifera* L., et une PGPR endophytique, *Burkholderia phytofirmans* souche PsJN, a été étudiée. Ceci a permis de caractériser des phénomènes de colonisation bactérienne, des réponses de défense de la plante, ainsi qu'une résistance systémique induite (ISR) permettant une protection contre l'agent pathogène *Botrytis cinerea* Pers.

L'association entre la vigne et la souche PsJN a tout d'abord été étudiée en conditions gnotobiotiques en utilisant des plantules *in vitro*, la souche bactérienne de type sauvage ou des dérivés génétiques (PsJN::*gfp*2x et PsJN::*gusA*11). Ceci a permis de déterminer une colonisation épi- et endophytique des racines de vigne par la souche PsJN ainsi qu'une migration de la bactérie de l'intérieur des racines jusqu'aux feuilles, par le biais du flux d'évapo-transpiration de la plante *via* les vaisseaux du xylème.

La colonisation de la vigne par la souche PsJN a ensuite été étudiée en conditions non stériles en utilisant des boutures fructifères dans le but de décrire une possible colonisation des inflorescences. Les résultats obtenus ont permis de montrer une colonisation épi- et endophytique du système racinaire par la bactérie et ensuite, une migration des racines jusque la rafle et les jeunes baies, en présence d'autres micro-organismes.

La mise en place de composés de défense ainsi qu'une ISR induite par la souche PsJN a été ensuite déterminée sur des boutures fructifères. Ceci a été caractérisé avant sa progression systémique et avec des conditions plus stériles. Des événements précoces tels que l'accumulation de peroxyde d'hydrogène et d'oxyde nitrique, ainsi que la synthèse de composés phénoliques ont été caractérisés au niveau racinaire. De plus, il est apparu, d'après des résultats préliminaires, que la souche PsJN induit, au niveau local ainsi qu'au niveau systémique, des gènes codants des protéines PR dont la signalisation dépend de la voie de l'acide salicylique et / ou de l'acide jasmonique. Ceci a permis de suggérer que la souche bactérienne induit des mécanismes communs des phénomènes de résistance systémique acquise (SAR) et d'ISR. Ces phénomènes permettent, par la suite, une protection de la vigne au niveau floral vis à vis de l'infection causée par *B. cinerea* Pers.

Mots Clés : *Vitis vinifera* L., *Burkholderia phytofirmans* souche PsJN, PGPR, endophyte, colonisation, défense, ISR, SAR, *Botrytis cinerea* Pers.