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Saccharothrix algeriensis NRRL B-24137: biocontrol properties, colonization and induced systemic resistance towards *Botrytis cinerea* on grapevine and *Arabidopsis thaliana*

JURY

M. AZIZ Aziz	Maître de Conférences, Université de Reims Champagne-Ardenne
M. REY Patrice	Professeur, Université de Bordeaux
Mme. MATHIEU Florence	Professeur, Université de Toulouse
Mme STRUB Caroline	Maître de Conférences, Université de Montpellier II
M. LEBRIHI Ahmed	Professeur, Université de Toulouse
M. COMPANT Stéphane	Maître de Conférences, Université de Toulouse

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Unité de recherche : LGC UMR 5503 (CNRS/UPS/INPT)
Directeur de Thèse : Pr. LEBRIHI Ahmed (INP-ENSAT)
Co-Directeur de Thèse : Dr. COMPANT Stéphane (INP-ENSAT)

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Summary

Résumé

Au cours de cette thèse, un isolat de sol de désert, *Saccharothrix algeriensis* NRRL B-24137, a été évalué pour ses propriétés bioactives contre le champignon phytopathogène *Botrytis cinerea,* pour sa colonization sur *Vitis vinifera* L., et *Arabidopsis thaliana* ainsi qu'en vue d'étudier les méchanismes de résistance systémique induite (ISR) contre *B. cinerea*.

Les résultats obtenus nous ont permis premièrement de montrer que *Sa. algeriensis* NRRL B-24137 peut présenter des activités antifongiques contre *B. cinerea* et que des métabolites peuvent être responsables de cette activité antifongique. Bien que ces métabolites soient encore en cours d'étude et que cette étude mérite d'être approfondie, nous avons démontré ensuite les propriétés de colonisation de l'isolat du sol du désert chez la vigne. Les résultats ont permis de montrer que la souche peut former des populations rhizosphèriques ainsi que des sous-populations endophytiques chez des plants de vigne (Cabernet Sauvignon sur porte-greffe 44-53 M) à des étapes précoces de colonisation. Puis nous avons démontré que la souche bénéfique peut induire une résistance systémique contre *B. cinerea*. Bien que les mécanismes impliqués ne soient pas encore compris, des parties préliminaires de ces travaux démontrent que les expressions de gènes responsables de la production de glucanase, chitinase ainsi qu'un inhibiteur de polygalacturonase ne semblent pas potentialisés pendant le phénomène de résistance systémique. Enfin nous avons démontré l'interaction entre *Sa. algeriensis* NRRL B-24137 et *Arabidopsis thaliana* qui résulte dans une association intime dûe également à colonisation rhizosphèrique et endophytique de la plante modèle. La souche bénéfique peut églement induire un phénomène de résistance systémique sur *A. thaliana* contre *B. cinerea* et les analyses de plantes mutées ont permis de determiner des parties des mécanismes impliqués dans l'ISR aini que des nouveaux mécanismes impliqués qui peuvent être induits par des microbes bénéfiques.

Mots clés : *Sa. algeriensis* strain NRRL B-24137, PGPR, endophyte, colonisation, *Vitis vinifera* L., *Arabidopsis thaliana*, défenses, ISR, *Botrytis cinerea*.

Abstract

In this thesis, the desert soil isolate, *Saccharothrix algeriensis* NRRL B-24137, has been evaluated for its bioactive properties towards the phytopathogenic fungus *Botrytis cinerea,* for its colonization of *Vitis vinifera* L., and *Arabidopsis thaliana* as well as to study the mechanisms of induced systemic resistance (ISR) towards *B. cinerea*.

The results obtained allowed us firstly to show that *Sa. algeriensis* NRRL B-24137 can exhibit strong antifungal properties towards *B. cinerea* and that some metabolites can be responsible of this antifungal activity. Although these metabolites are still under consideration and that this study needs further works, we have demonstrated then the colonization properties of the desert soil isolate with grapevine plants. The results showed that the strain can form rhizospheric as well as endophytic subpopulations with grapevine plants (Cabernet Sauvignon cultivar graffed on 44-53 M rootstock) at early step of colonization. Then we have demonstrated that the beneficial strain could induce a systemic resistance towards *B. cinerea*. Although the mechanisms are not yet well understood, preliminary parts of this work demonstrated that the genes responsible of glucanase production, chitinase as well as inhibitor of polygalacturonase activity do not seems to be primed during the systemic resistance phenomenon. Finally we demonstrated that the interaction between *Sa. algeriensis* NRRL B-24137 and *Arabidopsis thaliana* plants results in a close association due also to a rhizo- and endophytic colonization of the model plant. The beneficial strain can also induce a systemic resistance in *A. thaliana* towards *B. cinerea* and analyzes of plant mutants have allowed to determine parts of the mechanisms involved in ISR as well as new mechanisms that could be trigerred by beneficial microbes.

Keywords: *Sa. algeriensis* strain NRRL B-24137, PGPR, endophyte, colonization, *Vitis vinifera* L., *Arabidopsis thaliana*, defence, ISR, *Botrytis cinerea*.

List of Abbreviations

ACC: 1-aminocyclopropane-1-carboxylate

BABA: β-aminobutyric acid

BC: *Botrytis cinerea*

CWDE: Cell-Wall Degrading Enzyme.

DAPG: 2,4-DiAcetylPhloroGlucinol

EPR: Emergence-Promoting Rhizobacteria

ET: Ethylene

FISH: Fluorescence *In Situ* Hybridization

GUS: β-glucuronidase

GFP: Green Fluorescent Protein

H_2O_2: Hydrogen peroxide

ISR: Induced Systemic Resistance

JA: Jasmonic Acid

LOX: Lipoxygenase

LPS: Lipopolysaccharides

MAMP: Microbial Associated-Molecular Pattern

PAL: Phenil Alanine ammonia-Lyase

PGPR: Plant Growth-Promoting Rhizobacteria

ROS: Reactive Oxygen Species

SA: Salicylic Acid

SAR: Systemic Acquired Resistance

UV: Ultra-Violet

YIB: Yield Increased Bacteria

Foreward

Before to explain why the strain *Saccharothrix algeriensis* NRRL B-24137 was used in this thesis in order to determine new bacterial metabolites having direct biocontrol properties towards *Botrytis cinerea*, to determine its colonization behavior on grapevine and *Arabidopsis thaliana*, its systemic impact of on *B. cinerea* and the mechanism involved, a considerable survey of the literature will be presented. This will be correlated to rhizobacteria, endophytic bacteria, their microbial ecology, colonization behaviour, functions on plant growth, biocontrol properties, and mechanisms involved.

Why to talk about rhizobacteria and endophytes? Nowadys, there is a current need to use non chemical pesticides, non chemical phytostimulators, fertilizators for a sustainable management of agriculture due to problem of their uses on the Human health as well as on the environment. Among the solutions proposed exists the use of beneficial microorganisms such as some bacteria. These beneficial bacteria can come from different environments as well as colonize various hosts and help the growth of the plants and reduce pathogens pressure. These bacteria can be present in the rhizosphere as well as from inside plants and could be used for crop improvement as we will see in the following introduction.

Chapter I

Introduction

I. Generality on rhizospheric and/or endophytic bacteria

1. Definition and History

The rhizosphere is about 1-5 mm wide, but has no distinct edge and is different of the bulk soil (Lines-Kelly, 2004). This is the narrow region of soil surrounding the root system where the biology and chemistry of the soil are influenced directly by root secretions, the root systems and associated soil microorganisms (Figure 1). This concept of rhizosphere was firstly given by Lorenz Hiltner (Figure 2) in 1904. Later on many studies have been performed on the rhizosphere biology, as well as on microbial ecology of this zone (Hiltner, 1904; Smalla *et al.,* 2006; Hartmann *et al.,* 2008).

Since 1904 various studies have described the taxonomy of bacteria inhabiting the rhizosphere that are called rhizobacteria, which may be neutral, pathogenic or beneficial to their hosts (Raaijmakers *et al.,* 2009). Among them, exist PGPR (plant growth-promoting rhizobacteria). Different names such as for instance YIB (yield increased bacteria), EPR (emergence promoting bacteria) have been also often used to describe these beneficial rhizobacteria during the history of researches (Johri *et al.,* 2003; Kennedy *et al.,* 2006).

PGPR were firstly described by Kloepper and Schroth in 1978 and PGPR are known as exerting beneficial effects on plant development, health, yield *via* direct or indirect mechanisms (Compant *et al.,* 2005a). This is the case under natural conditions but also following use on some crops of non natural PGPR.

Although PGPR can be present in the rhizosphere under natural conditions or following their use, a subpart of their populations can not only be present in the rhizosphere, but could also enter inside the plant and colonize various plant parts (Compant *et al.,* 2005a, 2010a). As postulated by Galippe already in 1887 (Galippe, 1887; reviewed in Smith, 1911 and Compant *et al.,* 2010a), some soil bacteria can indeed enter plants. For a long time, the work of Galippe was not recognized (Compant *et al.,* 2012), although A. di Vestea confirmed Galippe's work (di Vestea, 1888; Compant *et al.,* 2010a). A recent publication made by Compant *et al.* (2012) highlights this history of Pionners working on endophytes, and participates in the rehabilitation of the pionner work of Galippe. Although this is highly interesting, this subject will not be overviewed in this thesis. Rather, we can ask ourselves on what is an endophyte.

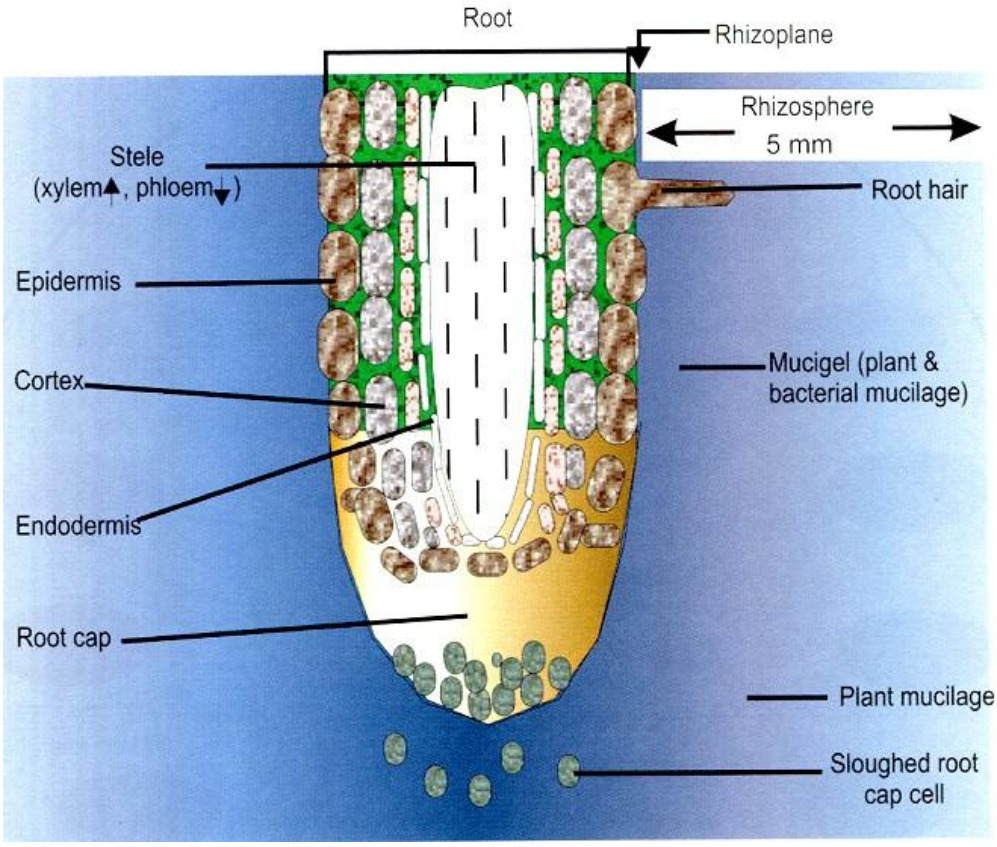

Figure 1. The rhizosphere. Drawing from Maier *et al.* (2000).

Figure 2. Dr Lorenz Hiltner
(pionner in rhizosphere biology)

What is indeed an endophyte? Defined literally, the word endophyte means inside the plants (endon Gr.= within, phyton = plant). The term endophyte was coined by A. de Bary in 1866 for pathogenic strains and the definition was then extended to all microorganisms living inside plants (reviewed in Wilson, 1995). This term could be used in a broad spectrum for different microorganisms, e.g. bacteria (Zinniel *et al.,* 2002), fungi (Cannon and Simmons, 2002), but also for plants (Marler *et al.,* 1999), insects inside plants (Feller, 1995), and algae within algae (Trémouillaux-Guiller *et al.,* 1991). However, the term endophyte has been also defined in several ways, and the definitions have been modified as the researches have advanced (Chanway, 1996).

In 1995 Wilson proposed to define endophyte as "fungi or bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease." It has been also defined as “bacteria that live in plant tissues without doing substantive harm or gaining benefit other than residency” for endophytic bacteria (Kobayashi and Palumbo, 2000; Kado *et al.,* 1992). In 2000, Bacon and White defined endophytes as “Microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects”. Various investigators have defined therefore endophytes in different ways that are usually dependent on the perspective from which the endophytes were being isolated and subsequently examined. However and although all definitions can have respects, the definition of J. Hallmann will be used in this thesis. Hallmann *et al.*, in 1997 defined endophytic bacteria as “bacteria detected inside surface-sterilized plants or extracted from inside plants and having no visibly harmful effects on plants”. This definition includes internal colonists with apparently neutral behavior as well as symbionts and is widely used among researchers working on endophytes.

2. Sources and Niches of colonization of PGPR and endophytes

Under laboratory or managed and natural conditions, PGPR and endophytes can help plants by providing nutriment to their hosts or by reducing abiotic and biotic stresses. These PGPR and/or endophytes can be isolated from natural hosts but also from other environments. They can be isolated indeed from crops, various other plants (Lugtenberg and Kamilova, 2009) but also researches of new competent PGPR and endophytes have led to the discovery that some strains could be also isolated from harsh environments such as desert soil and could be used for crop’s improvement (discussed in Compant *et al.,* 2010a; 2010b).

Under natural conditions or following inoculation, PGPR and endophytes have been tracked to know their niches of colonization. Various studies have focused on the microbial ecology of these beneficial bacteria. Their sources, niches of colonization, as well as colonization behaviours can explain why some of these beneficial bacteria can colonize some host plants before to exerce beneficial effects on them.

To study all these processes of colonization by PGPR and/or endophytes different tools have been even used. This was the case with the plate counting method to monitor populations in different plant parts. Metagenomic analyses have been also used to describe all the communities as certains strains could have enter in a viable but not cultivable state within the plants (Compant *et al.,* 2010a). To visualize colonization various microscopic tools have been also employed such *gfp*, *gus*A, DsRed, derivated markers, electron microcopy as well as Fluorescence *in situ* hybridization (FISH; Gamalero *et al.,* 2003).

By using ones or several of the techniques described before, many studies have focused on the colonization by PGPR on the plant rhizosphere and rhizoplane to explain pre-steps involved in beneficial effects by PGPR. Benizri *et al.,* (2001) described for instance the interaction between plant hosts and different members of *Pseudomonas*, *Bacillus*, *Pantoea*, *Burkholderia* genera to give information on the colonization processes. It has been shown that in the case of PGPR, these microbes could colonize the rhizosphere and then colonization may occur on the surface of some rhizodermal cells on the rhizoplane (root surface) after soil or root inoculation (Benizri *et al.,* 2001). Following rhizosphere colonization bacterial cells have been visualized as single cells attached to the root surfaces, and then as doublets on the rhizodermis, forming a string of bacteria as observed by *Peudomonas fluorescens* DF57 in barley root (Hansen *et al.,* 1997). In cotton, *Enterobacter asburiae* JM22 cells colonization in root have been also observed. Many bacteria were found to be located on the root surface, concentrated in the grooves between epidermal cells (Quadt-Hallmann *et al.,* 1997). Compant *et al.* (2005b) observed also that the plant growth-promoting bacterium *Burkholderia phytofirmans* strain PsJN::*gfp*2x on the rhizosphere of the grapevine plantlets. It has been observed that the rhizoplane of grapevine plantlets was rapidly colonized by PsJN::*gfp*2x cells immediately after rhizosphere inoculation. Microscopic observations of grapevine roots after 96 h of PsJN::*gfp*2x inoculation revealed colonization on both primary and secondary roots. PsJN cells congregated in high numbers at the sites of lateral root emergence on plantlets were observed at 96 h inoculation and bacterial cells were also found close to the cell walls of the rhizodermal cells as well as on the whole outline of some

rhizodermal cells (Compant *et al.,* 2005b). Various other examples have showed the colonization on the root surfaces by beneficial bacteria. However although colonization of plants by beneficial bacteria may occur on all the surface of some rhizodermal cells (Benizri *et al.,* 2001), it can be found that the root system is not colonized in a uniform manner by different bacterial strains in plants growing in the fields or under laboratory conditions. As described by Gamalero *et al.,* (2004) with *P. fluorescens* strain A6RI and tomato roots, the distribution and density of the inoculant strain could varie according to the root zone. Just after bacterial inoculation, bacteria were randomly distributed as single cells along the whole primary root. Microscopic tools such as *gfp* markers have also demonstrated that bacterial cells were distributed both in apex and in elongation zone and same time bacterial population has been observed in the root hairy zone (Figure 3). Bacterial cells have been also observed to be located closely to the longitudinal junctions between epidermis cell walls (Gamalero *et al.,* 2004).

As described before several PGPR do not only colonize the rhizosphere and the rhizoplane but also enter plants and colonize internal tissues (Compant *et al.,* 2005a; Hallmann and Berg, 2006; Figure 3). Indeed several recent studies confirmed that plants host a large number of endophytic communities that derive from the soil environment (Berg *et al.,* 2005b). Bacterial endophytes actively colonize various plant tissues, establish long-term associations, actually lifelong natural associations (Hardoim *et al.*, 2008).

Endophytes can be detected inside the root system firstly following rhizosphere and rhizoplane colonization. This has been demonstrated with various genera of bacteria as reviewed recently by Rosenblueth and Martinez-Romero (2006) and this is the case for some of the bacteria such as *Burkholderia phytofirmans* strain PsJN, *Enterobacter asburiae* JM22 described before (Quadt-Hallmann *et al.,* 1997; Compant *et al.,* 2005b). However a lot of other bacteria could also enter the endorhiza and could be endophytic inside plants.

To enter inside the root system, the penetration process by endophytes does not require automatically any active mechanism and almost all rhizosphere bacteria can be indeed endophytics at any stage of their life (Hardoim *et al*., 2008). Penetration can take place at cracks, such as those occurring at root emergence sites or created by deleterious microorganisms, as well as by root tips (Reinhold-Hurek and Hurek, 1998; Figure 3).

When bacteria colonize roots, they can invade root cells inter- and/or intracellularly and can penetrate into central tissue. In this way, they might reach central cell layers before

differentiation of the endodermis although endodermis can also be reached *via* secretion of cell-wall degrading enzymes (Compant *et al*., 2010a). Another route of entry appears to be the points of emergence of lateral roots (Figure 3), where bacterial cells have been detected between the cell layers of the lateral root and the cortex of the main root as observed for *Azospirillum* spp. (Umali-Garcia *et al*., 1980).

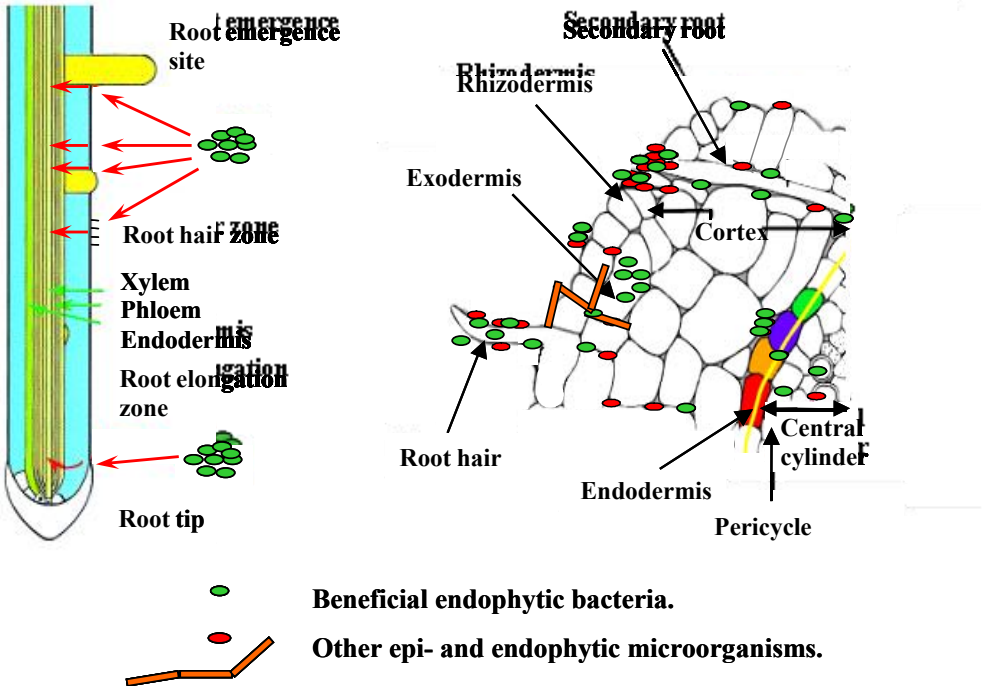

Figure 3. Sites of plant colonization by endophytic bacteria. Drawing from Compant *et al.* (2010a).

The secretion of cell-wall degrading enzymes (CWDEs) can be involved in bacterial penetration and spreading within the plant. Production of cell wall degrading enzymes has been detected in PGPR and/or endophytic bacteria gained entry inside plant *via* hydrolytic enzymes secretion (Hallmann *et al.,* 1997). Endoglucanase, cellulase and pectinase enzymes produced by numerous endophytic bacteria such as *Azoarcus* sp. strain BH72 (Hurek *et al.,* 1994), *Azospirillum irakense* strain KBC1 (Khammas and Kaiser, 1991), and *Pseudomonas fluorescens* strain 89B-61 (Benhamou *et al.,* 1996; Quadt- Hallmann *et al.,* 1997) have been indeed correlated to the entry of endophytes inside the root system. Enzymatic degradation of plant cell-walls by these bacteria was however only observed when they colonized the root epidermis but never after colonizing intercellular spaces of the root cortex, suggesting that

endophytes may induce production of cellulases and pectinases only for penetration into the host plant.

Some of the PGPR can not only enter cortical parts of the root interior but some are also able to cross the endodermis barrier, crossing from the root cortex to the vascular system (Compant *et al.*, 2010a; Figure 3). Colonization processes of the PGPR following vascular system colonization have been demonstrated and explained why endophytes could be found in the aerial plant parts. It was indeed demonstrated that bacterial endophytes could be found in different vegetative parts of plants, such as roots, tubers but also inside stems and/or leaves (Hallmann, 2001; Gray and Smith, 2005; Compant *et al.*, 2005b). Presence of endophytes in plant reproductive organs, such as flowers and fruits has also been reported (Misaghi and Donndelinger, 1990; Bacon and Hinton, 2006 ; Compant *et al.,* 2008; 2011) and xylem colonization in such organs have been demonstrated (Compant *et al.,* 2008; 2011). Some endophytes can use the lumen of xylem vessels to spread throughout the plant (Figure 3; Compant *et al.,* 2005b, 2010a). However, only few endophytes are able to colonize aerial vegetative and reproductive plants parts due to presence of several barriers (Hallmann, 2001). In another way, it has been suggested that some endophytes colonize the intercellular spaces of the plant and use it to spread inside the plants (Dong *et al.,* 1994), demonstrating two ways of colonization.

Although the two ways of colonization could explain the presence of endophytes inside the aerial parts of the plants, other sources such as the caulosphere for stem endophytes, the phyllosphere for leaf endophytes, the anthosphere for the ones from flowers as well as the carposphere for those colonizing fruits and the spermosphere for seed endophytes have been reported (Hallmann *et al.,* 1997; Hurek *et al.,* 2002). However endophytes related to these sources are rare and not reported widely. Endophytic bacteria mostly derive indeed from the rhizosphere as discussed before (Compant *et al.,* 2005a) and colonize various plant parts. However only specific systemic colonizers can reach aerial plant parts whereas others can be restricted in the endorhizal part (Compant *et al.,* 2010a).

In case of study the effect of a plant growth-promoting bacterium on a host plant (PGPR and/or endophyte) or on a new host (for crop improvement), it is interesting to study the colonization process of the bacterium and it is the case why we described the colonization process before. However to visualize the colonization process, the tools available are not sufficients sometimes to track the microbes on and inside plants and need to be improved.

This is dependent of the strain used as well as of the plant-microbe interaction. However there is a requisite to study the behaviour of such strains, firstly to know more about the niches of colonization in a microbial ecology viewpoint but also because colonization can be linked to the functions of rhizo- and endophytic bacteria.

3. Functions of rhizo- and endophytic bacteria

Plant growth-promoting rhizobacteria (PGPR) and/or endophytic bacteria are associated with almost all plant species and present in many environments of temperate regions, harsh environments, and various other environments. Despite their different ecological niches, free-living rhizobacteria and/or endophytic bacteria can enhance plant growth and control phytopathogens on different plants including crops (Gholami *et al.,* 2009), and it is a reason why they are used or can be used for agriculture. This plant growth-promotion as well as reduction of pathogens on the plants can be done *via* different direct or indirect mechanisms (Nelson *et al.,* 2004).

3.1: Direct plant growth promotion by PGPR and endophytic bacteria

Once inoculated on soil of plants, PGPR and/or endophytes have been reported to directly enhance plant growth by a variety of mechanisms: (1) fixation of atmospheric nitrogen that is transferred to the plant, (2) production of allelochemicals including siderophores that chelate iron and make it available to the plant root, (3) solubilization of minerals such as phosphorus, (4) synthesis of phytohormones (Arora *et al.,* 2001, Egamberdiyeva *et al.,* 2005). This has been demonstrated with different strains colonizing the rhizosphere or entering plant tissues (Lodewyckx *et al*., 2002).

(a) Biological nitrogen fixation

Inoculation of free-living N_2-fixing bacteria corresponding to diazotrophs have been shown to produce beneficial effects on plant growth (Kloepper *et al.,* 1980; Bashan and Holguin, 1998). The use of bio-fertilizers and bio-enhancers such as N_2 (nitrogen) fixing bacteria and beneficial microorganisms is of special importance as they can reduce chemical fertilizer applications and consequently lower production cost. Plants inoculated with some PGPR showed increased growth of inoculated plants that is associated with higher N accumulation by PGPR and better root growth, which promoted the greater uptake of water

and nutrient (Mia *et al.,* 2010). The higher N- incorporation can increase the formation of proteins and enzymes for better physiological activities and also contributed to the formation of chlorophyll, which consequently increased the photosynthetic activity (Raja *et al.,* 2006). Strains of *Pseudomonas putida* (G11-32 and 31-34) and *Pseudomonas fluorescens* (36-43) have been found to increase the nodulation and nitrogen fixation in *Glycine max* (L.) at a low root zone temperature (Zhang *et al.,* 1996). Some studies also indicate that co-inoculation of *Bradyrhizobium japonicum* (strains 532C and USDA110) and *Serratia liquefaciens* (2-68) and *Serratia proteamaculans* (1-102) strains can positively affect symbiotic nitrogen fixation by enhancing both root nodule number or mass, dry weight of nodules, yield components, grain yield, soil nutrient availability and by increasing the nitrogenase activity in soybean (Zhang *et al.,* 1997; Dashti *et al.,* 1998). However PGPR and/or endophytes are also known not only as helping the host growth *via* N fixation but also throughout different plant growth properties.

(b) Solubilisation of phosphorus.

Phosphorus (P) is major essential macronutrients for biological growth and development. It has been demonstrated that some PGPR/endophytes depending of their niches of colonization can offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil and make it available to the plants. The ability of some PGPR/endophytes to convert insoluble phosphorus (P) to an accessible form, like orthophosphate, is in fact an important trait for increasing plant yields (Rodrıguez *et al.,* 2006, Zaidi *et al.,* 2009). Within rhizobia, two species nodulating chickpea, *Mesorhizobium ciceri* strain RCAN08 and *Mesorhizobium mediterraneum* strains (PECA12, PECA03) are known for instance as good phosphate solubilizers (Rivas *et al.,* 2006). Bacterial strain isolates *Pseudomonas* sp. (*P. putida* PH6) and *Azospirillum* sp. (*A. brasilense* ATCC 29145) from the rhizosphere of Soybean have been also found to solubilise P *in vitro* along with other plant growth-promoting traits and increase the soybean growth (Cattelan *et al.,* 1999). It has been also demonstrated that an endophytic strain of *Gluconacetobacter diazotrophicus* strain PAl- 5 and one of *Bradyrhizobia* (*Bradyrhizobium japonicum* strain USDA 110) could increase the P uptake in soybean that contribute to plant growth enhancement (Son *et al.,* 2006). In fact, many beneficial bacteria can help the plant throughout P solubilization.

(c) Phytohormone production

Various studies have demonstrated that PGPR and/or endophytes can stimulate plant growth through the production of phytohormones that could be produced by bacteria such as auxins (indole acetic acid) (Spaepen *et al.,* 2009), gibberellines (Bottini *et al.,* 2004) and cytokinins (Timmusk *et al.,* 1999), or by regulating the high levels of endogenous ethylene in the plant (Glick *et al.,* 1998). Use of a PGPR strain, UMCV1 of *Bacillus megaterium* that promoted growth of *A. thaliana* and *P. vulgaris* seedlings was found to be dependent of cytokinin signaling as revealed by increased biomass production (Oritz-Castro *et al.,* 2008) for instance. A strain of *Paenibacillus polymyxa* strain B2 which synthesize auxins and cytokinins or that interfere with plant ethylene synthesis have been also identified (Timmusk *et al.,* 1999). Among PGPR species, *Azospirillum* (*A. brasilense strain* SM, Az39 and Sp245) is also known as one of the best studied IAA producers (Dobbelaere *et al.,* 1999; Smets *et al.,* 2004) and positive effect of *Bacillus subtilis* IAA producing strains CM1-CM5 on the edible tubercle *Dioscorea rotundata* L. have been also studied (Swain *et al.,* 2007). There is numerous example of correlation of phytohormone production and plant growth by PGPR and/or endophytes. However to regulate the ethylene level in plants, some PGPR/endophytes can reduce the level of ethylene reducing root growth *via* the enzyme ACC deaminase (Glick *et al.,* 1998) and this can be linked to a plant growth promotion. Ghosh *et al.,* (2003) found ACC deaminase activity in three *Bacillus* species (*Bacillus circulans* DUC1, *Bacillus firmus* DUC2 and *Bacillus globisporus* DUC3), which stimulated root elongation of *Brassica campestris* plants. For some species, up to 7 folds the level required for plant growth-promotion have been found in some strains such as in *Burkholderia phytofirmans* strain PsJN (Sessitsch *et al.,* 2005), an endophyte enhancing plant growth of tomato, grapevine, potato as well as many vegetables (Nowak *et al.,* 1995). However mechanism others that ACC deaminase has been also demonstrated and could explain plant growth-promotion by specific strains.

(d) Production of sulphur

The element sulphur present in the soil must be transformed or oxidized into sulphate by the bacteria before it could be available for plants. PGPR/endophytes could offer a biological rescue system capable of solubilising the insoluble inorganic S of soil and make it available to the plants (Chen *et al.,* 2006, Liu *et al.,* 1992). In this way it has been

demonstrated that a strain of *Delftia acidovorans* RAY209 isolated from a Canadian soil, can increase the canola plant growth by increasing the availability of sulphur to the plant (Banerjee and Yesmin, 2002). Similarly, sulphur oxidizing plant growth-promoting rhizobacteria *Achromobacter piechaudii* RAY12, *Agrobacterium tumefaciens* RAY28, *Stenotrophomonas maltophilia* RAY132 have been identified. As a result of this arrangement, plants are able to grow more efficiently and effectively and have enhanced growth characteristics (Banerjee, 2009).

3.2: Indirect effect by PGPR and/or Endophytic bacteria

PGPR and/or endophytic bacteria do not only stimulate the plant growth but also can reduce the phytopathogenic infections on plants. This is of special interest to reduce agrochemicals currently used in the fields to control phytopathogens. There are different ways of mechanisms of biocontrol reported by PGPR like competition for an ecological niche and nutrients, role of siderophores, antibiotic production and induced systemic resistance mechanisms. All these mechanisms will be presented in the following paragraphs.

II. Use of rhizosphere and endophytic bacteria for biocontrol of phytopathogens

PGPR and/or endophytes can offer an environmentally sustainable approach to increase crop productions and to control pathogens. In the recent years, scientists have focused their attention towards exploring the potential of beneficial microbes, for plant protection measures. Different strains from the rhizo and/or the endosphere of plants have shown their prooves to reduce various phytopathogenic infections (Table 1).

Mechanisms leading to biological control include antibiosis, nutrient or niche competition, induction of systemic resistance, and predation or parasitism (Cook and Baker, 1983; Weller *et al.*, 1988). However, the importance of each mechanism is determined by the physical and chemical state of the phytosphere (Weller *et al.,* 1988; Andrews, 1992). Different types of mechanisms will be discussed in this introduction and are shown in Figure 4.

Table 1: Some examples of rhizo- and/or endophytic bacteria having biocontrol properties against different pathogens in different plants.

PGPR/Endophytes	Target pathogen/Diseases	Plants	References
Pseudomonas aeruginosa 7NSK2	*Botrytis cinerea*	*Phaseolus vulgaris*	De Meyer and Höfte, 1997
Pseudomonas fluorescens strain WCS374	Fusarium wilt	*Raphanus sativus*	Leeman *et al.,* 1995
S. marcescens 90 – 166, *Bacillus pumilus* SE34, *P. fluorescens* 89B61, *Bacillus pasteurii* C9, *Paenibacillus polymyxa* E681, *Bacillus subtilis* GB03, *Bacillus amyloliquefaciens* IN937a, *Enterobacter cloacae* JM-22 and *Bacillus pumilus* T4	*P. syringae* pv. tomato DC3000 and *P. syringae* pv. Maculicola ES4326	*Nicotiana tabacum, Capsicum annuum, Cucumis sativus, Solanum lycopersicum, Arabidopsis thaliana*	Wei *et al.,* 1991, 1996; Kloepper, 1996; Raupach *et al.,* 1996; Zehnder *et al.,* 1999; Yan *et al.,* 2002; Zhang *et al.,* 2002; Ryu *et al.,* 2003
Pseudomonas fluorescens strain WCS374	*Colletotrichum falcatum*/red rot disease	*Saccharum officinarum*	Viswanathan and Samiyappan, 1999
Pseudomonas sp. strain WCS 417r	*Fusarium oxysporum* f. sp. Dianthi	*Dianthus caryophyllus*	Van Peer *et al.,* 1991
Pseudomonas putida strain 89B-61, *Serratia marcescens* strain 90-166, *Flavomonas oryzihabitans* strain INR-5, *Bacillus pumilus* strain INR-7	*P. syringae* pv. lachrymans/angular leaf spot	*Cucumis sativus*	Van loon *et al.,* 1998
Pseudomonas putida strain 89B-27 and *Serratia marcescens* strain 90-166	*Fusarium oxysporum* f. sp. cucumerinum	*Cucumis sativus*	Liu *et al.,* 1995
Bacillus pumilus strain SE 34	*F. oxysporum* f. sp. radicis-lycopersici	*Solanum lycopersicum*	Benhamou *et al.,* 1998
P. fluorescens strain 63-28	*Pythium ultimum*	*Pisum sativum*	Benhamou *et al.,* 1996
P. fluorescens strain 63-28	*F. oxysporum* f. sp. radicis-lycopersici	*Solanum lycopersicum*	M'Piga *et al.,* 1997
Bacillus cereus	*F. solani, Sclerotium rolfsii*	*Gossypium hirsutum , Phaseolus vulgaris*	Pleban *et al.,* 1995
P. fluorescens strain EP1	*Colletotrichum falcatum*	*Saccharum officinarum*	Viswanathan *et al.,* 1999
Serratia marcescens 90-166, *Bacillus pumilus* and *Pseudomonas fluorescens* 89B-61	*P. tabacina*	*Nicotiana tabacum*	Zhang *et al.,* 2002
Pseudomonas aeruginosa 7NSK2	*Botrytis cinerea*	*Solanum lycopersicum*	Audenaert *et al.,* 2002

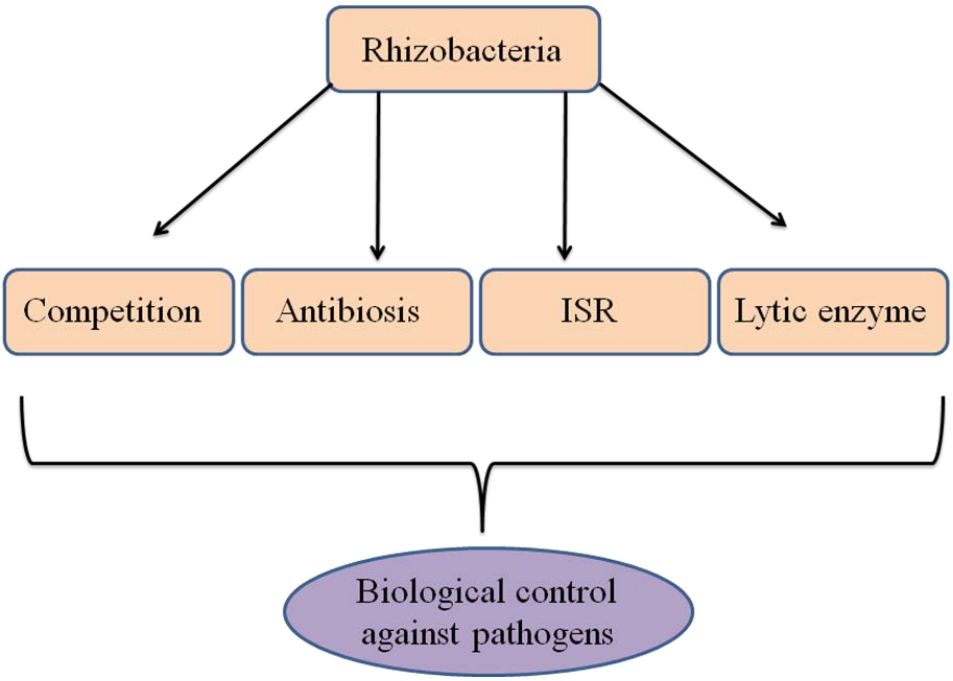

Figure 4. Different types of mechanisms of beneficial rhizobacteria finally leading to Biological control in plants against pathogens.

1. Competition for Space and Nutrients.

The root surface and the surrounding rhizosphere are rich in root exudates and sources of carbon. Thus, along root surfaces there are also suitable nutrient-rich niches that attract a great diversity of microorganisms, including phytopathogens (Compant *et al*., 2005a). Competition for these nutrients and niches is a fundamental mechanism by which PGPR protect plants from phytopathogens as well as endophytes when they are also present at the rhizosphere level before to enter plant tissues. Chemotaxis towards carbon, sugars, vitamins, amino acids that are exuded in the rhizosphere by the host plants could explain competition at the rhizosphere level (Compant *et al*., 2005a). Up to 40 % of photosynthate can be present at the root level. This implies that PGPR should have strong chemotactic abilities to reach exudates components before pathogens to protect the plants (Lugtenberg and Kamilova, 2009).

Another example of nutrient competition is the production of siderophores by certain bacteria as described firstly by J. Kloepper in 1980. Siderophores sequester iron (III) from the rhizosphere and once the iron is sequestered the siderophore is used exclusively by the

microbe that produced it and by certain plants (Datnoff *et al.,* 2007). Because this iron supply can only be used by the microbe that produced it, it limits the availability to other microbes and therefore the pathogen growth is suppressed (Kloepper *et al.,* 1980). Various bacteria are able to produce siderophores such as for instance strains of *Streptomyces* spp. e.g. *S. fulvissimus* ATCC 27431, *S. griseus* st-21-2 and *S. tanashiensis* IAM0016 (Yamanaka *et al.,* 2005; Bendale *et al.,* 2010), *Sinorhizobium meliloti* DM4 (Reigh and Connell, 1993), *Rhizobium leguminosarum* A775 (Dilworth *et al*., 1998), *Pseudomonas* spp. e.g. *P. putida* WCS358, NCIM 2847 and *P. fluorescens* Pf-5, NCIM 5096 (kojic *et al.,* 1999; Sayyed *et al.,* 2005; Paulsen *et al.,* 2005) and *Bradyrhizobium japonicum* (Datnoff *et al.*, 2007). Some of them are restricted to the rhizosphere whereas others can be endophytic in various plants and organs (Rosenblueth and Martinez-Romero, 2006).

2: Antibiosis.

Antibiotics are chemically heterogeneous group of organic, low molecular weight compounds produced by microorganisms (Raaijmakers *et al.,* 2002) which at low concentrations result in harmful effects to other microorganisms (Fravel, 1988; Pal and McSpadden Gardener, 2006). Some microorganisms, both rhizospheric and endophytic ones, are able to produce a broad collection of antibiotics and some antibiotics are produced by several bacteria (Table 2). For example, pyrrolnitrin is produced by some *Burkholderia* and *Pseudomonas* species (Raaijmakers *et al.,* 2002). This antibiotic has shown activity over *Rhizoctonia solani, Botrytis cinerea, Verticillium dahliae,* and *Sclerotinia sclerotiorum* (Ligon *et al.,* 2000). A wide variety of antibiotics have been also 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 (Defago, 1993; de Souza *et al.,* 2003; Nielsen *et al.,* 2002; 2003; Raaijmakers *et al.,* 2002) and oligomycin A, kanosamine, zwittermicin A, and xanthobaccin produced by *Bacillus*, *Streptomyces*, and *Stenotrophomonas* spp. (Hashidoko *et al.,* 1999; kim *et al.,* 1999; Milner *et al.,* 1996 ; Nakayama *et al.,* 1999).

Table 2: Some Antibiotics produced by rhizo and endophytic bacteria.

Antibiotics	Source	Target pathogen	Disease	References
2,4-diacetyl-phloroglucinol	*Pseudomonas fluorescens* F113	*Pythium* spp.	Damping off	Shanahan *et al.*, 1992
Agrocin 84	*Agrobacterium radiobacter*	*Agrobacterium tumefaciens*	Crown gall	Kerr *et al.*, 1980
Oomycin A	*P. fluorescens* Hv37a	*Pythium ultimum*	Damping off	Gutterson *et al.*, 1986
Pyoluteorin	*P. fluorescens* CHA0	*Thielaviopsis basicola, Pythium ultimum*	Black root rot	Keel *et al.*, 1992
Pyrrolnitrin	*P. fluorescens* BL915	*Rhizoctonia solani*	Damping-off, Stem cankers	Ligon *et al.*, 2000
Pyrrolnitrin	*Serratia* spp.	*Verticillium dahliae, Sclerotinia sclerotiorum*	Pink rot	Kalbe *et al.*, 1996
2,3-de-epoxy-2,3-didehydro-rhizoxin	*P. borealis* MA342	*Pyrenophora teres, Tilletia caries*	Damping off	Hokeberg *et al.*, 1998
Viscosinamide	*P. fluorescens* DR54	*Rhizoctonia solani*		Nielsen *et al.*, 1998
Butyrolactones	*P. aureofaciens* 63-28	*Phytophthora cryptogea*	Damping off	Gamard *et al.*, 1997
N-BBS	*Pseudomonas* sp. AB2	*Rhizoctonia solani, Botrytis cinerea*	Grey mold	Ki Kim *et al.*, 2000
AFA	*S. violaceusniger* YCED-9	*Pythium ultimum*	Damping off	Trejo-Estrada *et al.*, 1998
Pantocin A and B	*P. agglomerans* EH318	*Erwinia herbicola*	Erwinia disease	Wright *et al.*, 2001
Xanthobaccins	*Stenotrophomonas* SB-K88	*Pythium ultimum*	Damping off	Nakayama *et al.*, 1999
AFC-BC11	*B. cepacia* BC11	*Rhizoctonia solani*	Bacterial soft rot	Kang *et al.*, 1998
Kanosamine	*B. cereus* UW85	*Phytophthora medicaginis*	Damping off	Milner *et al.*, 1996
Zwittermycin A	*B. cereus* UW85	*Phytophthora medicaginis*	Damping off	Silo-Suh *et al.*, 1994; Smith *et al.*, 1993
Bacillomycin D	*Bacillus subtilis* AU 195	*Aspergillus flavus*	Aflatoxin contamination	Moyne *et al.*, 2001
Bacillomycin, fengycin	*Bacillus amyloliquefaciens* FZB42	*Fusarium oxysporum*	Wilt	Koumoutsi *et al.*,2004
Xanthobaccin A	*Lysobacter* sp. strain SB-K88	*Aphanomyces cochlioides*	Damping off	Islam *et al.*, 2005

Table 2: (continued) Some antibiotics produced by rhizobacteria and endophytic bacteria.

Antibiotics	Source	Target pathogen	Disease	References
Herbicolin	*Pantoea agglomerans* C9-1	*Erwinia amylovora*	Fire blight	Sandra *et al.,* 2001
Iturin A	*B. subtilis* QST713	*Botrytis cinerea* and *R. sonai*	Damping off	Paulitz and Belanger, 2001; Kloepper *et al.,* 2004
Mycosubtilin	*B. subtilis* BBG100	*Pythium aphanidrmatum*	Damping off	Leclere *et al.,* 2005
Phenazines	*P. fluorescens* 2-79 and 30-84	*Gaeumannomyces graminis*	Take-all	Thomashow *et al.,* 1990

3: Lytic enzymes secretions

It has been demonstrated also that PGPR can secrete not only antibiotics but also lytic enzymes such as chitinases, cellulases, amylases and 1,3-β glucanases enabling to reduce the growth of various phytopathogens. For examples, biocontrol of *Phytophthora cinnamomi* was obtained by using a cellulose-producing isolate ATCC 39149 of *Micromonospora carbonacea.* Control of *Phytophthora fragariae* causing raspberry root rot was suppressed by 1,3-β glucanases producing actinomycete isolate (EF-72, EF-22, and EF-97 (Valois *et al.,* 1996). Chitinolytic enzymes produced by *Bacillus cereus* strain 65 also appear to be involved in biocontrol of *Rhizoctonia soloni* (Pleban *et al.,* 1997). Similarly, in the case of biocontrol of *Pythium ultimum* in the rhizosphere of sugar beet by *Stenotrophomonas maltophila* W5 was due to the production of extracellular proteases (Dunne *et al.,* 1997).

Various mechanisms of biocontrol exist therefore as described before. However the list is not exclusive and each time a strain is described as a biocontrol agent towards one phytopathogen, some new metabolites could be characterized.

4: Induced Systemic Resistance.

PGPR and endophytes do not only secrete antibiotics, nor lytic enzymes but can also protect systemically the plants towards phytopathogenic infections (Van Loon *et al.,* 1998).

This however implies that the pathogen and the biocontrol microorganism are not in contact (van Loon *et al.,* 1998).

SAR and ISR are the two types of systemic resistances that are activated in plants during stress. The capacity of a plant to develop a broad-spectrum, systemic acquired resistance (SAR) after primary infection with a necrotizing pathogen is well known (Durrant and Dong, 2004). But when the plant roots colonized by specific PGPR develops a phenotypically similar form of protection, it is called rhizobacteria-mediated induced systemic resistance (ISR) (Van Loon *et al*., 1998). However ISR developed by endophytic strain has been also described (this is however not surprising as almost endophytes derive from the soil environment and be present on the root surfaces before to enter plant tissues as described before).

Rhizobacteria and/or endophytes-mediated ISR has been reported for bean, carnation, cucumber, radish, tobacco, tomato, the model plant *Arabidopsis thaliana*, as well as many other plants. This ISR is effective against different types of plant pathogens. In this respect, ISR resembles pathogen-induced systemic acquired resistance (SAR), which renders uninfected plant parts more resistant towards a broad spectrum of phytoathogens (Ryals *et al.,* 1996; Sticher *et al.,* 1997). However unlike SAR, ISR does not involve the accumulation of pathogenesis-related proteins (Pieterse *et al.,* 2001; Yan *et al.,* 2002). And in contrast to pathogen-induced SAR, which is regulated by SA, beneficial bacteria-mediated ISR is controlled by a signaling pathway in which ET and JA play key roles (Pieterse *et al*., 1998; Figure 5).

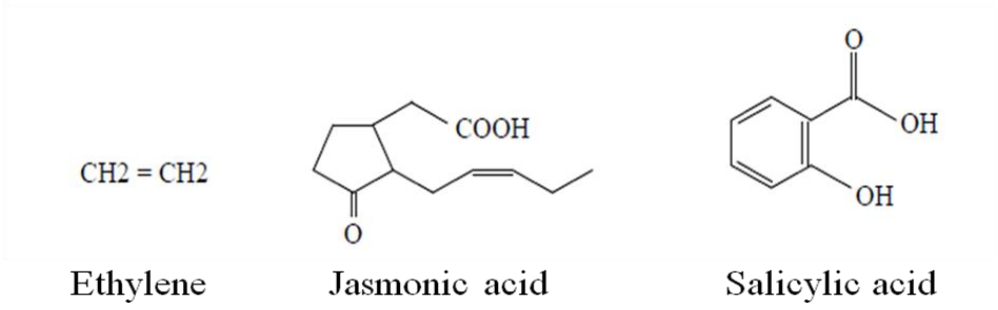

Figure 5: Three main components of resistance in plants against biotic and abiotic stress.

Exceptions have been demonstrated however on this generality of PGPR/endophytes inducing ISR and this seems to be more complex than previously thought. This depends in

fact to the pathosystem used as well as the beneficial strains, rhizospheric stricts or endophytics. Noneless of this, rhizobacteria and endophytes can induce defenses but also could enhance the plant's ability to suppress future pathogen attacks (Conrath *et al.,* 2002; Jakab *et al.,* 2001, 2005; Pozo *et al.,* 2004; Ton *et al.,* 2005; Zimmerli *et al.,* 2000; Figure 6). For instance challenge inoculation of plants with a leaf pathogen e.g., *P. syringae* pv. tomato, showed that ISR-positive plants with *Pseudomonas fluorescens* strain WCS417r were „primed' i.e., they reacted faster and more strongly to pathogen attack by inducing defense mechanism (Verhagen *et al.,* 2004; Figure 6).

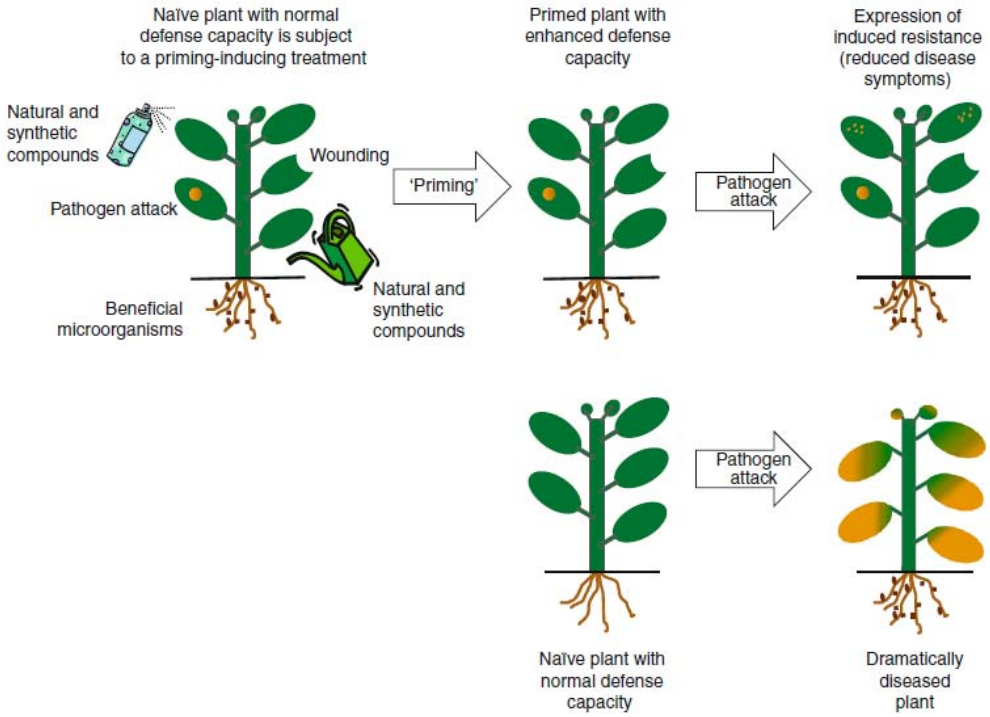

Figure 6: Beneficial rhizobacteria induce resistance against pathogen (Conarth, 2009). Plants inoculated with beneficial bacteria showing resistance (ISR) against phytopathogens.

Although effects of PGPR/endophytes are known on different plants, the mechanisms of ISR and SAR have been clearly differentiated by using different *Arabidopsis* mutants. The role of different defense related genes is demonstrated here and represented in Figure 7. All the current known components of ISR described with *A. thaliana* will be described.

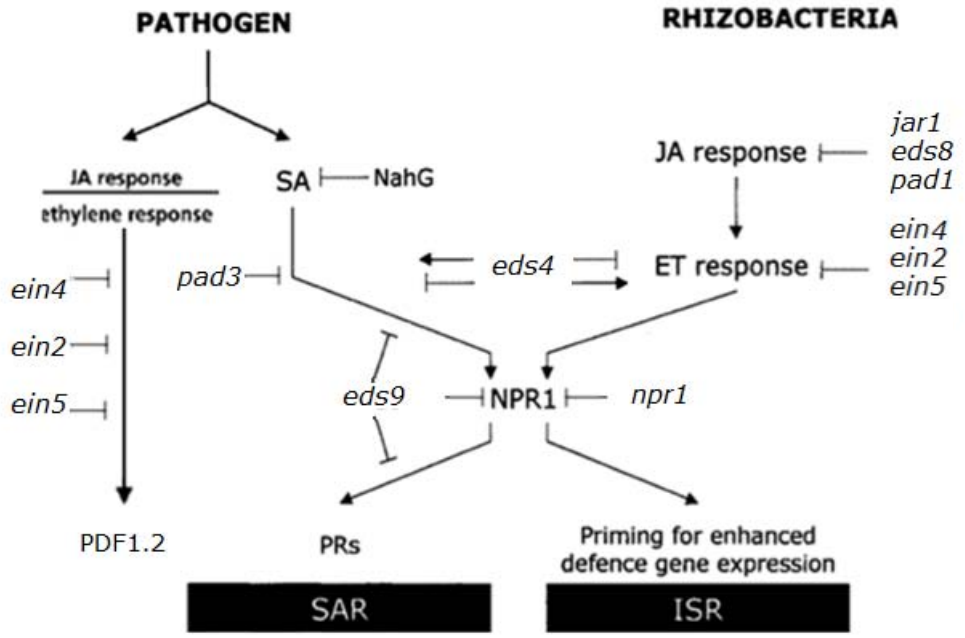

Figure 7: A model for ISR in *A. thaliana* adapted from Okubara, 2005; Pieterse, 1998 and Ton *et al.,* 2002.

Analysis of mutants impaired in jasmonate biosynthesis as well as in signaling has shed light on the complexity of a role for jasmonates as signaling compounds in ISR. Jasmonate have been demonstrated as involved in ISR *via jar-1*, *aos*, and *coi1-16* mutants. For example, the *coi1* mutation defines an *Arabidopsis* gene that functions in the jasmonate signaling pathway required for defense against pathogens or insects. It was reported that the COI1 gene encodes a protein containing leucine-rich repeats and a F-box motif (Xie *et al.,* 1998).

It has been found that ethylene acts in jasmonic acid (JA)-dependent pathways that are distincts from the salicylic acid (SA)-dependent SAR pathway (Piertese *et al.,* 1999). Ethylene seems to play an important role in various plant disease resistance pathways. However, depending on the type of pathogen and plant species, the role of ethylene can be dramatically different. Plants deficient in ethylene signaling may show either increased susceptibility or increased resistance. For example, in soybean, mutants with reduced ethylene sensitivity produce less severe chlorotic symptoms when challenged with the virulent strains *Pseudomonas syringae* pv. *glycinea* and *Phytophthora sojae*.

EIN2, an integral membrane protein of unknown function, is a major positive regulator of the ethylene pathway because loss-of-function mutations result in complete ethylene insensitivity (Alonso *et al.,* 1999). *ein2* (*Arabidopsis* plants with defects in ethylene perception), results in enhanced susceptibility toward *Alternaria brassicicola* (Penninckx *et al.,* 1998). *ein2* develops only minimal disease symptoms as the result of enhanced disease tolerance when infected by virulent *P. syringae* pv *tomato* or *Xanthomonas campestris* pv *campestris* (Bent *et al.,* 1992). However, the *ein2* mutant also displays enhanced susceptibility to the necrotrophic fungus *Botrytis cinerea* (Thomma *et al.,* 1999). *ein2* plants treated with the PGPR strain SE34 showed reduced systemic resistance against *P. syringae* pv. tomato (Ryu *et al.,* 2003). Mutations in *EIN2* result in the complete loss of ethylene responsiveness throughout plant development, suggesting that EIN2 is an essential positive regulator of ISR (Wang *et al.,* 2002).

ein3 encodes a nuclear-localized protein that belongs to a multigene family in *Arabidopsis*. Genetic epistasis analysis of ethylene response mutants has shown that EIN2 acts upstream of EIN3 (Wang *et al.,* 2002). EIN3 is a key positive switch in ethylene perception. For example, mutants *ein3* have reduced responses to ethylene, whereas overexpression of *ein3* results in ethylene hypersensitivity or a constitutive ethylene response (Roman *et al.,* 1995; Chao *et al.,* 1997). The *ein3* mutant was unable to express *Pseudomonas fluorescens* WCS417r-mediated ISR against *Pseudomonas syringae* pv. *tomato* (Pst) (Knoester *et al.,* 1999). Mutants *ein3* plants also showed more susceptibility to *P. syringae* bacteria showing its involment in ISR (Chen *et al.,* 2009).

Interestingly, recent studies have suggested that EIN3 protein levels rapidly increase in response to ethylene and that this response requires several ethylene signaling pathway components, including the ethylene receptors EIN4, EIN2, EIN5, and EIN6 (Resnick *et al.,* 2006).

To investigate the role of ethylene in the expression of ISR, the ethylene signaling mutants *ein4, ein5* and *ein7* were also tested in different studies. Similar results were obtained with the ethylene-insensitive mutants *ein4, ein5, ein7,* indicating that the expression of ISR requires the complete signal-transduction pathway of ethylene. None of the ethylene-insensitive mutants *ein2-1, ein3-1, ein4-1, ein5-1,* and *ein7* expressed ISR in response to treatment with *Pseudomonas fluorescens* WCS417r against *Pseudomonas syringae* pv. *tomato* (Pst) (Knoester *et al.,* 1999), demonstrating their roles in ISR.

EIR1 is a membrane-bound protein localized exclusively in roots and was proposed to have a root-specific role in the transport of auxin (Luschnig *et al.,* 1998). The *Arabidopsis* mutant *eir1* is insensitive to ethylene at the root level (Roman *et al.,* 1995). *Pseudomonas fluorescens* WCS417r was not able of inducing resistance in this mutant against *Pseudomonas syringae* pv. *tomato* (Pst) (Knoester *et al.,* 1999). Similary, *eir1* was unable to mount *Pseudomonas fluorescens* strains CHA0r-induced ISR against *Peronospora parasitica*. So it is summarized that EIR1 is required for ISR (Lavicoli *et al.,* 2003).

Further analyses showed that ethylene acts downstream of jasmonic acid, and upstream of NPR1, in the ISR pathway (Pieterse *et al.,* 1998).

The defense regulatory protein NONEXPRESSOR OF PR GENES1 (NPR1) has been described to have role in SAR but it was tested in ISR bioassay that clearly showed that WCS417r-mediated ISR is dependent of NPR1 defense response (Pieterse *et al.,* 1998; Van Wees *et al.,* 2000). It was identified as a key signaling node in the interaction between the SA and JA pathways, because mutant *npr1* plants were blocked in SA-mediated suppression of JA-responsive genes (Spoel *et al.,* 2003). Further analysis of the ISR signal-transduction pathway revealed that NPR1 acts downstream of the JA and ET signal pathway (Pieterse *et al.,* 1998). These results suggest that the NPR1 protein is important in regulating different hormone-dependent defense pathways.

Different *Arabidopsis* mutants of Enhanced disease susceptibility (EDS) have also been reported in ISR. *eds4-1* and *eds8-1 mutants* were nonresponsive to induction of ISR by *Pseudomonas fluorescens* WCS417r. Indeed EDS4 and EDS8 are required for ISR by *Pseudomonas fuorescens* WCS417r against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and act in either the jasmonate response (EDS8), the ethylene response (EDS4) in the ISR signaling pathway (Ton *et al.,* 2002). In comparison mutant *eds5-1* was nonresponsive to induction of pathogen-induced SAR by *Pseudomonas syringae* pv. tomato DC3000 (Ton *et al.,* 2002), and *eds5-1* is known to be blocked in the synthesis of SA.

Phytoalexin deficient mutants *pad1, pad3 and pad4* are also found to be involved in defence signaling pathway involving JA or SA. PAD1 is JA dependant whereas PAD3 and PAD4 have been demonstrated as SA signaling dependants (Zhou *et al.,* 1998; Glazebrook *et al.,* 2003). It has been shown that *PAD4* is a regulator of defense responses and acts upstream from SA to affect expression of *PR-1* and camalexin synthesis (Zhou *et al.,* 1998). Different results have also indicated that *pad3* mutation depends on SA-dependent resistance (SAR). *pad1* and *pad4* mutations not only regulate camalexin production but also control other

defense pathways that can contribute to resistance. The pleiotropic nature of *pad1* is indeed evidenced by its effect on leaf morphology (Glazebrook *et al.,* 1997) and that of *pad4* by a block in the production of salicylic acid (Zhou *et al.,* 1998).

Although all these gene products described before have been demonstrated as involved in ISR or not, it is possible that other mechanisms could be correlated to ISR. For instance mutant *ups1,* which has reduced expression of phosphoribosylanthranilate transferase, a tryptophan biosynthetic enzyme, is defective in a wide range of defence responses after infection with *Pseudomonas syringae* or *Botrytis cinerea* (Denby *et al.,* 2005). It has been also demonstrated that there is a disruption of SA, JA and Ethylene-dependent pathways in the *ups1* mutant, suggesting a role for UPS1 upstream of the three major signaling pathways (Jason and Denby, 2000). It is therefore possible that UPS1 could be required for ISR in case of an induction by a beneficial bacterium although this needs to be demonstrated. The expression of genes regulated by both the salicylic acid and jasmonic acid/ethylene pathways is reduced in *ups1* compared with wild type. Reactive oxygen species (ROS)-mediated gene expression is also compromised in this mutant indicating that this mutant is defective in signalling pathways activated in response to both biotic and abiotic stress (Glawischnig, 2007). This could allow suggesting ourself on a role of ROS in ISR if UPS1 is required. To describe this, *Atrboh* (*A. thaliana* respiratory burst oxidase homolog) mutants could be used as the genes products are known as involved in the production of ROS during the fungal infection (Sagi and Fluhr, 2001). Reactive oxygen species (ROS) were initially recognized as toxic by-products of aerobic metabolism, removed by means of antioxidants and antioxidative enzymes. In recent years, it has become apparent that ROS play an important signaling role in plants controlling processes such as growth, development, response to biotic and abiotic environmental stimuli, and programmed cell death (PCD) (Foreman *et al.,* 2003; Sagi *et al.,* 2004; Torres *et al.,* 2005).

H_2O_2 has been proposed as a systemic signal (Alvarez *et al.,* 1998). H_2O_2 is a signalling molecule of widespread importance in plant responses to various biotic and abiotic stimuli that include pathogen challenge, drought stress, exposure to atmospheric pollutants, extremes of temperatures, gravitropism, hormones, cell development and senescence (Apel and Hirt, 2004; Laloi *et al.,* 2004; Neill *et al.,* 2002).

The recent identification of ROS-generating enzymes, such as the plant homolog of respiratory-burst NADPH oxidases has led to the demonstration that plant cells can initiate and most likely amplify ROS production for the purpose of signaling (Kwak *et al.,* 2003;

Gerber and Dubery 2004; Maruta *et al.,* 2010). Reactive oxygen species (ROS) could be integrated with several different signaling pathways as they can activate both SA- and JA/ethylene-mediated signalling in plants. It has been found that ROS play a role in the establishment of systemic acquired resistance (SAR) (Durrant and Dong, 2004). SA and ROS are interconnected because ROS accumulation is potentiated by very small doses of SA in Soybean against *Pseudomonas syringae* pv. *glycinea* (Shirasu *et al.,* 1997) and ROS also induce SA accumulation (Chamnongpol *et al.,* 1998; Eckey-Kaltenbach *et al.,* 1997; Sandermann *et al.,* 1998; Sharma *et al.,* 1996; Yalpani *et al.,* 1994). However what else for ISR? ROS are also induced by beneficial bacteria as demonstrated by Hubalek in 2009 with *B. phytofirmans* strain PsJN as their production are primed when plants are challenged with *Phytophtora infestans*. There is however limited researches on the possibility that ROS can be linked to ISR. Some studies describe that ROS are not involved, but that ROS can be primed once pathogens infected a plants. Other described that ROS are involved during interaction between a host and a beneficial microbe (see or instance the work described by the group of Puppo with rhizobia).

Other mechanisms can be studied. This could allow to determine new mechanisms of ISR after use of one strain. However everyone knows that *Arabidopsis thaliana* plants are used for fundamental researches to dissect more mechanisms. It is interesting to note that when a PGPR and/or an endophyte is used, it is important to evaluate also its potential on a cultivated plant. Indeed use of these beneficial bacteria can give a service for the Agriculture.

In the following paragraph we will amplify the need to describe the use of beneficial bacteria, both rhizo- and endophytic strain on grapevine. Why this crop? It is one of the plants for which there is a current need to have a portfolio of beneficial bacteria to use on.

III. Use of rhizospheric and endophytic bacteria and their secondary metabolites to protect grapevine towards diseases

Grapevine, *Vitis vinifera* L., is one of the major cultivated crops. It can be however subjected to many pathogens diseases. This leads to yield losts as well as problems of grape quality (Evans *et al.,* 2011). Phytosanitary products have been extensively used to remedy this problem (Rosslenbroich and Stuebler, 2000). However risks of pollution, problems on human

health and apparition of pathogen resistance have been correlated to use of such chemical products (Wightwick *et al.,* 2008). This has lead to find new alternative strategies to protect the plants (Janisiewicz and Korsten, 2002; Leroux, 2004; Spadoro and Gullino, 2005). Among solutions, uses of beneficial bacteria from the rhizosphere as well as endophytic bacteria have been proposed (Hallmann *et al.,* 1997; Bloemberg and Lugtenberg, 2001). The role of both rhizobacteria and endophytes in biocontrol of plant diseases or for a sustainable management of agriculture has been highlighted. A large number of rhizobcteria and endophytes have been used in grapevine against different plant pathogens. Strains used, mechanisms involved and level of protection are summarized and discussed in the following publication.

Review

Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases

Stéphane Compant[1†*], Günter Brader[2†], Saima Muzammil[1†], Angela Sessitsch[2], Ahmed Lebrihi[1] and Florence Mathieu[1]

[1] Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Département Bioprocédés et Systèmes Microbiens, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1, France

[2] AIT Austrian Institute of Technology GmbH, Health & Environment Department, Bioresources Unit, 3430 Tulln, Austria

† authors contributed equally

* Corresponding author: Dr. Stéphane Compant, Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Dépt. Bioprocédés et Systèmes Microbiens, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1, France. Tel: +33 (0) 5 34 32 39 68. E-mail: scompant@ensat.fr

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Abstract

Grapevine is one of the most important economic crops yielding berries, wine products as well as derivates. However, due to the large array of pathogens inducing diseases on this plant, considerable amounts of pesticides -with possible negative impact on the environment and health- have been used and are currently used in viticulture. To avoid negative impacts of such products and to ensure product quality, a substantial fraction of pesticides needs to be replaced in the near future. One solution can be related to the use beneficial bacteria inhabiting the rhizo- and/or the endosphere of plants. These biocontrol bacteria and their secondary metabolites can reduce directly or indirectly pathogen diseases by affecting pathogen performance by antibiosis, competition for niches and nutrients, interference with pathogen signaling or by stimulation of host plant defenses. Due to the large demand for biocontrol of grapevine diseases, such biopesticides, their modes of actions and putative consequences of their uses need to be described. Moreover, the current knowledge on new strains from the rhizo- and endosphere and their metabolites that can be used on grapevine plants to counteract pathogen attack needs to be discussed. This is in particular with regard to the control of root rot, grey mould, trunk diseases, powdery and downy mildews, pierce's disease, grapevine yellows as well as crown gall. Future prospects on specific beneficial microbes and their secondary metabolites that can be used as elicitors of plant defenses and/or as biocontrol agents with potential use in a more sustainable viticulture will be further discussed.

Keywords: *Vitis vinifera* L., diseases, biocontrol, beneficial bacteria, secondary metabolites.

Introduction

Grapevine is one of the most important economic crops, mainly because of the use of their berries for red, white, and rosé wine. This represents more than 7.5 million ha of cultivated surfaces in the world with 27 million t of wine produced by year as described for 2009 (FAOSTAT 2011). However, grapevine plants can be infected and colonized by a large variety of pathogenic microorganisms such as deleterious fungi, oomycetes and bacteria (Gouadec et al. 2007). These vine diseases can have drastic effects on the host plants, on berries, but also on wine qualities and their sensorial and organoleptic properties (Gouadec et al. 2007), resulting in economic losses for the wine growers and producers (van Helden 2008).

Pesticides have been or are currently applied in the vineyard to avoid the outbreak of vine pests or diseases, to manage the surrounding flora, to increase grape yield and to ensure wine quality (Leroux 2003; Pezet et al. 2004). As for instance in France more than 30, 000 t / year of fungicides and bactericides have been used for grapevine production (FAOSTAT 2011). For Europe, the International Organization of Vines and Wine estimates that 70, 000 t of fungicides are used annually

on around 3.8 million hectares of land dedicated to viticulture (http://www.endure-network.eu/). Worldwide, on average 35% of all pesticides are used for viticulture. The continuous use of phytosanitary products during the last decades has been, however, accompanied by an increasing awareness of the problems arising from intensive pesticide use. Consequences of intensive pesticide use include their persistence in soils, contamination of the environment, as well as appearance of resistant pathogenic strains (Leroux 2004). Additionally, specific pesticides have been withdrawn from the market due to their negative impact on human health and the environment (Amaro and Mexia 2003). Development of new active molecules targeting vine pests without undesired impact is possible. However, due to increasing cost to develop these new molecules, other alternative solutions have also been proposed.

To reduce the use of phytosanitary products, genetically modified (GM) plants have been propagated to control vine pests and diseases (see for examples the studies of Ferreira et al. 2004; Agüero et al. 2005; Vidal et al. 2006; The Local Monitoring Committee et al. 2010). However, this alternative strategy has not been and is still not widely accepted. So far, no GM grapevine has been commercialized (The Local Monitoring Committee et al. 2010). Many regions, especially in Europe, are generally not in favour of cultivation of GM crops (Marshall 2009), so there is a need for other solutions.

One of the alternative strategies to reduce the use of pesticides in grapevine production corresponds to the use of beneficial bacteria as biocontrol agents (Bent 2006). Since the rhizosphere concept of Lorenz Hiltner describing that the soil surrounding roots is influenced by plants and by microorganisms (Hiltner 1904; Hartmann et al. 2008), a large number of studies have demonstrated that part of the rhizobacteria inhabiting the rhizosphere can stimulate plant growth (plant growth-promoting rhizobacteria; PGPR) as well as protect plants against pathogen infections (biocontrol strains) (Berg 2009; Lugtenberg and Kamilova 2009). Plant growth promotion (e. g. achieved by hormone stimulation or changed nutrient availability) and biocontrol activities of particular rhizobacteria strains are distinct issues, however, in practice this is often hard to dissect as bacteria can show both activities. Also, particularly in field or in greenhouse trials, biocontrol bacteria might promote plant growth by reducing pathogenic pressure. Biocontrol by beneficial bacteria might be achieved by direct antibiosis, competition for niches and nutrients, interference with pathogen signalling or by inducing plant resistance (Figure 1, Berg 2009; Lugtenberg and Kamilova 2009). Moreover biocontrol might be achieved by degradation of virulence factors or phytotoxins of pathogens, thereby leading to reduction of disease symptoms (Compant et al., 2005a). Considerable literature information has shown that rhizobacteria can secrete various secondary metabolites (SMs). Both rhizobacteria and SMs produced by them can act on pathogens by depriving the pathogens of nutrients (competition), lysing cells and/or blocking specific functions related to pathogen growth (antibiosis) and act therefore as biocontrol agents (Berg 2009; Compant et al. 2005a; Lugtenberg and Kamilova 2009). Rhizobacteria and their SMs are also known to induce plant defense reactions

leading to a systemic resistance or priming of above ground parts to be more resistant to subsequent pathogen infection (Berg 2009; van Loon 2008; van Loon and Bakker 2005), and this can be used for grapevine protection against phytopathogenic diseases.

Already since the 19th century with the description of bacteria-like structures by Woronin (the so-called *Frankia* sp.) and the work of Galippe and di Vestea (see Compant et al. 2010a; 2012) with bacteria other than root nodulating strains, it has been widely accepted that specific microsymbionts can also colonize different host plants and plant parts. Although sources of colonization of these endophytic bacteria could be the anthosphere, the caulosphere, the phyllosphere or the spermosphere, the prevailing opinion suggests colonization of a large fraction of the endophytic population from the rhizosphere as described by microscopic, genetic as well as metagenomic evidence (Hallmann 2001; Hallmann and Berg 2007; Compant et al. 2010a).

As rhizobacteria, also endophytes are known to stimulate host plant growth and can act as biocontrol agents to alleviate infection by pathogenic strains, in particular cases even to higher levels than root-restricted bacteria (Welbaum et al. 2004; Hallmann and Berg 2007). Bacterial endophytes inhabiting plant internal tissues are also a source of SMs that may act as elicitors of plant defenses or as antimicrobial agents with potential use to control disease (Qin et al. 2011).

Different elicitors of plant defenses are known from beneficial bacteria, both from the rhizo- and the endosphere of plants. This includes a variety of primary bacterial constituents such as flagella (flagellin) or lipopolysaccharides (LPS) but also SMs with high structural diversity specific for certain strains (Qin et al. 2011; van Loon and Bakker 2005). In addition, continuous research and discovery of novel elicitors and strains from different environments, particularly from harsh ecosystems, will likely yield novel strains and elicitors capable of triggering plant defenses and enabling resistance. This is especially interesting for the reduction of the use of pesticides in viticulture, where – in France - up to 50% of the total pesticide entry is used for only 3.3 % of cultivated surfaces and in EU 3.5% of the cultivated land receives 15% of the total pesticide entry representing 20-22 kg of pesticide /ha used for grapevine (Compant 2011; Compant and Mathieu, 2011).

The role of both rhizobacteria and endophytes in biocontrol of plant diseases or for a sustainable management of agriculture has been highlighted (van Loon and Bakker 2005; Lugtenberg and Kamilova 2009) and information on the usage of beneficial microbes in viticulture is currently emerging. Research performed on specific strains have moreover allowed the description of SMs secreted by specific strains (both rhizo- and endosphere colonizing bacteria), which may be responsible for their effects on pathogen targets and/or on resistance mechanisms of grapevine plants (Compant and Mathieu 2011). Additionally, new beneficial bacterial strains and SMs to control plant diseases with potential use in viticulture are continuously described (Compant 2011). Nevertheless, a better understanding of how and which microorganisms or bacterial metabolites can be used to reduce disease pressure in grapevine plants is needed. In this review, the use of beneficial bacteria and their

metabolites used to control various grapevine diseases caused by fungi, oomycetes or bacteria is described. This also includes the description of mechanisms involved in plants, on phytopathogen diseases reduction, but also on the origin of strains and metabolites used to control grapevine diseases. Future prospects for a better delivery of inoculants or elicitors are also provided. Understanding the mechanisms through which beneficial bacteria and their metabolites act on phytopathogens and plant responses is a pre-requisite for a better delivery of bacterial microsymbionts in the field, but also for fundamental research or bioprocesses development.

Beneficial bacteria and biocontrol of grapevine fungal and oomycetes diseases

The research performed so far has demonstrated that specific strains of both rhizo- and endophytic bacteria as well as some of their secreted secondary metabolites can inhibit pathogens affecting grapevine (Figure 1). In the following paragraphs the focus will be on fungal trunk diseases, Fusarium root rot, grey mould, powdery and downy mildew as serious diseases affecting viticulture and on beneficial bacteria strains reducing these diseases (Table 1). Their effects under controlled and field conditions are discussed.

Biocontrol of wilt and root rot caused by *Fusarium* spp.

Wilts and root rots of grapevine caused by fungal pathogens such as *Armillaria* spp. *Fusarium* spp. and *Verticillium* dahlia Kleb. have been occasionally reported (Garrido et al. 2004; Gubler et al. 2004; Zhang et al. 2009; Ziedan et al. 2011). In the following part we will exemplify the biocontrol of wilt and root rot caused by *Fusarium* spp., which are of regional importance, particularly in warm vine regions such as Australia, Brasil, Egypt (Garrido et al. 2004; Highet and Nair 1995; Ziedan et al. 2011) and may also cause problems in combination with phylloxera feeding (Granett et al. 1998). Depending on the rootstock (Omer et al., 1999), *Fusarium oxysporum* E.F. Sm. & Swingle (Nectriaceae) can cause reduced plant growth, affects the survival of young plants and the yield and productivity of grapevine (Highet and Nair 1995). Incidences on vineplants suffering from this fungus have been described recently in Egypt, where *F. oxysporum* isolates on grapevine plants (Cv. crimson) caused vascular wilt (on 66.7% of the cases) and root-rot syndrome (33.3%) (Ziedan et al. 2011). Another species of *Fusarium*, *F. solani* Sny. & Hans. can also lead to rootstock deficiency (Andrade 1993; Grasso 1984; Gugino et al. 2001). To tackle the problem of *Fusarium* infections in grapevine, Ziedan et al. (2010) studied biocontrol bacteria to alleviate vine plant infections by *Fusarium* spp. Seven strains of *Streptomyces* spp. isolated from grapevine rhizospheric soil, were screened for antagonistic activities towards *F. oxysporum*. All isolates showed antifungal activities. One isolate identified as *Streptomyces alni* exhibited the highest activity, which was correlated to an inhibition of fungal growth, malformation, lysis of hyphae as well as inhibition of normal branches and conidia of

conidiophores on dual culture plates. This indicates a direct antibiosis effect of this biocontrol strain, potentially mediated by the effect of a hitherto uncharacterized antibiotic (Ziedan et al. 2010). Under greenhouse and field conditions, the use of *S. alni* was associated with a reduction of root rot infection. An increase of grape yield of cv. Superior was also noted. In combination with the biofertiliser "Rhizobacterin" containing the *Klebsiella planticola* strain BIM B-161 the *S. alni* strain was even more effective (Ziedan et al. 2010). The obtained results suggest that the *S. alni* strain could be successfully used in combination with biofertilisers for controlling root-rot of grapevine, especially in organic farming systems.

In addition to *S. alni*, the *Pseudomonas fluorescens* isolate NRC10, a rhizobacterial strain isolated from the grapevine root environment, might have the potential to control *Fusarium* rot in grapevine plants (Ziedan and El-Mohamedy 2008). A number of fluorescent Gammaproteobacteria such as *P. fluorescens* are well known to act as biocontrol or PGPR agents as well inhabiting the rhizosphere of grape plants (Svercel et al. 2009; 2010). For strain NRC10 it was demonstrated that it can attach or adhere fungal hyphae of *Fusarium* spp. It can also penetrate fungal cell walls and can be responsible for morphological changes of fungal hyphae, and conidiospores as well as of partial degradation of fungal cell walls and sclerotia (Ziedan and El-Mohamedy 2008). Mechanistically, both production of lytic enzymes by the biocontrol bacteria or production of antifungal metabolites have been discussed, as such mechanisms and modes of actions have been described for closely related *P. fluorescens* strains (Ziedan and El-Mohamedy 2008). Soil treatment of cv. Thompson Seedless with *P. fluorescens* NRC10 can significantly reduce additionally root rot percentage and disease severity in the field. It has been further shown that inoculation of *P. fluorescens* NRC10 on soil of grape plants induced an increase of fruit yield in an Egyptian vineyard (Ziedan and El-Mohamedy 2008). This demonstrates the potential of this isolate for application directly in the field.

Both examples cited before show that there are alternatives to pesticide use to control *Fusarium* sp. contamination on vine plants. However, considerable information is still required on how these strains can protect grape plants against root rot disease. In particular it is not clear at the moment if and which SMs are involved in the root rot inhibition. Additionally, activation of plant defense reactions leading to resistance may play a role in the reduction of the infection. It may be speculated that jasmonate and ethylene dependent induced resistance is important in enhanced grapevine resistance to Fusarium rot – at least after *P. fluorescens* treatment - since the contribution of these signal pathways in enhanced resistance in *Arabidopsis* after treatment with different *P. fluorescens* strains is well established (van der Ent et al. 2009; van Wees et al. 2008).

Biocontrol of fungal trunk diseases

Trunk diseases can be caused by various fungal taxa and have been widely reported as severe diseases infecting grapevine plants. The diatrypaceous fungus *Eutypa lata* (Pers.) Tul. & C. Tul. is known to cause one of the major symptoms, the Eutypa dieback. Other fungi of this family have been

also shown to be associated with the disease, and have been isolated from necrotic tissues in shoots, at margin of canker in cordons, trunks, spurs or from surface of decorticated bark or wood of grapevines (Trouillas et al. 2010). Associated species are *Cryptovalsa ampelina* (Incertae sedis) (Nitschke) Fuckel, *Diatrype stigma* (Hoffm.) Fr. and *Eutypa leptoplaca* (Mont.) Rappaz causing vascular necrosis (Trouillas and Gubler 2010) as well as *Cryptosphaeria pullmanensis* Glawe, *Cryptovalsa ampelina*, *D. stigma*, *D. whitmanensis* J.D. Rogers & Glawe, and *E. leptoplaca* infecting and causing lesions in green shoots (Trouillas and Gubler 2004; 2010; Trouillas et al. 2010). Reassessment of concept of *Eutypa lata* has allowed to support that another associated fungus, *E. armeniaceae* Hansf. & M.V. Carter, is synonymous of *E. lata* (Rolshausen et al. 2006). Eutypa dieback results in significant economic damage on grapevine plants. Infected grapevines show a wedge-shaped staining of dead wood, gradually decline in productivity and eventually die. Dieback can also lead to stunted grapevine shoot, cupped and chlorotic leaves with necrotic margins, as well as to reduced qualitative yield productivities (Carter 1991; Kotze 2008).

Historically, management of Eutypa dieback relied on sanitary practices as well as the protection of the surface area of pruning woods by phytosanitary products (Carter and Price 1974; Rolshausen and Gubler 2005; Bester et al. 2007). At the moment, apart from fungicide use, various *Trichoderma* strains are in discussion as potential biocontrol agents for dieback (John et al. 2004; Halleen et al. 2010; Kotze 2008). However, also an endophytic strain of *Bacillus subtilis*, which was isolated from grape wood arm of cv. Chenin Blanc infected with *E. lata*, was under discussion as it can reduce the pathogen infection, colonization as well as the disease (Ferreira et al. 1991). This strain can inhibit mycelial growth, induce malformation of hyphae as well as reduce ascospore germination in *in vitro* tests indicating a direct antibiosis effect of the strain. Interestingly, it has been further demonstrated that spraying a suspension of this strain on grape wood reduces infection with the pathogenic agent (with a 100% reduction; Ferreira et al. 1991). This demonstrates the potential of a beneficial endophytic bacterium to control *E. lata* infection. Other potential biocontrol bacteria also exist. Following the study of Ferreira et al. in 1991, Munkvold and Marois (1993) tried to identify effective bacterial strains to control *E. lata* in the field. However, only a small fraction of three strains of more than 150 active strains in the laboratory on wood has been tested in the field in these experiments and tests failed to find a biocontrol agent (Munkvold and Marois 1993). In 2001, it has been demonstrated that 121 isolates (from different origins, belonging to Actinomycetes, *Bacillus* spp., *Erwinia herbicola* and *Pseudomonas* spp.) of 188 tested could exhibit antagonistic activity towards *E. lata in vitro* (Schmidt et al. 2001). One *B. subtilis* strain (B1α), two *E. herbicola* strains (JII/E2 and JII/E4) and one actinomycete (strain A123) have shown the highest degree of antagonism on grape wood discs. The use of such strains could allow a reduction of 70 to 100% of the pathogen infection and its colonization over a four week period as demonstrated by the experiments. *Erwinia herbicola* JII/E2 and *B. subtilis* B1α inhibited growth of six different *E. lata* isolates on wood. Moreover, inhibition of the fungus by these strains correlated with a reduction in fungal hydrolase activity, which

is highly correlated with mycelial growth in wood, demonstrating the strong ability of these strains to reduce *E. lata* growth and their potential for application (Schmidt et al. 2001). What could be verified is if bacterial biocontrol strains are also effective against *E. lata* in the field. Nevertheless, an effective biocontrol strain against Eutypa dieback has high potential in application, especially if this strain could also control a number of other fungi causing similar symptoms/ other trunk diseases. These include for instance members of Botryosphaeriaceae.

Botryosphaeria dothidea (Moug.) Ces. & De Not*., Diplodia seriata* De Not*.,* and *B. stevensii* Shoemaker are the cause of "Black Dead Arm" (BDA) in France (Larignon and Dubos 2001). The disease is characterized by wood streaking and red patches at the margin of the leaves, and large areas of chlorosis and deterioration between the veins (Larignon and Dubos 2001). However the occurrence of the Botryosphaeriaceae is not always linked to BDA disease. Virulence and symptoms of Botryosphaeriaceae have been reported as different according to cultivars and countries. For example, no symptoms of BDA were found associated with the same species on grapevines in Portugal (Phillips 2002). Nevertheless Botryosphaeriaceae members have been frequently isolated from grapevines showing decline or dieback symptoms in different countries as in Egypt (El-Goorani and El Meleigi 1972), California (Gubler et al. 2005), Arizona, Mexico (Leavitt 1990), Europe (Hungary, France, Italy, Portugal, Spain; Rovesti and Montermini 1987; Lehockzky 1974; Phillips 1998; Larignon and Dubos 2001; Luque et al. 2005), South Africa (Van Nierdeck et al. 2004), Chile (Auger et al. 2004), and Australia (Castillo-Pando et al. 2001).

Although it is often difficult to distinguish symptoms of Botryosphaeriaceae from the ones caused by other fungal pathogens such as *E. lata*, *E. leptoplaca* and *Phomopsis viticola* (Sacc.), a number of different members have been associated with the disease such as *Diplodia seriata, Neofusicoccum australe* Slippers, Crous & M.J. Wingf*., B. dothidea, N. luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips*,, N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips*, B. stevensii, Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Úrbez-Torres et al. 2006) and the anamorphs *Diplodia sarmentorum* (Fr.:Fr.) Fr., *D. porosum* Niekerk & Crous, *Fusicoccum viticlavatum* Niekerk & Crous, and *F. vitifusiforme* Niekerk & Crous (van Niekerk et al. 2004). Recent advances in control of Botryosphaeriaceae infection have shown that beneficial microbes could control some of the species mentioned above. In particular, *in vitro* assays have shown that the heat stable metabolites of *Bacillus subtilis* AG1 can inhibit the growth of *Lasiodiplodia theobromae* (Alfonzo et al. 2009). Recent screening also shows that a considerable number of bacterial isolates from the rhizosphere and/or endosphere from grapevine, as well as from harsh environments, can reduce *in vitro* growth of *D. seriatia* and *N. parvum* (unpublished information). However there is still as yet no work related to determine the potential of beneficial bacteria to control Botryosphaeriaceae infection in the field. This is partly due to the fact that beneficial bacteria acting as a biocontrol agent should not only reduce Botryosphaeriaceae infection but also other fungi responsible for trunk diseases.

Esca (also known as black measles in the USA) is attracting more consideration in viticulture and has long been considered a single disease, which normally affects adult or old vines. Although different fungi have been correlated with the disease, three main fungi, *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams and *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai (corresponding to causal agents of petri disease) together with *Fomitiporia mediterranea* M. Fisch., have been mainly associated with esca (Surico et al. 2008). However these fungi can lead to five related syndromes. This forms the esca disease complex with potentially dramatic consequences up to death of the grapevine plant (Graniti et al. 2000). The syndromes are brown wood streaking of rooted cuttings, Petri disease with brown wood streaking in young vines, young esca (also recently called phaeotracheomicosis), white rot, and esca proper (addition of young esca with white rot; Gramaje and Armengol 2011; Graniti et al. 2000; Mostert et al. 2006; Surico et al. 2008). The three main fungi *Pa. chlamydospora*, *Pm. aleophilum* and *F. mediterranea* are generally spread by spores released from infected vines or other host plants during wet conditions and are dispersed by wind currents. Infection on fresh pruning wounds is believed to be the main cause of entrance for fungi causing trunk disease symptoms (Graniti et al. 2000). Although some pesticides have been employed to reduce infection of these diseases, commercial use has been restricted and has been disputed in the case of the use of sodium arsenate (Chiarappa 2000). Researches on biocontrol agents have started to find alternative strategies to reduce petri disease, young esca, white rot and esca proper. This has been concentrated on beneficial fungi such as *Trichoderma* spp. strains (Fourie and Halleen 2006; Halleen et al. 2010; Kotze 2008), but beneficial bacteria have been studied as well. In particular, *in vitro* assays have shown that metabolites of *Bacillus subtilis* AG1 described above can -in addition to *Lasiodiplodia theobromae* - inhibit the growth of fungi involved in trunk diseases such as *Pm. aleophilum,* and *Pa. chlamydospora* (Alfonzo et al. 2009). Other bacteria are currently being tested as biocontrol agents to reduce diseases caused by the fungi (unpublished results). Although the first results in lab showed promising findings to protect the decline of vine resulting from trunk diseases, more work is required for the use of such strains or metabolites. Especially, additional testing in plants and long term management in the field is needed to ensure the required level of protection.

Searching the mechanism involved is needed for a better delivery of bacterial inoculants and for the application of bacterial metabolites in the field. Some of those so far tested biocontrol strains and their metabolites seem to have a direct effect on the growth of fungi in grapevine wood, either by growth inhibition or by inhibition of fungal enzymatic activities. What should be studied further is how far activation of plant defenses is also playing a role in bacterial biocontrol of trunk diseases. Search for strains with the potential to degrade phytotoxic disease factors of Eutypa dieback and esca disease pathogens (Christen et al. 2005) might provide an additional strategy, how bacterial strains could control trunk diseases. Since only limited means for the control of trunk disease exist,

development of bioncontrol strains will be an important factor in the future for controlling trunk disease in viticulture.

Biocontrol of grey mould caused by *Botrytis cinerea*

Grapevine is not only infected by fungi affecting trunks and roots, but also by fungi deteriorating fruit setting and quality such as *Botrytis cinerea* Pers. (Sclerotiniaceae). *B. cinerea* is known to be responsible for grey mould and Botrytis bunch rot affecting young fruit, during the ripening process and making the grapes inappropriate for wine making. The potential of specific strains to control grey mould has been demonstrated by a number of beneficial bacteria. Strains belonging to Pseudomonadaceae, Bacillaceae, Enterobacteriaceae, Actinobacteria as well as Burkholderiaceae have been shown to have a positive effect on grey mould control (Compant et al. 2011).

An endospore forming bacterial strain (GI 070) belonging to the species *Bacillus circulans*, was described as antagonistic to *B. cinerea* (Paul et al. 1997). The bacterial culture and its filtrate can completely suppress the fungus in Petri-dishes and reduce grey mould symptoms on grapevine *in vitro* plantlets (Paul et al. 1997). In another study, Krol (1998) studied 17 isolates on 282 leaf-derived endophytic strains exhibited antagonistic activities to *B. cinerea*. However, only two isolates (one *Bacillus* sp. and one *P. fluorescens* strain) limited the disease development on grapes (Krol 1998). Both studies demonstrate that different bacteria have the potential to control grey mould symptoms on grapes, but also show that *in vitro* antagonistic activities have limited prediction in activities *in planta* and that induced plant resistance might play a major role in the observed effects.

In another study the potential of different bacteria isolated from the rhizosphere or the endosphere of different plant parts of healthy field-grown grapevine plants cv. Chardonnay was evaluated for biocontrol of grey mould symptoms (Trotel-Aziz et al. 2006; 2008). Twenty-six out of 282 bacterial strains, all of them isolated from vineyards and belonging to *Pseudomonas, Bacillus, Acinetobacter* and *Pantoea* demonstrated protective activity (85-100%) against *Botrytis cinerea* on dual culture plates. The biocontrol activity of the bacteria *Pseudomonas fluorescens* PTA-268, PTA-CT2*, Bacillus subtilis* PTA-271, *Pantoea agglomerans* PTA-AF1 and PTA-AF2, as well as *Acinetobacter lwoffii* PTA-113 and PTA-152 was moreover demonstrated on *in vitro* plantlets cv. Chardonnay. Differential induction of defense-related responses such as lipoxygenases, phenylalanine ammonia-lyases and chitinases in grapevine leaves was correlated with the protection (Trotel-Aziz et al. 2006; 2008). Moreover treatment with the strains *P. agglomerans* AF2, *B. subtilis* 271, *A. lwoffii* 113 and *P. fluorescens* CT2 enhanced oxidative burst and production of the phytoalexin resveratrol in grapevine leaves, which was well correlated with the enhanced resistance to *B. cinerea* (Verhagen et al. 2011). Verhagen et al. (2010) also showed that *Pseudomonas aeruginosa* (7NSK2), *P. fluorescens* (strains CHA0, Q2-87 and WCS417) and *P. putida* (WCS358) could induce resistance to *B. cinerea* in grapevine, which was correlated to a different extent with phytoalexins and oxidative burst production.

The authors showed that inducing resistance in the plant is a major mechanism observed in protection against *B. cinerea* and also demonstrated that the bacterial metabolites salicylic acid (SA), 2,4-diacetylphloroglucinol (DAPG), pyochelin and pyoverdin contributed to this resistance, but are not the only chemical factors involved.

In field experiments during four consecutive years, the potential of the beneficial strains described before were also demonstrated, and the severity of grey mould disease on grapevine leaves and berries was reduced (Magnin-Robert et al. 2007). This was correlated to different levels of protection, depending on the bacterial strain used (in total 7) and of the inoculation method (Magnin-Robert et al. 2007). The state of plant resistance was associated with a stimulation of plant defense responses such as chitinase and β-1,3-glucanase activities (with known botryticidal activities) in both leaves and berries (Magnin-Robert et al. 2007), again indicating a major contribution of enhanced plant resistance in response to the biocontrol strains. Highest activities were, howewer, dependent on plant organs. *Acinetobacter lwoffii* PTA-113 and *Pseudomonas fluorescens* PTA-CT2 showed highest protection in leaves, and *A. lwoffii* PTA-113 or *Pantoea agglomerans* PTA-AF1 in berries, suggesting that different strains can be more appropriate for treatment of specific organs (Magnin-Robert et al. 2007).

Use of the endophytic plant growth-promoting bacterium, *Burkholderia phytofirmans* strain PsJN (Sessitsch et al. 2005), isolated from onion root infected with *Glomus vesiculiferum* in Germany (Nowak et al. 1995) has been demonstrated as enabling the reduction of infection of *B. cinerea* on grapevine plants (Ait Barka et al. 2000; 2002). It has been additionally shown that this □-proteobacterium could improve host plant growth as well as establishes rhizospheric and endophytic subpopulations in various organs and systemically spread inside grapevine plants (Compant et al. 2005b; 2008a; 2008b). Although no experiment was done in the field to evaluate the potential of such strains under natural conditions as well as its persistence inside soil and internal tissues during a long period, a recent study has demonstrated that the species *B. phytofirmans* could be naturally present in the vineyard (Lo Picollo et al. 2010). It can establish subpopulations in leaves of grapevine plants as demonstrated in Italy (Lo Picollo et al. 2010) and could therefore be used for application on grape although this needs to be tested under field conditions.

Attempts to use members of the Actinomycetales such as *Streptomyces* spp. or *Micromonospora* spp. to control *B. cinerea* have also been studied (Loqman et al. 2009; Lebrihi et al. 2009a; 2009b). Some soil strains of these bacteria can allow grapevine *in vitro* plantlets to withstand grey rot (Loqman et al. 2009). Experiments corresponding to the use of other *Streptomyces* sp. strains have also shown that a protection can occur under greenhouse conditions (Lebrihi et al. 2009a; 2009b). Moreover, cyclic bacterial metabolites (tetracyclopeptides) secreted by these latter strains can induce protection directly by antibiosis or indirectly by inducing various plant defense responses leading to protective effects (Lebrihi et al. 2009a; 2009b). However, due to large arrays of various

Actinomycetes secreting bio-active compounds, further experiments need to be conducted with attempts to find new bioactive compounds as well as new strains for *B. cinerea* control.

Research on new elicitors secreted by bacteria has recently also demonstrated that not only microbes can reduce infection *B. cinerea* but also their SMs alone. Glycolipids biosurfactants such as rhamnolipids secreted by *Pseudomonas aeruginosa* used in food protection, in cosmetology and for industrial applications can reduce grapevine disease such as the Botrytis rot. The effect of rhamnolipids was recently assessed on *B. cinerea* as well as on grapevine using cell suspension cultures and *in vitro*-plantlets of cv. Chardonnay (Varnier et al. 2009). Rhamnolipids can have direct antifungal properties by inhibiting spore germination and mycelium growth of the fungus. They can also efficiently protect grapevine against the disease. Defenses were associated to a Ca^{2+} influx, mitogen-activated protein kinase activation and reactive oxygen species production as early events (Varnier et al. 2009). Induction of plant defenses including expression of a wide range of defense genes, hypersensitive response (HR)-like response explained parts of the mechanisms involved in plant resistance. Additionally, rhamnolipids potentiated defense responses induced by chitosan elicitor and by the culture filtrate of *B. cinerea* (Varnier et al. 2009), suggesting that the combination of rhamnolipids with other effectors could participate in grapevine protection against the grey mould disease.

A recent study demonstrated another possibility to control *B. cinerea* caused diseases. An important virulence factor of *B. cinerea* with broad activity is oxalic acid. Schoonbeek et al. (2007) therefore investigated an interesting approach to reduce *B. cinerea* caused symptoms by looking for bacteria capable of degrading oxalic acid. The authors found an active oxalic acid degrading strain named oxB, which is closely related to *Cupriavidus campinensis.* Strain oxB could limit grey mould symptoms on leaves and strongly reduce disease symptoms in inflorescences under laboratory conditions.

In summary, biocontrol of *B. cinerea* by beneficial bacteria seems to be achieved mainly by activation of induced resistance in the plants. A number of strategies using beneficial bacteria to fight *B. cinerea* are in discussion and application potential seems to be higher than for the other diseases discussed. However, this is partly owed to the fact that the *B. cinerea* phytopathosystem is easy to study under laboratory conditions. Widening the search for new active strains and bacterial metabolites should allow developing an even broader portfolio of biocontrol strains, which would allow a more stable usage under different conditions, with different cultivars as well as allowing a better rotation system to overcome reduction of efficiency.

Biocontrol of powdery mildew (*Erysiphe necator*)

Powdery mildew of grapevines (*Erysiphe necator* Schw*.,* syn. *Uncinula necator,* anamorph *Oidium tuckeri*) spread from America to Europe in the mid of the 19th century has ever been since a serious issue for the European wine industry causing loss and diminished quality of grapevine fruits.

E. necator is known as infesting all green tissues and typically grows in round areas on young leaves, which become chlorotic and can become senescent and fall of prematurely. Inflorescences and young berries may become completely covered by the mildew (Gadoury et al. 2012; Pearson and Goheen 1988). Elder berries become more resistant to *E. necator,* but even low number of *E. necator* might have an effect on subsequent grey mould infestations (Ficke et al. 2002). Control of *E. necator* is mainly achieved by the use of an array of fungicides, but also by a number of inorganic substances, above all sulphur. Attempts to use biological control include various fungi, parasitic fungi such as *Ampelomyces quisqualis* and the mycophagous mite *Orthotydeus lambi* (Gadoury et al. 2012; Kiss 2003). However, bacteria such as some *Bacillus* strains have been tested for their capability to restrict the growth of *E. necator*. Seedlings of cv. Chardonnay were protected by *B. pumilus* B-30087 almost as effectively as the chemical fungicide myclobutanil at 25ppm, althought *in vitro* growth of a number of different fungi was not affected by this bacterium. This indicates either a specific direct inhibition mechanism or a defense activation effect allowing the plant to successfully combat *E. necator* infections. It has been suggested therefore that a water soluble antifungal metabolite smaller than 10000 Daltons and different from zwittermicin A may play a role in the effects of *B. pumilus* B-30087 (Lehmann et al. 2000).

Other *Bacillus* strains have also been patented to fight against *E. necator*. The *Bacillus* strains ATCC 55608 and 55609 were almost as effective against *E. necator* as metalaxyl in assays in grapevine plants. These strains produce antifungal substances including zwittermicin-A, which might play a vital role in the interaction (Marrone et al. 1999). More recently, Sawant et al. (2011) conducted field studies with Milastin K, a formulation of *B. subtilis*, over 3 years with cv. Thompson seedless. They observed that under low and medium *E. necator* pressure the pathogen could be controlled effectively, while under high pathogen pressure the effect was not as effective as sulphur.

While putatively effective and good candidates are known for bacterial biocontrol of *E. necator,* which has potential to be used for specific applications, what as to be studied is whether if these can compete however with cheap and effective sulphur treatments. However, *Bacillus* strains and bacterial SMs acting as bioeffectors may also have the advantage to be used in combination with synthetic or inorganic antifungal compounds. These combinatory applications are however more difficult with sensitive mycophagous mites and parasitic fungi.

Biocontrol of downy mildew (*Plasmopara viticola*)

Plasmopara viticola (Berk. and Curt.) Berl. and de Toni is another problematic grapevine pathogen introduced to Europe from America in the second half of the 19th century. It is the causative agent of downy mildew resulting in severe losses in grapevine production especially in more humid areas of Europe and North America. Pathogen infection results at first as yellow spots on leaf surfaces and growth of sporophores on the opposite lower leaf sides can be observed. Later on, it can cause losses through defoliation and killing of shoots and deteriorating fruit quality. In favorable weather

conditions and without protective measurements losses may rise up to 75% (Gessler et al. 2011; Pearson and Goheen 1988). *P. viticola* is an oomycete and relies as such on a zoospore stage, at which grapevine plants are invaded *via* stomata (Riethmueller et al. 2002). This entry mechanism may play a role in the effectiveness of biological control of the disease with oligosaccharides such as oligogalacturonides (OGA), which affects stomata regulation; nevertheless other defense mechanism must be induced by certain oligosaccharides since PS3 (sulfated laminarin) induces protection to *P. viticola*, but does not affect stomatal closure (Allègre et al. 2009). Also bacteria and their SMs have been patented as potential inhibitors of oomycetes including *P. viticola.* The effect of *Serratia marcescens* MSU-97 specifically on oomycetes have been shown *in vitro.* The active SM is a small cyclic peptide named serratamolide with membrane activity inhibiting oomycetes (Strobel et al. 2005). More recently, a terrestial actinomycete, *Streptomyces* sp. ANK313 was shown to produce the chinone khatmiamycin, which shows motility inhibition and causes lysis of zoospores of *P. viticola* (Abdalla et al. 2011). It remains to be seen, if these and other beneficial bacteria also have a positive effect on downy mildew control *in planta* and in vineyards and if biocontrol strains can also boost grapevine defence against *P. viticola.* Future applications of any of the biocontrol measurements can help to reduce the intensive use of copper and pesticides required for downy mildew control.

The majority of information on bacterial biocontrol of diseases caused by fungi and oomycetes can be found for grey disease caused by *Botrytis cinerea*. This does not necessarily reflect a limitation of the use of bacterial biocontrol for severe grapevine diseases such as powdery mildew, downy mildew and trunk diseases, but might also simply reflect the easiness of screenings for activity against *B. cinerea* and the widespread use of *B. cinerea* as test fungus in a number of laboratories. Future research for the use of bacteria for biocontrol should also focus on downy mildew and trunk diseases. Of course, different types of strains might be effective against these pathogens, also due to their different life conditions and location *in planta*, but for a broader practical application of biocontrol strains a wider portfolio and/or combinatory use of strains with the ability to control major grapevine diseases are necessary.

Beneficial bacteria and biocontrol of grapevine bacterial diseases

In addition to phytopathogenic fungi, bacteria infecting grape plants are the causal agents of severe diseases: *Agrobacterium vitis* causes crown gall (Süle and Burr 1998; Stafford 2000; Escobar and Dandekar 2003), *Candidatus* Phytoplasma vitis and *C.* Phytoplasma solani cause flavescence dorée (FD) and bois noir (BN) (Constable 2010), *Xylophilus ampelinus* arms bunches (Ridé 1996) and *Xylella fastidiosa* causes Pierce's disease (Hopkins 1989). Although different strategies have been used to control them, research of biocontrol agents to control these vine diseases has shown the potential of different bacterial strains to reduce bacterial infections (Table 1, Figure 1). This is

especially important for bacterial diseases that are difficult to treat with conventional pesticides and localized in the phloem or xylem vessels.

Biocontrol of *Agrobacterium vitis*

Crown gall disease of grapevines occurs especially in climates where cold winter temperatures can cause wounds, which are the main entry points for the pathogen. The disease incidence can be very high in affected vineyards and nurseries resulting in reduced growth and potentially the death of the plants (Burr and Otten 1999; Creasap et al. 2005). Currently few strategies for disease management of *A. vitis* exist. As an example for biocontrol of bacterial disease, a non-tumorigenic strain (F2/5) of *Agrobacterium vitis* has been shown to inhibit the *in vitro* growth of 21 of 25 *A. vitis* and two of 10 *A. tumefaciens* biovar 1 pathogenic strains (Burr and Reid 1994). When applied to wounds on potted woody grape trunks (*Vitis vinifera* L. cvs. Chardonnay and Riesling) in the greenhouse, the gall sizes were moreover significantly reduced for seven of 10 *A. vitis*, one of two *A. tumefaciens* biovar 1 and one of one biovar 2 strains, demonstrating the potential of a non–tumorigenic strain for field application. Co-inoculation of F2/5 with the pathogen was moreover at least as effective as pre-inoculation with F2/5. When the pathogen was inoculated prior to F2/5, the level of control was however greatly reduced (Burr and Reid 1994). However, caution should be taken in the application of strains belonging to species containing pathogenic strains. Burr and Reid (1994) demonstrated that the biocontrol strain was non-tumorigenic and that none of the three plasmids of strain F2/5 can hybridize with a probe consisting of the T-DNA from *A. tumefaciens* strain C58. However, the use of close relatives of pathogenic strains for biocontrol presents the risk that non-pathogenic biocontrol strains might mutate or acquire virulence plasmids, especially if the exact mechanisms of protection are not well understood (Seemüller and Harries 2010).

To investigate the mechanisms involved in biocontrol by the strain F2/5, agrocin-minus mutants were constructed. The mutants of strain F2/5 controlled grape crown gall as well as the wild-type strain (Burr et al. 1997), indicating that agrocin is not a major factor in the mechanism of biological control. Tumorigenic *Agrobacterium* strains attach to grapevine cells before infection. Therefore a competition of biocontrol strains for attachment sites may reduce therefore infection pressure of pathogenic strains (Shim et al. 1987). Attachment of tumorigenic strains (CG49 and K306) and biological control strains (F2/5 and the agrocin-minus mutant 1077) was also often reduced when mixtures of the strains were applied, but high concentrations of all strains attached, suggesting that competition for attachment sites is however not a factor involved in the mechanism of biological control (Burr et al. 1997). Transfer of T-DNA to grape by CG49 was prevented or greatly inhibited in the presence of F2/5 or 1077, although the Ti plasmid virulence genes of the phytopathogens were induced by exudates from grape shoots that had been previously inoculated with F2/5 (Burr et al. 1997). Alternative mechanism of plant protection by non-tumorigenic strains might include induced resistance of the plants or bacterial signal interference. Although the mechanism of how F2/5 could

control crown gall clearly needs further investigation, non-pathogenic *Agrobacterium* strains promise interesting strategies to control the disease.

Other non-tumorigenic strains have also been used on grapevine plants such as *Agrobacterium vitis* strain E26 or VAR03-1 (Kawaguchi et al. 2007; 2008; Wei et al. 2009). In biological control tests strain VAR03-1 was especially effective in reducing the incidence of gall formation on grapevine and reduced gall size by 84%–100% in comparison to the positive control (Kawaguchi et al. 2005; 2007; 2008). To minimize the potential risks of using biocontrol *Agrobacterium* strains, polymerase chain reaction and Southern blot analyses were used to determine that five essential virulence genes (*virA*, *virG*, *iaaH*, *iaaM* and *ipt*) were not present in strain E26 controlling crown gall disease (Wei et al. 2009). This suggests that this strain is unlikely to elicit crown gall symptoms in either host or non-host plants.

Not only non-tumorigenic strains of *Agrobacterium* spp. could control crown gall disease, but also strains from other taxa. *Pseudomonas aureofaciens* B-4117, *P. fluorescens* CR330D and 1100-6, *Bacillus subtilis* EN63-1, *Bacillus* sp. EN71-1, as well as *Rahnella aquatilis* HX2, can inhibit for instance the growth of a wide range of plant pathogens, including *A. tumefaciens*, when tested on agar media or on grapevine plants. The *P. aureofaciens* strain B-4117 persisted moreover on the root surfaces of inoculated vine cuttings and in non-sterile soil (Khmel et al. 1998). In growth chamber studies, *P. fluorescens* „1100-6' that reduce crown gall disease was also found to survive in the rhizoplane of grapevines for 6 months and predominantly occupied xylem and pith tissues (Eastwell et al. 2006), demonstrating a rhizo- and endosphere competence of this beneficial strain. With *Rahnella aquatilis* HX2, it has been shown in field trials that immersion of the basal ends of grape cuttings with HX2 cell suspension inhibited or even completely prevented crown gall formation caused by *A. vitis* K308 (30.8% compared to 93.5% in plants without HX2). Strain HX2 was found in the grape rhizosphere, grown under field conditions, for up to 90 days after inoculation and did not influence the mean population sizes of selected members of the microflora (Chen et al. 2007).

The production of an antibacterial substance ("ABS") was suggested to be an important factor in the biocontrol process by strain HX2 used to control crown gall as described by Chen et al. (2009) and Guo et al. (2009). ABS is a thermostable and alkali-sensitive substance containing sugar(s) and an unknown moiety with an absorption maximum at 285-nm. ABS displays a broad activity spectrum against 13 test isolates of phytopathogenic bacteria including *Agrobacterium*. *Agrobacterium* spp. strains were additionally more sensitive to ABS than other tested strains, with larger inhibition zones and lower minimal inhibitory concentration. The metabolite did not cause bacterial cell lysis, no leakage of cytoplasmic materials from cells of *A. vitis* but it rather inhibits RNA and protein synthesis in tumorigenic *A. vitis* (Chen et al. 2009).

Although the extent of disease control depends on the grape variety tested, the results suggest that there is potentially beneficial effect in using the antagonists to diminish the influence of latent rootstock infection of crown gall. Other bacteria preventing crown gall of grapevine are endophytes of

xylem sap of vine plants grown in Nova Scotia, Canada. Despite variation was noted in performing *in vitro* antibiosis, 24 strains were catalogued to have a strong inhibitory effect on *A. vitis* (Bell et al. 1995). This includes strains of *Enterobacter agglomerans*, *Rahnella aquatilis*, and *Pseudomonas* spp. Soil microcosm studies with a *xylE*-marked *A. vitis* strain showed in particular that one of these endophytes (an isolate of *P. corrugata*) is able to control population numbers of agrobacteria *in situ. In planta* trials with *V. vinifera* cv. Chardonnay showed that less than 47% in comparison to the positive control treatment produced galled vines, demonstrating significant biocontrol of the disease by three of the endophytes (Bell et al. 1995).

Biocontrol of grapevine yellows caused by Phytoplasmas

In grapevine, infections with phytoplasmas 16S rDNA group I, II, III, V and XII-A and XII-B corresponding to different *Candidatus* Phytoplasma species have been described and economically most important are *Ca.* Phytoplasma australiense (16S rDNA group XII-B) causing Australian grapevine yellows, *Ca.* Phytoplasma solani (XII-A, Stolbur) causing bois noir (BN) and *Ca*. Phytoplasma vitis (V) causing flavescence dorée (FD) (Constable 2010). In Europe, BN and FD frequently occur in wine producing countries; infection of plants results in reddening (red varieties) or yellowing of leaves, backward curling of leaf edges, shoots failing to harden off, shoots may die back and berries may shrivel and dry early. BN and FD are transmitted by phloem sucking insects, but with distinct epidemiology. FD is transmitted by the leafhopper *Scaphoideus titanus,* which is monophagous on grapevine in Europe and can transmit FD from grapevine to grapevine. BN on the other hand is transmitted by the planthopper *Hyalesthes obsoletus,* not able to fulfill a lifecycle on grapevine. The insects feed on herbs including nettle and bindweed, which are believed to be the main reservoir hosts of BN. Transmission to grapevine from these hosts is believed to be rather an accident (Constable 2010; Maixner 2011). Alternative vectors of BN have however also been discussed (Constable 2010; Riedle-Bauer et al. 2008). The different epidemiology has an impact on disease management, which relies on viticultural practices and insecticide treatments to reduce vector pressure, since no practical methods except the largely banned and expensive antibiotic treatments are available to treat Phytoplasma infected plants at the moment.

A potential mechanism of how bacterial diseases can be controlled is by cross protection with mild or avirulent strains of the disease causing agents (Seemüller and Harries 2010). Such cross protection with avirulent strains has been observed with phytoplasma (*Ca.* Phytoplasma prunorum) infected apricots, where infections with avirulent or mild strains seem to have a pre-immunizing effect (Seemüller and Harries 2010), either competing with disease causing phytoplasmas or enhancing the resistance of colonized plants. Given the risks of such cross protection and the limited knowledge how cross protection is achieved, application of this strategy is limited. Nevertheless there is an interest for such biocontrol applications in bacterial diseases difficult to control, especially in areas where disease pressure is very high.

Established beneficial bacteria like *Bacillus* spp. or *Pseudomonas* spp. cannot directly compete with phytoplasmas due to their different *in planta* location. However, since beneficial bacteria can prime plants and may induce resistance to a wide array of pathogens including nightshade viruses (Kloepper et al. 2004, van Loon 2007), also an effect on phloem colonizing phytoplasmas can be expected. In this respect it is interesting to note that in all grapevine yellows, spontaneous remission and recovery has been described (Constable, 2010). Bulgari et al. (2011) recently demonstrated that lower diversity of endophytic bacteria exists in Phytoplasma infected leaves of grapevine plants. This can be the results of a direct interaction between phytoplasmas and endophytic bacteria or a phytoplasma mediated plant response that restructured endophytic bacterial community. Isolation of endophytic bacteria in healthy, or especially in plants showing remission and their uses on grapevine could be therefore interesting for biocontrol of the disease.

Repeated biocontrol treatment with various inducers of plant resistance such as benzothiadiazole and glutathione/oligosaccharines mixtures lead to enhanced remission in BN affected grapevines (Romanazzi et al. 2009). Very recently, the concept of inducing enhanced resistance to phytoplasma with beneficial bacteria has been however evaluated using *Chrysanthemum* as model organism. Results showed that pretreatment with *Pseudomonas putida* S1Pf1Rif decreases the negative effects on plant growth infected with chrysanthemum yellows phytoplasma (CYP), but had no effect on CYP viability and proliferation (Gamalero et al. 2010). A combination treatment of *P. putida* S1Pf1Rif and the fungus *Glomus mossae* BEG12 resulted in slightly increased resistance and a delay of symptoms in CYP infected and non-resistant plants (D'Amelio et al. 2011). *G. mossae* could also reduce symptoms of the stolbur phytoplasma causing BN in grapevine in tomato (Lingua et al. 2002). It would be interesting to see if beneficial microorganisms also have an effect on symptom reduction of phytoplasma disease in grapevine plants under greenhouse and field conditions.

Biocontrol of *Xylella fastidiosa*

Pierce's disease has been well described in South-Eastern US and occurs in several regions in North- and Central America (Hopkins 2005). The causal agent of this disease is *X. fastidiosa*, which colonizes intensively xylem vessels after being transmitted by a sharpshooter (Cicadellidae). Symptoms on affected grapevines include yellow and brown color on leaves and eventually a sudden collapse of the foliage or a gradual death over a period of 1 to 5 years after plantation, with strong impact on the ability to produce wine in the affected regions (Almeida et al. 2005; Baumgartner and Warren 2005; Chatterjee et al. 2008; Hopkins 2005). This has lead to study potential solutions for control.

Several strains of avirulent endophytic *X. fastidiosa* can provide reduction in symptom development as described with cv. Carignane in greenhouse and field experiments (Hopkins 2005). In a 2-year assay on cv. „Himrod' in the vineyard, strain Syc86-1 (isolated from sycamore), but not strain PD-1 (derived from grapevine), was moreover effective in limiting the development of Pierce's

disease. In tests on new vineyard plantings of cv. Flame Seedless and cv. Cabernet Sauvignon, six non pathogenic strains of *X. fastidiosa* were evaluated for biological control of the natural progression of Pierce's disease (Hopkins 2005). However, only one strain (EB92-1) provides good control of the disease. Genome sequencing of strain EB92-1 revealed its very close resemblance to pathogenic *X. fastidiosa* strains, but lacks 10 putative virulence genes (Zhang et al. 2011). Grape strain PD95-6 showed lower disease severity in Flame Seedless when compared with non-treated vines. Strain PD91-2 delayed symptoms in Cabernet Sauvignon for 12 to 18 months, and strain EB92-1 (isolated from elderberry) but not strain Syc86-1 indeed allowed reduction of the disease in both cultivars. Biological control by inoculation of susceptible grapevines with benign strains of *X. fastidiosa*, especially strain EB92-1, appears therefore to possibly control Pierce's disease in commercial vineyards in Florida as well as other areas (Hopkins 2005) where the disease occurs or could appear in the future. The use of avirulent strains closely related to pathogenic *X. fastidiosa* strains cross protecting grapevine against Pierce's disease might bear risks as avirulent strains may mutate or acquire virulence genes. In areas such as the southeastern United States where Pierce's disease strongly limits grapevine production (Hopkins 2005), these risks might be acceptable.

Several biocontrol agents have been tested or under consideration for biocontrol of the discussed bacterial diseases. The effect of avirulent strains of these pathogens might be the result of niche competition and/or interference of signals with aggressive pathogens strains. Alternatively and additionally, effects of these biocontrol strains on enhanced plant resistance and plant immunity must be taken into consideration. This type of mechanism is also more likely involved in the biocontrol ability of bacteria inhabiting distinct habitats in the plant than the respective plant disease causing bacteria. Little evidence exists so far for direct antibiotic effects of biocontrol SM on bacterial pathogens; however SMs might also change plant defence mechanisms leading to altered resistance to bacterial pathogens. Future research will show, which of the discussed mechanism is of major importance for application of biocontrol strains in the control of bacterial grapevine diseases.

Conclusions and future prospects

Considerable information on the possibility to use biocontrol agents of bacterial origin to fight a variety of grapevine diseases affecting yield and productivity has become available. In this review we focused on fungi responsible for trunk diseases, root rot by *F. oxysporum*, grey mould induced by *B. cinerea,* powdery mildew caused by *Erysiphe necator,* downy mildew caused by the oomycete *Plasmopara viticola* as well as on the bacterial pathogens *X. fastidiosa*, *Ca.* Phytoplasma spp. and *A. vitis*. Continuous research for effective beneficial bacteria, associated SMs and study of their mechanism is very important to allow the development of effective biocontrol agents and to allow sufficient disease management for these and other grapevine diseases in viticulture. There are not

enough examples of biocontrol agents and SMs used for grapevine in our opinion. A current need for practical use of beneficial bacteria or their metabolites corresponding to a portfolio of different products would allow a more efficient disease treatment. The research for mechanisms involved can be of high importance for a better understanding of the processes involved and should subsequently also lead to better applications in disease management. Only few mechanisms enabling vine plant resistance have yet been demonstrated. For a number of bacterial metabolites their antifungal or antibacterial properties to vine pathogens have not even been tested yet. Additionally studying effect of new biocontrol bacteria as well as new metabolites having the abilities to control crop disease or to stimulate plant defense reactions is therefore of special importance for fundamental knowledge and development. In case of a climate change scenario (Compant et al. 2010b), some strains isolated from desert soil can be promising agents as they are adapted to more extreme conditions (unpublished results). However, the colonization process, the persistence in soil, as well as the mechanisms allowing host plant protection should be obligatory studied before field delivery and marketing.

A natural microflora can inhabit the vine host plants, both in the rhizosphere and the endosphere of various plant organs. Any application of specific microbe(s) should lead to study its behaviour inside grape plants and also the interaction with the natural microflora. The intensive use of pesticides in viticulture may also have a strong impact on endophyte composition; nevertheless the aspect of potential alteration of microflora by biocontrol agents shall not be neglected. All these aspects should be considered for both fundamental knowledge in beneficial bacteria – plant interactions as well as for further improvement of bacterial biocontrol in the vineyard, *i.e.* for a sustainable management of viticulture.

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Table 1: List of examples of biocontrol beneficial strains having biocontrol properties on phytopathogens of grapevine diseases.

Table 1: Continued.

Biocontrol strain	Mechanisms described	Phytopathogen	Disease	Evidence	References
Streptomyces spp.	antibiosis	*Fusarium oxysporum* E.F. Sm. & Swingle	Fusarium wilt and root rot	Field	Ziedan et al. 2010
Pseudomonas fluorescens isolate NRC10	antibiosis + ISR	*Fusarium oxysporum* E.F. Sm. & Swingle	Fusarium wilt and root rot	Field	Ziedan and El-Mohamedy 2008
Bacillus subtilis strain	antibiosis	*Eutypa lata* (Pers.) Tul. & C. Tul.	Eutypa dieback	*in planta*	Ferreira et al. 1991
B. subtilis strain B1α	antibiosis + interference with virulence factors	*Eutypa lata* (Pers.) Tul. & C. Tul.	Eutypa dieback	on wood disks	Schmidt et al. 2001
Erwinia herbicola strains JII/E2 and JII/E4	antibiosis + interference with virulence factors	*Eutypa lata* (Pers.) Tul. & C. Tul.	Eutypa dieback	on wood disks	Schmidt et al. 2001
Erwinia herbicola strains JII/E2 and JII/E4	antibiosis + interference with virulence factors	*Eutypa lata* (Pers.) Tul. & C. Tul.	Eutypa dieback	on wood disks	Schmidt et al. 2001
Actinomycete strain A123	antibiosis + interference with virulence factors	*Eutypa lata* (Pers.) Tul. & C. Tul.	Eutypa dieback	on wood disks	Schmidt et al. 2001
Bacillus subtilis AG1	antibiosis	*Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.	grapevine canker	on wood disks	Alfonzo et al. 2009
Bacillus subtilis AG1	antibiosis	*Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams	esca disease	*in vitro*	Alfonzo et al. 2009
Bacillus subtilis AG1	antibiosis	*Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai	esca disease	*in vitro*	Alfonzo et al. 2009
Bacillus circulans GI 070	antibiosis	*Botrytis cinerea* (Pers.)	grey mould	*in vitro*	Paul et al. 1997
Bacillus subtilis AG1	antibiosis	*Botrytis cinerea* (Pers.)	esca disease	*in vitro*	Alfonzo et al. 2009
Bacillus sp.	antibiosis + ISR	*Botrytis cinerea* (Pers.)	grey mould	*in planta*	Krol 1998
Bacillus circulans GI 070	antibiosis	*Botrytis cinerea* (Pers.)	gray mould	*in vitro*	Paul et al. 1997
Bacillus sp.	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	*in planta*	Krol 1998
Pseudomonas fluorescens strain PTA-CT2	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	*in planta*	Krol 1998
Pseudomonas fluorescens PTA-268, PTA-CT2	antibiosis + ISR	*Botrytis cinerea* (Pers.)	grey mould	Field	Magnin-Robert et al. 2007; Trotel-Aziz et al. 2006; 2008; Verhagen et al., 2011
Pseudomonas fluorescens PTA-268, PTA-CT2	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	Field	Magnin-Robert et al. 2007; Trotel-Aziz et al. 2006; 2008; Verhagen et al., 2011
Bacillus subtilis PTA-271	antibiosis + ISR	*Botrytis cinerea* (Pers.)	grey mould	*in planta*	Trotel-Aziz et al. 2006; 2008; Verhagen et al., 2011
Bacillus subtilis PTA-271	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	*in planta*	Trotel-Aziz et al. 2006; 2008; Verhagen et al., 2011
Pantoea agglomerans PTA-AF1 and PTA-AF2	antibiosis + ISR	*Botrytis cinerea* (Pers.)	grey mould	Field	Magnin-Robert et al. 2007; Trotel-Aziz et al. 2006; 2008; Verhagen et al., 2011
Pantoea agglomerans PTA-AF1 and PTA-AF2	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	Field	Magnin-Robert et al. 2007; Trotel-Aziz et al. 2006; 2008; Verhagen et al., 2011
Acinetobacter lwoffii PTA-113 and PTA-152	antibiosis + ISR	*Botrytis cinerea* (Pers.)	grey mould	Field	Magnin-Robert et al. 2007; Trotel-Aziz et al. 2006; 2008; Verhagen et al., 2011
Acinetobacter lwoffii PTA-113 and PTA-152	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	Field	Magnin-Robert et al. 2007; Trotel-Aziz et al. 2006; 2008; Verhagen et al., 2011
Pseudomonas aeruginosa (7NSK2)	ISR	*Botrytis cinerea* (Pers.)	gray mould	*in planta*	Verhagen et al., 2010
P. fluorescens (strains CHA0, Q2-87 and WCS417)	ISR	*Botrytis cinerea* (Pers.)	gray mould	*in planta*	Verhagen et al., 2010
P. putida (WCS358)	ISR	*Botrytis cinerea* (Pers.)	gray mould	*in planta*	Verhagen et al., 2010

Biocontrol strain	Mechanisms described	Phytopathogen	Disease	Evidence	References
Pseudomonas aeruginosa (7NSK2)	ISR	*Botrytis cinerea* (Pers.)	grey mould	*in planta*	Verhagen et al. 2010
Streptomyces spp.	antibiosis	*Fusarium oxysporum* E.F. Sm. & Swingle	Fusarium wilt and root rot	Field	Ziedan and El-Mohamedy 2008
P. fluorescens (strains CHA0, Q2-87 and WCS417)	ISR	*Botrytis cinerea* (Pers.)	grey mould	*in planta*	Verhagen et al. 2010
Pseudomonas fluorescens isolate NRC10	antibiosis + ISR	*Fusarium oxysporum* E.F. Sm. & Swingle	Fusarium wilt and root rot	Field	Ziedan and El-Mohamedy 2008
P. putida (WCS358)	ISR	*Botrytis cinerea* (Pers.)	grey mould	*in planta*	Verhagen et al. 2010
Bacillus subtilis strain	antibiosis	*Eutypa lata* (Pers.) Tul. & C. Tul.	Eutypa dieback	*in planta*	Ferreira et al. 1991
Burkholderia phytofirmans strain PsJN	ISR	*Botrytis cinerea* (Pers.)	grey mould	*in planta*	Ait Barka et al. 2000; 2002
B. subtilis strain B1α	antibiosis + interference with virulence factors	*Eutypa lata* (Pers.) Tul. & C. Tul.	Eutypa dieback	on wood disks	Schmidt et al. 2001
Streptomyces spp.	antibiosis	*Botrytis cinerea* (Pers.)	grey mould	*in vitro*	Loqman et al. 2009
Micromonospora spp.	antibiosis	*Botrytis cinerea* (Pers.)	grey mould	*in vitro*	Loqman et al. 2009
Erwinia herbicola strains JII/E2 and JII/E4	antibiosis + ISR	*Eutypa lata* (Pers.) Tul. & C. Tul.	Eutypa dieback	on wood disks	Schmidt et al. 2001; Lebrihi et al. 2009a, 2009b
oxB related to *Cupriavidus* sp.	degradation of virulence factors	*Botrytis cinerea* (Pers.)	grey mould	on leaves	Schoonbeek et al. 2007
Actinomycete strain A123	antibiosis + interference with virulence factors	*Eutypa lata* (Pers.) Tul. & C. Tul.	Eutypa dieback	on wood disks	Schmidt et al. 2001
B. pumilus B-30087	antibiosis + ISR	*Erysiphe necator* Schw.	Powdery mildew	*in planta*	Lehmann et al. 2000
Bacillus strains ATCC 55608 and 55609	antibiosis	*Erysiphe necator* Schw.	Powdery mildew	Field	Sawant et al. 2011
Bacillus subtilis AG1	antibiosis	*Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.	grapevine canker	*in vitro*	Alfonzo et al. 2009
Serratia marcescens MSU-97	antibiosis	*Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni	downy mildew	*in vitro*	Strobel et al. 2005
Bacillus subtilis AG1	antibiosis	*Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams	esca disease	*in vitro*	Alfonzo et al. 2009
Streptomyces sp. ANK313	antibiosis	*Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni	downy mildew	*in vitro*	Abdalla et al. 2011
a non-tumorigenic strain (F2/5) of *Agrobacterium vitis*	competition, signal interference + ISR	*Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai	crown gall	*in planta*	Burr and Reid 1994; Burr et al. 1997
Bacillus subtilis AG1	antibiosis		esca disease	*in vitro*	Alfonzo et al. 2009
Bacillus circulans GJ 070	competition, signal interference + ISR	*Botrytis cinerea* (Pers.)	gray mould	*in vitro*	Kawaguchi et al. 2007; Paul et al. 2008
Agrobacterium vitis strain E26 or VAR03-1	antibiosis + ISR	*Agrobacterium vitis*	crown gall	*in planta*	Paul et al. 1997
Bacillus sp.	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	*in planta*	Krol 1998
Pseudomonas aureofaciens B-4117	antibiosis	*Agrobacterium vitis*	crown gall	*in planta*	Khmel et al. 1998
P. fluorescens strain	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	*in planta*	Krol 1998
P. fluorescens CR330D and 1100-6	antibiosis, competition	*Agrobacterium vitis*	crown gall	*in planta*	Khmel et al. 1998
Pseudomonas fluorescens PTA-268, PTA-271	antibiosis	*Agrobacterium vitis*	crown gall	*in planta*	Khmel et al. 1998
Bacillus subtilis EN62-1	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	Field	Magnin-Robert et al. 2007; Trotel-Aziz et al. 2006; 2008; Verhagen et al., 2011
Bacillus sp. EN71-1	antibiosis	*Agrobacterium vitis*	crown gall	*in planta*	Khmel et al. 1998
Rahnella aquatilis HX2	antibiosis	*Agrobacterium vitis*	crown gall	Field	Khmel et al. 1998
Strains of *Enterobacter agglomerans*, *Bacillus subtilis* PTA-271, *Rahnella aquatilis*, and *Pseudomonas* spp.	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	*in planta*	Trotel-Aziz et al. 2006; Bell et al. 1995; 2008; Verhagen et al., 2011
X. fastidiosa Syc86-1, EB92-1, PD95-6, PD91-2, EB92-1	competition + ISR	*Xylella fastidiosa*	Pierce's disease	Field	Magnin-Robert et al. 2007; Hopkins 2005
Pantoea agglomerans PTA-AF1 and PTA-AF2	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	Field	Trotel-Aziz et al. 2006; 2008; Verhagen et al., 2011
Acinetobacter lwoffii PTA-113 and PTA-152	antibiosis + ISR	*Botrytis cinerea* (Pers.)	gray mould	Field	Magnin-Robert et al. 2007; Trotel-Aziz et al. 2006; 2008; Verhagen et al., 2011
Pseudomonas aeruginosa (7NSK2)	ISR	*Botrytis cinerea* (Pers.)	gray mould	*in planta*	Verhagen et al., 2010
P. fluorescens (strains CHA0, Q2-87 and					

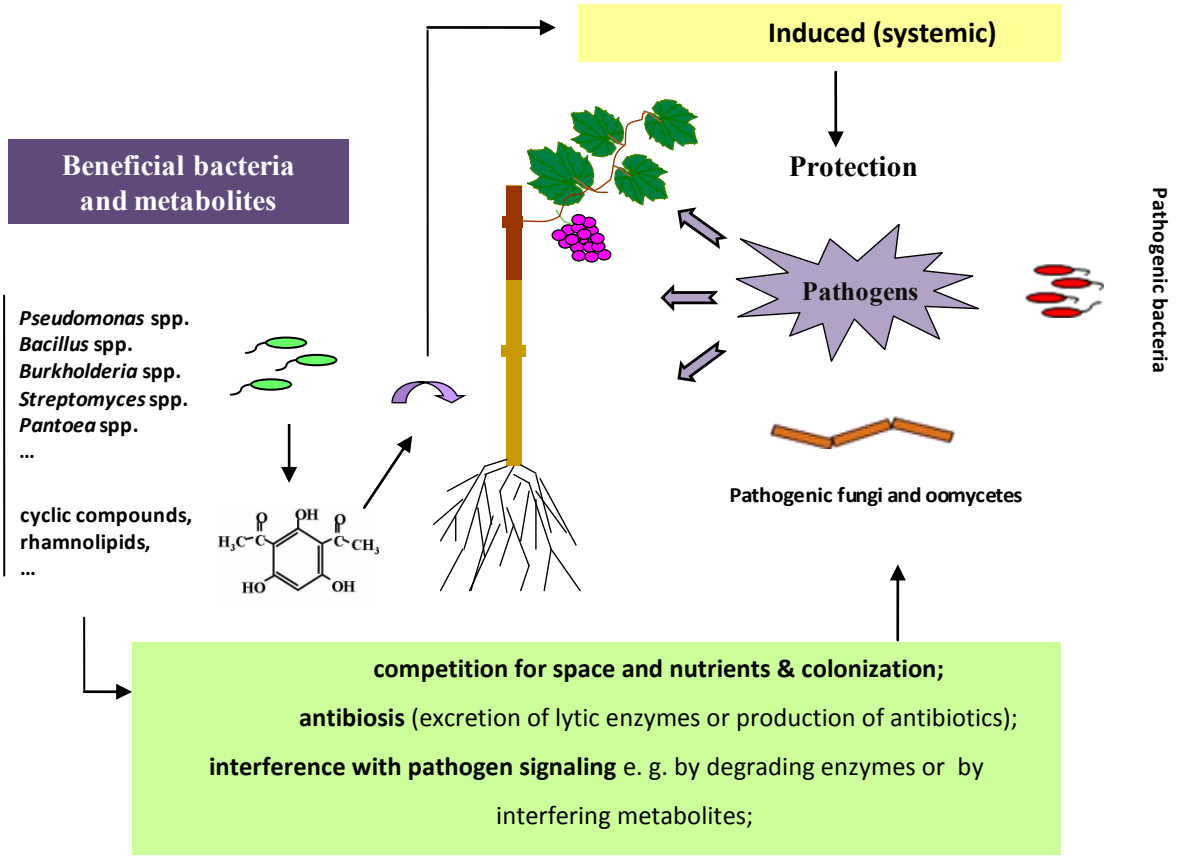


Figure 1: Drawing summarizing the potential mechanisms involved in the control of grapevine pathogen diseases by beneficial bacteria and their secondary metabolites.

IV. The case of rhizo- and endophytic actinobacteria and rare species for plant health: an interesting area for biocontrol studies

It has been described in the review that there is a current need to develop a portfolio of beneficial bacteria to control grapevine pathogen diseases. Actinomycetes are of special interest in the rhizosphere and also as endophytes. They could be used as biocontrol agents towards several grapevine diseases as well as to study ISR mechanisms on plants such as *Arabidopsis thaliana*. It is also interesting to track them on and inside plants and to study the colonization process.

1. Interesting genera and species for biocontrol and secretion of bioactive products

Actinomycetes have received considerable attention as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters (Doumbou *et al.,* 2002; Shahrokhi *et al.,* 2005; Hamdali *et al.,* 2008b). In soil, production of secondary metabolites acting as antibiotic metabolites and antimicrobial compounds (Sabaratnam and Traquair, 2002; Berdy, 2005; Hyang *et al.,* 2005; Lehman *et al.,* 2005) make actinomycetes able to restrict the attack by pathogenic organisms (Beom *et al.,* 1999; El-Tarabily *et al.,* 2000). Some reports showed well the agricultural implications of these microorganisms in biological control of plant pathogens (Cao and Forrer, 2001; Bressan, 2003; Ghorbani *et al.,* 2005) and to initiate defense responses in native hosts or non host plants to cope with the stresses at cell, tissue and organ level following inoculation of these organisms (Hasegawa *et al.,* 2006).

Biological control activities by rhizo- and endophytic actinomycetes have been reported against different pathogens and include the suppression of *Fusarium oxysporum* (Cao *et al.,* 2005), *F. pseudograminearum* (Franco *et al.,* 2007), *Verticillium dahliae* (Krechel *et al.,* 2002), *Rhizoctonia solani* (Krechel *et al.,* 2002; Cao *et al.,* 2004b; Coombs *et al.,* 2004), *Plectosporium tabacinum* (El-Tarabily, 2003), *Gauemannomyces graminis* (Coombs *et al.,* 2004; Franco *et al.,* 2007) and *Pythium* spp. (Franco *et al.,* 2007; El-Tarabily *et al.,* 2009). Additional examples on biocontrol of phytopathogens of grapevine can be found in the review described before.

Actinomycetes are of great interest because they are major producers of secondary metablites acting as bioactive molecules and enzymes. Actinomycetes are producing 45% of discovered bioactive molecules and enzymes (Figure 8).

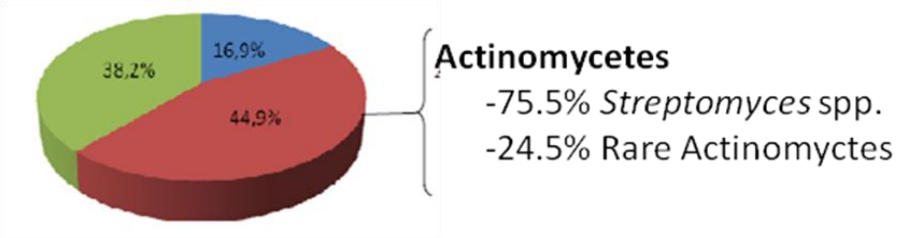


Figure 8: Origin of microbial bioactive molecules. Bioactive products not belonging to actinomycetes products belonging to Actinomycetes products from microscopic fungus (According to Berdy, 2005).

The property of bioactive molecule production against plant pathogens associated with actinomycetes explains their ability to act as biocontrol tools (Doumbou *et al.,* 2002). Over one thousand secondary metabolites from actinomycetes were discovered during the years 1988-1992 (Sanglier *et al.,* 1993). Actinomycetes produce a variety of antibiotics and secondary metabolites with diverse chemical structures that have antifungal, anti-tumor and immunosuppressive activities (Behal, 2000). Within actinomycetes, *Streptomyces* spp. have been investigated predominantly, mainly because of their dominance on, and the ease of isolation from, dilution plates and because of the commercial interest shown on the antibiotics produced by certain *Streptomyces* spp.

Streptomyces griseoviridis strain K61, isolated from *Sphagnum* peat (Tahvonen 1982a, b), is for instance a nice example of biocontrol agent. It has been reported to be antagonistic to a variety of plant pathogens including *Alternaria brassicola*, in cruciferous plants (Tahvonen, and Avikainen, 1987), *Botrytis cinerea* and *Rhizoctonia solani* in lettuce (Tahvonen, and Lahdenpera, 1988), and *F. oxysporum* in carnation (Tahvonen and Avikainen, 1987).

Streptomyces griseoviridis is used as the active ingredient in a biofungicide, Mycostop™ that is used against *Fusarium*, *Alternaria*, *Phytophthora* and *Pythium* which cause damping-off and root diseases (Mohammadi and Lahdenpera, 1992). This product is available in United States (Cross and Polonenko, 1996).

The actinomycetes isolates *Streptomyces toxytricini* vh6, *Streptomyces flavotricini* vh8 showed prominent antagonistic potential against *Rhizoctonia solani* and exhibited

significant level of accumulation of phenolic compounds in tomato plants under pathogenic stress (Patil *et al.,* 2011). *Streptomyces* sp. S-70 had also a potential to suppress infection of *Alternaria brassicicola* on chinese cabbage seedlings (Igarashi *et al*., 2002) and it has been found that such an effect was dependent on a novel plant bioactive compound, fistupyrone (an inhibitor for the formation of infection hypha that is necessary for *A. brassicicola* to accomplish the infection). Endophyte *Streptomyces* sp. TP-A0569 was also found to produce fistupyrone against *A. brassicicola* (Igarashi *et al*., 2000).

Sasaki *et al.,* (2001a) identified new bioactive compounds TPU-0031-A and B produced by actinomycetes, *Streptomyces* sp. TP-A0556 against *Aspergillus fumigatus* TFO 886. They also found Cedarmycins A, a new antimicrobial antibiotic from *Streptomyces* sp. TP A0456 and found it active against *Candida glabrata* IFO 0622, and *Cryptococcus neofonnans* ATCC90, *in vitro* (Saski *et al.,* 2001b). A new naphthoquinone antibiotic, alnumycin, was also reported in *Streptomyces* sp. DSM 11575 isolated from root nodules of *Alnus glutinosa* (Bieber *et al.,* 1998). Shimizu *et al*. (2004) also proved that the endophyte actinomycete, *Streptomyces galbus* strain R-5 produces actinomycin X2 and fungichromin having antibacterial and antifungal activities *in vitro* against *Pestalotiopsis sydowiana*, a major pathogen of rhododendron. *Streptomyces* sp. NRRL30562 was additionally studied and it has been shown to produce antibiotics designated as munumbicins A-D40. This antibiotic possessed a wide-spectrum activity against phytopathogenic fungi like *Rhizoctonia solani* (Castillo *et al*., 2002).

Streptomyces sp. NRRL30566, which was isolated from a fern-leaved grevillea (*Grevillea pteridifolia*) produced also novel wide-spectrum antibiotics named kakadumycins. This is found to be effective against *Bacillus anthracis in vitro* (Castillo *et al*., 2003).

The actinomycetes and the compounds mentioned above are a few examples of biocontrol actinomycetes and agroactive compounds isolated from actinomycetes. They are in fact several examples (see for instance also coronamycin secreted by the endophyte *Streptomyces* sp. MSU-2110 having bicontrol properties against phytopathogenic *Pythium* ultimum and *Rhizoctonia solani;* Ezra *et al.,* 2004). However actinomycetes not only secrete antibiotics but also cell wall degrading enzymes. *Streptomyces* sp. EF-14 has been identified as one of the most potent antagonists of *Phytophthora fragariae* var. rubi (the causal agent of raspberry root rot). This strain is able to lyse the mycelium of this pathogen *in vitro* as well as *in vivo* (Valois *et al.,* 1996) and produces β-1, 6-glucanase (Fayad *et al.,* 2001; Toussaint *et al.,* 1997). El-Tarabily (2003) reported that *Actinoplanes missouriensis*, endophytic

actinomycete in lupin roots produced also chitinase and inhibited the growth of *Plectosporium tabacinum*, the causal agent of lupin root rot in Egypt.

Although various examples have been cited above for actinomycetes, there is some rare bacteria that can be used also for metabolic secretion but may be also for crop protection, may be for grapevine, to study the mechanisms of ISR and to determine process of colonization.

The genus *Streptomyces* largely predominate among strains of Actinomycetes although other isolated genera such as *Nocardia* and *Micromonospora* are also relatively abundant (Sabaou *et al.,* 1998; Lechevalier and Lechevalier, 1967). Actinomycetes, which do not belong to the genus *Streptomyces*, which are low representative among isolates of actinomycetes, isolated with conventional isolation techniques, are considered rare. They belong to the genera *Microbispora, Microtetraspora, Amycolatopsis, Actinomadura* or *Saccharothrix* (Figure 9).

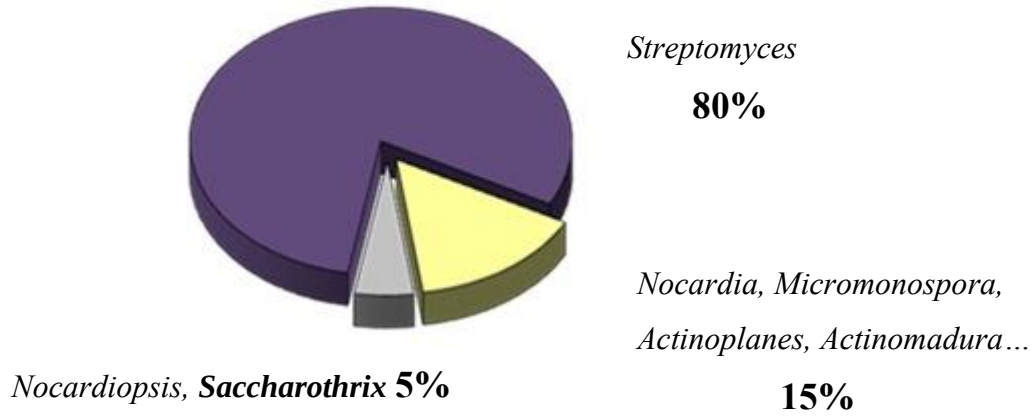


Figure 9: Percentage of different genera belonging to group Actinobacteria. genus *Streptomyces* genera *Nocardia, Micromonospora, Actinoplanes, Actinomadura* genera *Nocardiopsis* and *Saccharothrix.*

2. *Actinosynnemataceae* and *Saccharothrix* spp., uncommon bacteria

Among non Streptomyces exists members of the family Actinosynnemataceae. This family has been defined by Labeda and Kroppenstedt, 2000. The phylogenetic studies of this family based on the analysis of 16S rDNA sequences showed that it contains the genera

Actinokineospora (Hasegawa, 1988), *Actinosynnema* (Hasegawa *et al.,* 1978), *Lechevalieria* (Labeda *et al.,* 2001), *Lentzea* (Yassin *et al.,* 1995; Labeda *et al.,* 2001ll as *Saccharothrix* (Labeda *et al.,* 1984; Labeda and Lechevalier, 1989a). This family lies within the suborder Pseudonocardiniae in the class Actinobacteria (Stackebrandt *et al.,* 1997). Among these members belongs the genus *Saccharothrix*.

In the laboratory LGC UMR 5503, one strain of *Saccharothrix* genus was used for biomass production, metabolites secretion and characterization. However before to described the strain used (that is in fact one actinobacterial strain among others studied in the laboratory), it is intesresting to describe what is the genus *Saccharothrix*.

Earlier phylogenetic studies (Embley *et al.,* 1988; Warwick *et al.,* 1994) proposed that the genus *Saccharothrix* was associated with the family Pseudonocardiaceae, but this affiliation was not supported statistically in phylogenetic analyses, and diagnostic chemotaxonomic characteristics of *Saccharothrix* species were different from those of taxa whose placement in the Pseudonocardiaceae was well supported.

The original first strain *Saccharothrix australiensis*, was isolated from a soil sample from Australia. The genus appears to be ubiquitous in soils and has a worldwide distribution. Isolates described as members of this genus have come from soil samples collected in the United States, Japan, Panama, Africa, Russia and Algeria. The genus *Saccharothrix* have nine species as shown in table 4.

Table 4: Species belonging to genus *Saccharothrix*

Saccharothrix species	Strain number	References
Sa. algeriensis	NRRL-B 24137	Zitouni *et al.,* 2004
Sa. australiensis	ATCC 31497	Labeda *et al.,* 1984
Sa. coeruleofusca	ATCC 35108	Grund and Kropenstedt, 1989
Sa. espanaensis	ATCC 51144	Labeda and Lechevalier, 1989
Sa. longispora	ATCC 35109	Grund and Kropenstedt, 1989
Sa. mutabilis subsp. *capreolus*	ATCC 23892	Grund and Kropenstedt, 1989
Sa. mutabilis subsp. *mutabilis*	ATCC 31520	Grund and Kropenstedt, 1989
Sa. syringae	ATCC 51364	Grund and Kropenstedt, 1989
Sa. Texasensis	ATCC 51593	Labeda and Lyons, 1989
Sa. xinjiangensis	NBRC 101911	Hu *et al.,* 2004

3. *Saccharothrix algeriensis* NRRL B-24137: a model used for characterization of new bioactive metabolites

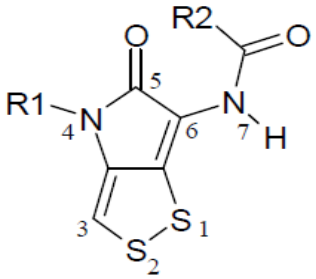
A large number of actinomycetes have been isolated and screened from soil that are source of 70-80% of commercially available secondary metabolites (Baltz, 2008). *Saccharothrix* strains secrete different type of antibiotics. However among them *Saccharothrix algeriensis* strain NRRL B-24137 was isolated from a Saharan soil sample collected at a palm grove in Adrar (South Algeria; Zitouni *et al*., 2004). This strain is known as having a broad spectrum of action against Gram-positive bacteria, Gram negative bacteria, yeasts and filamentous fungi (Lamari, 2006; Table 5) and secrete different kinds of secondary metabolites. This strain was one of the strains studied in the laboratory.

Table 5: Antibiotic activity of *Sa. algeriensis* NRRL B-24137, the activity was determined on ISP2 medium. The intensity of the antibiotic depends on inhibition zone in mm, No zone, -, Zone 2 to 9 mm, +, 10 à to 19 mm, ++, from 20 to 23 mm, +++, according to Lamari, 2006.

Target species	Antibiotic activity	Target species	Antibiotic activity
Gram positive bacteria		**Filamentous Fungi**	
Bacillus subtilis	++	*Alternaria* sp.	++
Micrococcus luteus	+++	*Aspergillus niger*	+
Staphylococcus aureus	+++	*Botrytis cinerera*	++
Enterococcus faecalis	+	*Fusarium oxysporum* f. sp. *albedinis*	++
Gram negative bacteria		*F. oxysporum* f. sp. *Ciceri*	+
Alcaligenes faecalis	+	*F. oxysporum* f. sp. *Lentis*	+
Escherichia coli	++	*F. oxysporum* f. sp. *Lini*	+
Klebsiella pneumoniae	+++	*F. oxysporum* f. sp. *Lycopersici*	++
Proteus mirabilis	+	*Fusarium culmorum*	+
Pseudomonas aeruginosa	-	*Fusarium graminearum*	+
Pseudomonas fluorescens	+	*Geotrichum candidum*	++
Serratia marcescens	+	*Mucor ramannianus*	+++
Yeasts		*Penicillium purpureum*	++
Candida albicans	+	*Penicillium* sp.	++
Debaryomyces subglosus	+	*Pythium irregulare*	++
Kluyveromyces lactis	+	*Rhizoctoria solani*	++
Rhodotorula mucilaginosa	+	*Sclerotium scleorotiorum*	++
Saccharomyces cerevisiae	++	*Thielaviopsis neocaledoniae*	+++
		Verticillium sp.	++

Saccharothrix algeriensis NRRL B-24137 is known to produce bioactive compounds belonging to the dithiolopyrrolone class of antibiotics (Lamari *et al.,* 2002a, b; Zitouni *et al.,* 2004). Dithiolopyrrolones are members of the pyrrothine class of naturally occurring antibiotics that contain N-acyl derivatives of 6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo[4,3- b] pyrrole.

From the culture of *Sa. Algeriensis* NRRL B-24137, Lamari *et al.,* (2002a, 2006) purified seven different compounds with antibiotic activity against *Bacillus subtilis* and *Mucor ramannianus* and six of them have been completely characterized. They belong to the family of dithiolopyrrolones. Their structure is shown in Figure 10.



R1 = CH_3; R2 = CH_3	Thiolutin
R1 = CH_3; R2 = $CH(CH_3)_2$	Iso-butyryl-pyrrothine (ISP)
R1 = CH_3; R2 = $(CH_2)_2$-CH_3	Butanoyl- pyrrothine (BUP)
R1 = CH_3; R2 = $CH=C(CH_3)_2$	Senecioyl- pyrrothine (SEP)
R1 = CH_3; R2 = $C(CH_3)=CH(CH_3)$	Tigloyl- pyrrothine (TIP)
R1 = CH_3; R2 = C_6H_5	Benzoyl- pyrrothine (BEP)

Figure 10: Structure of dithiolopyrrolone antibiotics produced by *Saccharothrix algeriensis* NRRL B-24137 (Lamari, 2006).

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Dithiolopyrrolone antibiotics have strong activities against a variety of Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi (Lamari *et al.,* 2002a; Webster *et al.,* 2002).

Different thesis have focused on the production of these secondary metabolites, as on modelisation of the production. However the use of this strain was not done in a biocontrol viewpoint, e.g. to reduce pathogen diseses of plants. A. Zitouni from Algeria used it to reduce a pathogen on plants (unpublished data). The potential of *Sa. algeriensis* should be explored more for the production of other secondary metabolites other than dithiolopyrrolones that could also be usefull for biocontrol of phytopathogens. It is also possible that the strain could be used on various plants to counteract pathgogen attack.

4. Does *Saccharothrix algeriensis* NRRL B-24137 can be used to protect plants towards phytopathogens? But it came from desert soil and can secrete some antibiotics!

In the introduction, it has been highlighted that some rhizo- and or endophytic bacteria could be used to protect plant diseases as well as increase host growth. Nowadays, a current gap is still missing regarding if some bacteria could colonize various host plants. Especially this is the case of microbes isolated from harsh environments (Compant *et al.,* 2010b). It is in particular unknown if *Saccharothrix algeriensis* NRRL B-24137 could protect some plants following use. The strain is known as an antibiotic producer. Under natural conditions, a lot of microorganisms can also secrete secondary metabolites having antibiotic activities (Compant *et al.,* 2011) and it could not be problematic following application as this exists under natural conditions.

The strain NRRL B-24137 has been isolated from desert soil and some scientists think that bacteria from desert soil can not protect crops towards pytopathogenic diseases. It has been however postulated that to find new plant growth-promoting bacteria or biocontrol agent, harsh environments could however provide a source of beneficial bacteria (Compant *et al.,* 2010b). It is becoming increasingly evident moreover that microbes from soil and plants growing in harsh environments such as desert soil may represent an enormous untapped genetic reservoir for plant improvement. It has been even recently postulated that transferring these microbes from native plants to non-host plants promises a revolutionary biotechnology to rapidly improve plant germplasm (Barrow *et al.,* 2008). This is may be the case for *Saccharothrix algeriensis* NRRL B-24137. However this needs to be tested and if positive results arrive, the mechanisms involved should be described. This could allow finding a new biocontrol agent for crop improvement, especially for grapevine. Microbial colonization of such strains on non host plants should be also studied, and also visualization of the process of colonization should be done if any application is made on crops growing in temperate conditions (Compant *et al.,* 2010b). Production of metabolites towards the pathogenic agent should be also determined. This is in order to better understand the association with a strain from desert soil with a crop plant.

Given the potential of *Saccharothrix algeriensis* NRRL B-24137, a beneficial actinomycete, against different fungi having antibiotic activities, it appeared necessary to explore its potential for biocontrol of phytopathogens. However to study its potential, a pathogen needed to be focused.

5. *Saccharothrix algeriensis* NRRL B-24137/grapevine-*Arabidopsis thaliana/Botrytis cinerea* as a model?

The fungus *Botrytis cinerea* was selected in this thesis because this fungus is widely used in pathosystems as well as allow to determine potential of biocontrol agents towards it.

Why *B. cinerea*? This fungus can cause lost of yield and can infect many plants. On grapevine for instance, it can lead to gray mould disease (Pezet *et al.,* 2004). This can be correlated to lost of qualitative and quantitative yield and up to 40% of lost of yield can be obtained when infection occur (Viniflhor, 2006). Gray mould can have impact on quality of wines (Marchal *et al.,* 2002). The plant itself can reduce infection by constituve defences as well as by a large array of induced defenses including chitinases, glucanases, phytoalexines, inhibitors of polygalacturonases as well other pathogenesis-related proteins that can restrict the infection but the defences of the plant are not sufficients to counteract totally the infection caused by this necrotrophic agent, and disease can occur (reviewed in Elmer and Reglisnki, 2006). There is some fungicides to control it that are currently replaced more and more by natural pesticides such as elicitors (chitosan, cyclodextrins, laminarin, ergostérol, BABA, UV), plant growth-promoting fungi (from the genera *Trichoderma*, *Ulocladium*, *Gliocladium*, *Epicoccum*, *Pichia*, and *Aureobasidium*; Elmer and Reglisnki, 2006), oomycetes (*Pythium* sp.) and also beneficial bacteria (*Pseudomonas* sp., *Bacillus* sp., *Streptomyces* sp. as well as others as you can have seen in the review presented in this introduction). However, there is a need to have a portfolio of biocontrol agents to reduce this phytopathogen. May be the desert soil isolate NRRL B-24137 can reduce the gray mould agent on plant such as grapevine but what can be the mechanisms involved, do the strain NRRL B-24137 can also colonize grapevine plants even if it has been isolated from desert soil? On *Arabidopsis thaliana* what can be its impact towards *B. cinerea*? What about the colonization behaviour and the mechanisms of protection involved?

Objectives of the thesis

The objectives of the thesis were:

a) to study and search new bioactive molecules secreted by *Sa. algeriensis* NRRL B-24137 having direct biocontrol activities against *Botrytis cinerea* (Figure 11a);

b) to characterize the epi- and/or endophytic colonization of *Sa. algeriensis* NRRL B-24137 in grapevine before to study a putative impact on reduction of symptoms of grapevine caused by *B. cinerea* (Figure 11b);

c) to study the potential of *Sacchrothrix algeriensis* NRRL B-24137 to induce systemic resistance allowing reduction of [illegible] by using model plant *A. thaliana* and to determine the mechanisms of resistanc[illegible]R) as well as putative new mechanisms of ISR (Figure 11c).

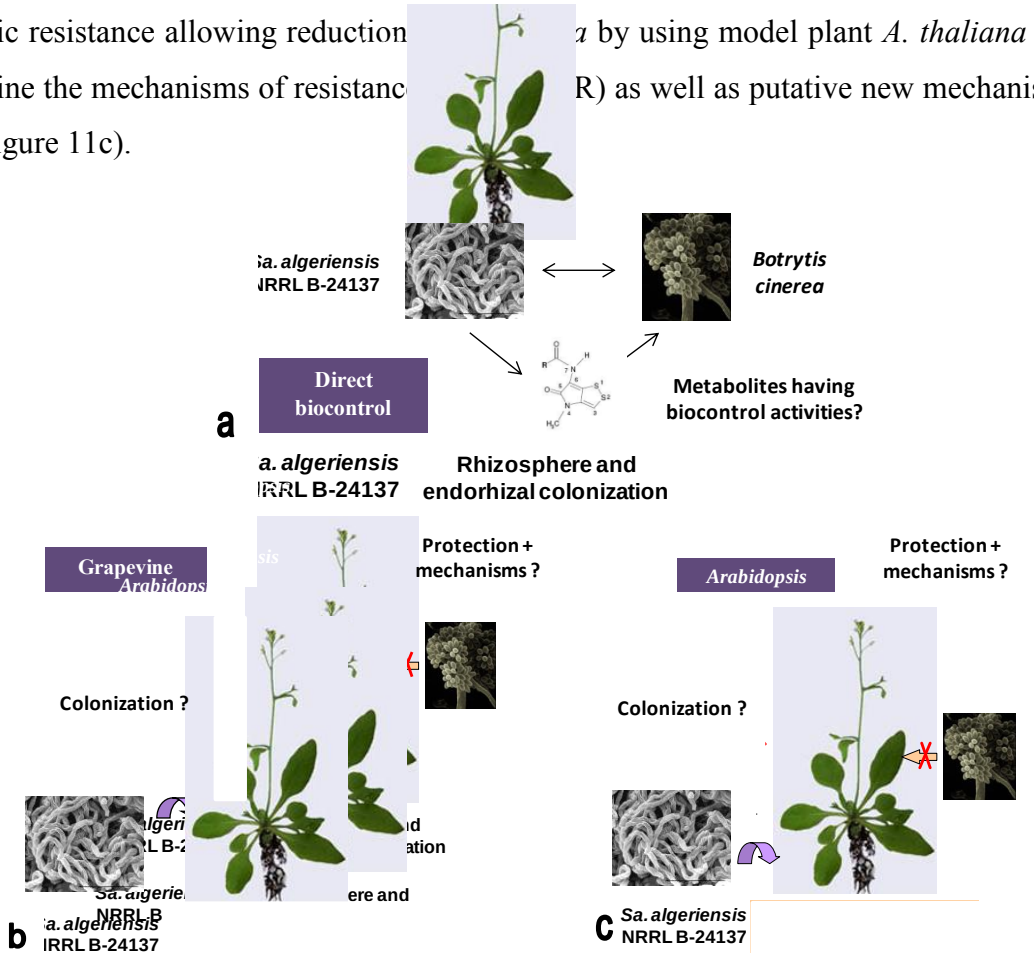

Figure 11: Drawings representing the objectives of the thesis.

Chapter II

Direct biocontrol activity of *Saccharothrix algeriensis* NRRL B-24137 towards *Botrytis cinerea* Pers.

Saima Muzammil[1], Florence Mathieu[1], Ahmed Lebrihi[1, 2], and Stéphane Compant[1]

[1] Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Dépt Bioprocédés et Systèmes Microbiens, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1, France.

[2] Université Moulay Ismail, Marjane II, BP 298, Meknès, MAROC

*Corresponding author: Dr. Stéphane Compant, Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Dept BIOSYM, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1, France. Tel: +33 (0) 6 43 44 00 60. E-mail: scompant@ensat.fr

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Abstract

In this study the direct biocontrol activity of the desert soil isolate *Saccharothrix algeriensis* NRRL B-24137 towards *Botrytis cinerea* BC1 was evaluated. The results showed that the actinobacterial strain can reduce the fungus on a dual culture plate with a specific medium allowing growth of both microorganisms. Data results showed also that a red pigmented compound is secreted by the beneficial bacteria and could be linked to the growth reduction of *B. cinerea* BC1 as revealed by TLC analysis. HPLC profil of metabolites revealed that metabolites secreted on the medium used for co-culture of NRRL B-24137 and *B. cinerea* BC1 including also the red pigmented compound could not however correspond to dithiolopyrollones previously described as secreted by NRRL B-24137 and bioactive towards different microorganisms, suggesting a switch of the physiological state of the beneficial strain on the medium used. Researches of the red metabolite as well as other putative metabolites secreted by the beneficial strain on the medium is under progress by analysis of compounds on HPLC with a program not corresponding to the one previously used for dithiolopyrrolones. Although it is still unknown what the compounds secreted could be, this study shows that *Sa. algeriensis* NRRL B-24137 can display a strong bioactivity against the fungal phytopathogen *Botrytis cinerea* and that some compounds other than the ones previously known as secreted by NRRL B-24137 could be also bioactives towards *B. cinerea*.

Keywords: *Saccharothrix algeriensis* NRRL B-24137*, Botrytis cinerea,* secondary metabolites, biocontrol.

Introduction

Beneficial bacteria can have impact on host plants physiology as well as reduce systemically symptoms caused by various phytopathogens (Lugtenberg and Kamilova, 2009). Some of these bacteria can also have direct biocontrol activities towards phytopathogens (Raaijmakers et al., 2002). This can be linked to secondary metabolites secretion having bioactives properties (Raaijmakers et al., 2002; Compant et al., 2005). However these secondary metabolites having biocontrol activities should be characterized.

In this study, *Saccharothrix algeriensis* NRRL B-24137, a filamentous bacterium belonging to the actinomycetes and isolated from Saharan soils in Algeria (Zitouni et al., 2004) has been used. It has been previously reported that the strain produces bioactive

metabolites belonging to the dithiolopyrrolone class of antibiotics (Lamari et al., 2002a, b; Zitouni et al., 2005). Dithiolopyrrolone antibiotics exhibit broad-spectrum antibiotic activity against a variety of Gram-negative and Gram-positive bacteria and fungi (Lamari et al., 2002a; Webster and Chen, 2000; Minamiguchi et al., 2001, Webster et al., 2002). Dithiolopyrrolones can reduce various microorganisms, and also *Botrytis cinerea.* However never an antibiosis phenomen has been performed between a strain secreting dithiolopyrrolones and *B. cinerea.*

The aim of this work was to investigate if *Saccharothrix algeriensis* NRRL B-24137 can have an antagonistic effect against the fungus *Botrytis cinerea* as well to described the secretion of bioactive molecules responsible of growth reduction of *Botrytis cinerea*.

Materials and methods

Bacterial culture

Sa. algeriensis NRRL B-24137 (= DSM 44581) was used for this study. *Sa. algeriensis* NRRL B-24137 was grown on International Streptomyces Project 2 (ISP2) agar plates (pH 7.0) containing per liter of distilled water: 4 g D(+) glucose (Acros organics), 10 g malt extract (Fluka), 4 g yeast extract (Fluka) and 18 g agar (Sigma) for 7 days at 30 °C.

Fungal culture

Botrytis cinerea strain BC1 (isolated by S. Compant from grapevine plants in 2008 in Austria) was grown on PDA (Potato Dextrose Agar) medium at 25°C under light conditions for 9 days.

Antifungal assay on petri dishes

For the biocontrol test and production of bio active molecules, different media were used in order that NRRL B-24137 and *B. cinerea* BC1 could grow. A medium consisting to half of Nutrient agar (NA, Fluka) and half PDA (Bacto) (14g of Nutrient agar, 12 g of PDB, 7g of agar, pH=5.10) has been among these media prepared.

Plugs of *Sa. algeriensis* NRRL B-24137 (grown on ISP2 medium) was placed in one corner and *Botrytis cinerea* BC1 (grown on PDA) was placed in opposite corner of the petri dish. Petri dishes without the *Botrytis cinerea* plug served as controls. All the plates were allowed to grow at 25°C for 10 days. The results of plates were visualized 10 day post inoculation.

Extraction of metabolites

Once microorganisms grown and that an inhibition zone appeared, the inhibition zone part of petri dishes were cut in small pieces and dissolved in methanol in the ratio of 5 mL of methanol for 1 g of medium. Then, the sample was filtered with 0.45 µm membrane filters (Advantec, Dublin, Ireland). The organic extract was evaporated to 1mL using speed-vac (Genevac, Ish, UK) at a temperature maintained under 30 °C. Same process was carried out with the medium containing metabolite secreted by *Sa. algeriensis* NRRL B-24137 without *Botrytis cinerea* BC1.

Separation of Bioactive compounds by TLC

The thin layer chromatography (TLC) of metabolites was performed on TLC: 20x20 cm RP-18F with chloroform/methanol (9:1, v:v) or n-butanol, acetic acid and water (3:1:1, v:v:v) as a solvent. Silica gel (SiO_2 x H_2O) coated aluminium sheets were used for TLC of metabolites secreted in the inhibition zone and secreted by *Sa. algeriensis* NRRL B-24137 alone in NA:PDA medium. 250 ul of each extract were spotted onto 20×20-cm silica gel plates (Merck Art. 5735, Kiesselgel 60F254) and then developed with N-butanol, acetic acid and water (3:1:1). The developed TLC plates were dried 1 hour at room temperature to remove all traces of solvent. Then they were observed under a lamp with a 365 nm light wavelength.

The TLC plates were placed in a plastic box and overlaid with 50 mL of ½ PDA (containing 7 g/l of agar) seeded with *Botrytis cinerea* (concentration of 6.5 x 10^5 spore/mL). Then, the box was covered with a saran film and incubated at 25^{o}C under light condition. The results were visualized 4 day post inoculation. The growth of fungus was visible on the plates and clear areas of inhibition of the fungal growth indicated the location of active metabolite compounds on the TLC plates.

High performance liquid chromatography analysis

HPLC of *Sa. algeriensis* NRRL B-24137 metabolites from dual culture plates was performed according to Lamari *et al.* (2002a) to determine if they were dithiolopyrrolones. Active metabolite was detected by HPLC (Bio-tek instruments, Milan, Italy). The analytical column was ProntoSIL 120-5-C18 SH, 150 × 4.6 mm (Bishoff chromatography, Leonberg, Germany) fitted with a guard column of 10 × 4 mm and detection was achieved with a diode array detector (UV-vis 545 V, Bio-tek instruments).

For detection of metabolites secreted by *Sa. algeriensis* NRRL B-24137 during biocontrol assay, analyses were performed in the following chromatographic conditions. Samples were analyzed by a linear gradient elution using a mixture of acetonitrile/bidistilled water (solventA/solvent B) as mobile phase and a flow rate of 0.8 mL min-1. Column temperature was maintained at 30 °C and injection volume was 50 µL. UV detection of antibiotics was carried out at 220 nm, 390 nm and at more wavelength.

Metabolite compounds secreted by *Sa. algeriensis* NRRL B-24137 were also separated by HPLC (waters: controller 600, pump 600, dual λ absorption detector 2487, Linear Recorder); column C18 (250 × 7.8 mm UP ODS) with a mobile phase: linear gradient of acetonitril–H_2O from 0 to 100% for 30 min, a flow rate: 0.8 ml/min, and different UV detection.

Partial separation of antifungal products of *Saccharothrix algeriensis* NRRL B-24137

Metabolite compounds secreted by *Sa. algeriensis* NRRL B-24137 were separated by HPLC (waters: controller 600, pump 600, dual λ absorption detector 2487, Linear Recorder); column C18 (250 × 7.8 mm UP ODS); mobile phase: linear gradient of acetonitril–H_2O from 0 to 100% for 30 min; flow rate: 0.8 ml/min, detection: UV at 220 nm. Five different peaks were collected separately.

Results

In vitro biocontrol activity of *Sa. algeriensis* NRRL B-24137

Sa. algeriensis NRRL B-24137 was evaluated for its activity towards the phytopathogenic fungus *Botrytis cinerea* BC1 by dual-culture *in vitro* assay. It has been found that *Sa. algeriensis* NRRL B-24137 exhibited a strong ability to inhibit the *Botrytis cinerea* growth. In this *Sa. algeriensis* NRRL B-24137 and *Botrytis cinerea* BC1 interaction, the development of the fungal strain was prevented in an area of at least 1 cm around the *Sa. algeriensis* NRRL B-24137 (Figure 1a-b). The region around the *Sa. algeriensis* NRRL B-24137 was found to be red/orange colored showing the secretion of some bioactive antifungal molecule by *Sa. algeriensis* NRRL B-24137.

Separation of metabolites by TLC

The thin layer chromatography technique was used to separate some of the compounds present in the media of the interaction (inhibition zone) of *Sa. algeriensis* NRRL B-24137 and *Botrytis cinerea* BC1. One red compound visible at room light and several other compounds were revealed by UVs. Figure 2 shows the active zone detected by TLC of the extract of inhibition zone by *Sa. algeriensis* NRRL B-24137 against *Botrytis cinerea* BC1. One active band of red color was obtained by developing the TLC with n-butanol, acetic acid and water (3:1:1). Chloroform/Methanol 9:1 v/v solvent was also tested for TLC but n-butanol, acetic acid and water (3:1:1) was found to be best for obtaining migration of the bands. The strain *Sa. algeriensis* NRRL B-24137 was found to possess the red-pigment like observed previously. This pigment was active against *Botrytis cinerea* BC1 (Figure 2).

High performance liquid chromatography analysis using the program for dithiolopyrrolones

The HPLC analyzis have been performed to determine the metabolic profiles of the methanol extracts of *Sa. algeriensis* NRRL B-24137 and *Botrytis cinerea* BC1 inhibition zone. The analysis with the same method as used for detection of dithiolopyrrolones was carried out. The profil obtained (Figure 3) showed one peak that seems to correspond to red-pigment like band with a retention time 4.5 min. However this metabolite does not correspond to dithiolopyrrolones and appeared before the retention time of these compounds.

High performance liquid chromatography analysis using another program

As the red metabolite did not correspond to dithiolopyrrolones and that its retention time was too early, another program of HPLC was used. Profil obtained indicates that 5 peaks appeared with the program used (Figure 4).

Recuperation of compounds with semi preparative High performance liquid chromatography

Each of the peaks were separated, harvested for forther purification. However GS-MS ws not performed in this study.

Discussion

Actinomycetes are of great interest for the production of a variety of bioactive compounds, such as antibiotics, antitumor agents, antiparasites, immunosuppressant agents, and several enzymes important in the food industry and other industries (Demain, 1999). There are several recent reports of the patterns of the production of antimicrobial and antifungal compounds by different actinomycetes from soils (Basilio et al., 2003; Lee and Hwang, 2002; Busti et al., 2006; Bredholdt et al., 2007; Sabaou et al., 1998). Secondary metabolites secreted by actinomycetes can also be responsible of reduction of fungal phytopathogens directly (Raaijmakers et al., 2002; Compant et al., 2005).

Dual-culture assays showed in our study that *Sa. algeriensis* NRRL B-24137 can reduce growth of *B. cinerea* BC1. The activity of *saccharothrix* against Gram-positive bacteria, Gram-negative bacteria and fungi has been widely published (Isshiki et al., 1989; Sabaou, 1998; Sugawara, 1999; Kinochita, 1999; Wang et al., 2001).

Production of secondary metabolites is a process influenced by several physico-chemical factors including nutrient supply, oxygenation, temperature and pH (Olano et al., 2008). In our study the selected medium for the production of antifungal compounds was the combination of nutrient agar and PDA. It could be assumed that this medium has influenced the production of some new metabolites other than dithiolopyrrolones. To our knowledge, *Sa. algeriensis* NRRL B-24137 has the ability to produce a wide range of dithiolopyrrolones with different radicals (Lamari et al., 2002a; 2002b; Bouras et al., 2006; Boura*s* et al., 2008; Merrouche et al., 2010). But the metabolite secreted in our work does not correspond to the dithiolopyrrolones known as secreted by NRRL B-24137 as compared with thiolutin (Retention time = 12.3 min) as shown by Chorin (2009). HPLC analysis performed for the red pigment like band showed the secretion of metabolite at very early stage (retention time= 4.5 min). By changing the HPLC program, we determine that some peaks related to the red compound can be obtained in presence of others.

Saccharothrix species have been reported to produce antibiotics belonging to glycopeptide (Takeuchi et al., 1992), carboxylic nucleoside (Bush et al,. 1993), dithiolopyrrolone (Lamari et al., 2002), heptadecaglycoside (Singh et al., 2000), anthracyclin (Zitouni et al., 2004), macrolide (Murakami et al., 2009) and angucycline (Kalinovskaya et al., 2010) families. We do not know yet what however can be the secondary metabolites secreted by strain NRRL B-24137 and responsible of the growth reduction of *B. cinerea* BC1. Therefore further experiments are needed to characterize these secondary metabolites.

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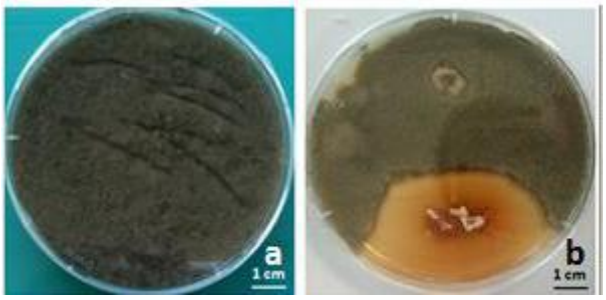

Figure 1: Petri dishes showing the activity of *Sa. algeriensis* NRRL B-24137 against *Botrytis cinerea* BC1 at 10 days (a) *Botrytis cinerea* BC1 alone (b) *Sa. algeriensis* NRRL B-24137 with *B. cinerea* BC1, showing the single inhibition zone.

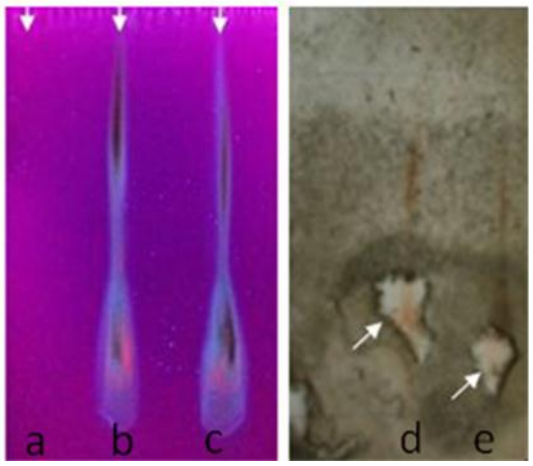

Figure 2: TLC analysis of the methanolic extraction of medium with metabolite secretion. Visualization at 365 nm. (a) NA/PDA medium as control (b) NRRL B-24137 metabolite secreted medium alone (c) of inhibition zone between NRRL B 24137 and *B. cinerea* (d-e) TLC plate of metabolites tested against *B. cinerea* showing the red pigment like having biocontrol activity.

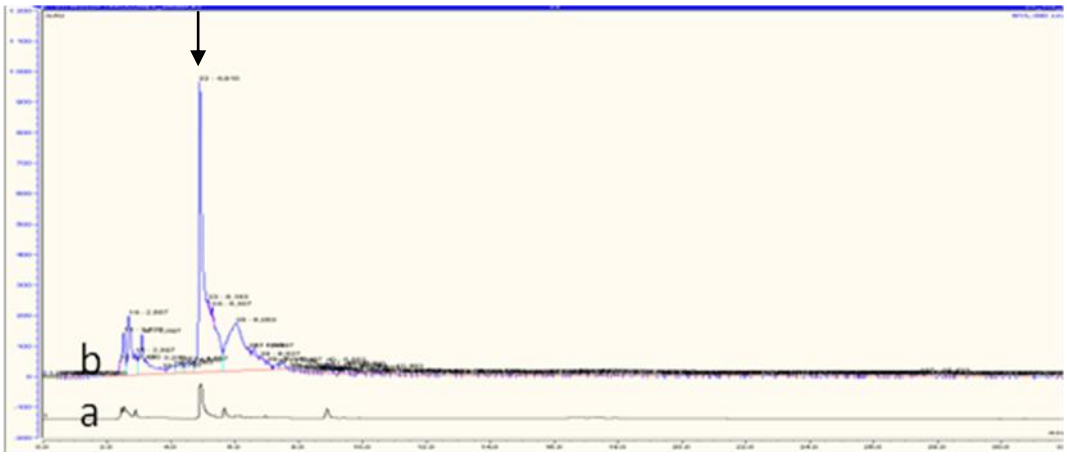

Figure 3: Metabolites detected by HPLC by using the program of dithiolopyrrolones with (a) nutrient agar medium as a control (b) metabolites from zone inhibition. Arrow shows the peak corresponding to the red pigmented metabolite.

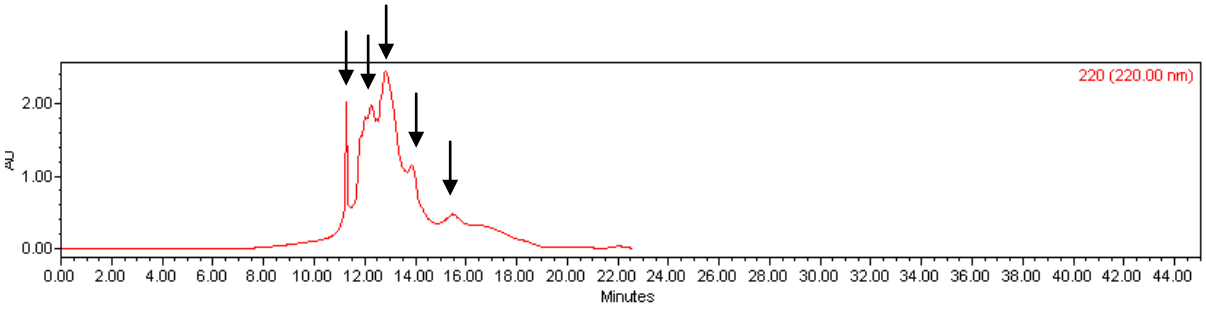

Figure 4: Metabolites (arrows) detected by using the second program of HPLC from the zone of inhibition.

In this work, the potential of strain NRRLB-24137 to reduce *Botrytis cinerea* growth was evaluated. This was carried out on petri dishes and results have shown that the strain isolated from desert soil can reduce the growth of the fungus. Further experiments have demonstrated that some metabolites can be responsible of the reduction of the fungus. Among them a red metabolite was demonstrated as involved in the reduction. However other metabolites can be also secreted and for the moment we do not yet know what can be all these metabolites. Due to the medium used, it seems however that they are not dithioopyrrolones, known as secreted by the beneficial strain. A switch of physiology may have occurred for the microbe with the medium used as when the bacterium was re-cultivated on ISP2 medium, the secretion of the red metabolite disappeared (data not shown).

Even if we do not know yet the metabolites responsibles of the reduction of *B. cinerea* growth, this part of the thesis demonstrated that strain NRRL B-24137 can have direct biocontrol activity towards *B. cinerea*.

In the next chapter we will focus on the colonization of grapevine plants by the beneficial strain.

Chapter III

Visualization of root colonization of grapevine by the Saharan soil isolate *Saccharothrix algeriensis* NRRL B-24137 by using DOPE-FISH microscopy and a probe specific to *Saccharothrix* spp.

Stéphane Compant[1], Saima Muzammil[1], Ahmed Lebrihi[1, 2], and Florence Mathieu[1]

[1] Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Dépt Bioprocédés et Systèmes Microbiens, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1, France.

[2] Université Moulay Ismail, Marjane II, BP 298, Meknès, MAROC

Running title: Visualization of colonization of grapevine by *Sa. algeriensis* NRRL B-24137 and DOPE-FISH microscopy

*Correspondence: Dr. Stéphane Compant, Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Dept BIOSYM, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1, France. Tel: +33 (0) 6 43 44 00 60. E-mail: scompant@ensat.fr

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Abstract

Background and Aim. There is currently a gap of knowledge if some beneficial bacteria isolated from desert soils can colonize epi- and endophytically plants of temperate regions. In this study the colonization process of the Saharan soil bacterium *Saccharothrix algeriensis* NRRL B-24137 was studied in grapevine to determine however if the beneficial strain can colonize a non natural host plant.

Methods. A probe specific to *Saccharothrix* spp. was designed and has allowed visualizing the colonization behavior of *Sa. algeriensis* NRRL B-24137 on and inside roots of grapevine plants. This was done by DOPE-FISH microscopy.

Results. The results showed ten days following inoculation that the strain could colonize the root hair zone, root elongation zone, as well as root emergence sites. Further observations showed that the strain could be also endophytic inside the endorhiza of grapevine plants crossing from the rhizodermis to cortical cell layers and therefore could establish endophytic subpopulations inside grapevine plants.

Conclusions. Taking into account of the natural niches of the beneficial strain, this study shows therefore that in spite of its isolation from desert soil the strain can be rhizo- and endophytic with grapevine plants.

Keywords: *Saccharothrix algeriensis* NRRL B-24137, desert soil, grapevine, colonization, DOPE-FISH

Introduction

Plant growth-promoting bacteria are known as helping their hosts by increasing directly plant growth and/or by protecting them towards pathogenic diseases directly or by inducing systemic resistance (Bakker et al. 2007; Lugtenberg and Kamilova 2009; Zamioudis and Pieterse 2012). Some of these bacteria could be isolated from the phyllosphere, anthosphere, carposphere as well as the caulosphere. However the majority of these bacterial microsymbionts are epiphytics and colonize the rhizosphere, which is a rich zone of colonization of microbes interacting with their hosts (Lugtenberg and Kamilova, 2009). A subset of the rhizosphere microflora can also enter inside plants, establishing subpopulations and proliferating within as endophytes (Rosenblueth and Martínez-Romero, 2006; Hallmann and Berg, 2007; Compant et al. 2010a; Reinhold-Hurek and Hurek 2011). Nowadays, there is however a current gap if some bacteria could colonize various host plants. Especially this is

the case of microbes isolated from harsh environments such as desert soils (Compant et al. 2010b). To find plant growth-promoting bacteria or biocontrol agent, harsh environments might however provide a rich source of beneficial bacteria (Compant et al. 2010b). Moreover, it is becoming increasingly evident that microbes from soil and plants growing in harsh environments such as desert soil may represent an enormous untapped genetic reservoir for plant improvement. It has been even recently postulated that transferring these microbes from native plants to non-host plants promises a revolutionary biotechnology to rapidly improve plant germplasms (Barrow et al. 2008). However, microbial colonization of such strains on non host plants should be studied, and also visualization of the process of colonization should be done if any application is carried out on crops growing in temperate conditions (Compant et al. 2010b; Compant et al. submitted). This is a pre-requisite for a better knowledge on how these microbes could interact with their hosts as well as if they could form epi- and endophytic populations.

In correlation to the search of beneficial microbes from harsh environments such as desert soil an actinomycete member of the Actinosynnemataceae family was isolated from desert soil in a palm grove of Adrar in Algeria, and identified as *Saccharothrix algeriensis* NRRL B-24137 (Zitouni 1995; Zitouni et al. 2004). Strain NRRL B-24137 is known as secreting various secondary metabolites, such as dithiolopyrrolones, with broad bioactive activities (Lamari et al. 2002a; 2002b; Zitouni et al. 2005). The strain is a biocontrol agent and reduces *Botrytis cinerea* infection on grapevine and on various plants under greenhouse and in field conditions (unpublished) under 25°C conditions but also even under high temperature conditions (Muzammil et al. in press). However, colonization processes of this strain should be studied to understand where the beneficial strain is localized following soil application as well as before to study mechanisms of plant resistance.

Generally, soil bacteria responsible of biocontrol activities may be endophytic following early steps of colonization (Compant et al. 2005; 2010a; Reinhold-Hurek and Hurek 2011). Such interactions have been demonstrated for some bacterial taxa but for others information on colonization capacity is still largely unknown (Rosenblueth and Martinez-Romero 2006). It is however of special interest to examine this kind of interactions to increase knowledge of how a beneficial bacterium can interact with its host, and niches of colonization could provide information regarding microbial ecology of such microbes.

To visualize colonization and to track microbes on and inside plants various tools such as for instance *gfp*, *dsred*, and *gus* markers as well as derivatives could be used (Larrainzar et al. 2005). However this implies that the microbe will be transformed before application,

which is difficult to achieve for some microorganisms. An alternative method is FISH (fluorescence *in situ* hybridization; Amman et al. 1990; Wagner et al. 2003), although it can suffer from some limited avantages (Wagner et al. 2003). Different improvements have been however published to increase the signal in FISH (Wagner and Haider 2012). In 2010, Stoecker et al. described for instance the use of DOPE-FISH corresponding to 5'- and 3'-doubly labelled probes instead of single labelled probes for FISH. It has been demonstrated that doubly labelled probes strongly increase *in situ* accessibility of rRNA target sites. This technique provides moreover more flexibility for probe design (Stoecker et al. 2010) and can allow visualizing microorganisms that could not be well visualized by single FISH or can not been transformed.

In this study we created a specific probe for *Saccharothrix* spp., as well as use DOPE-FISH technique to monitor the early colonization process of a beneficial strain on grapevine plants both in the rhizosphere and root endosphere of plants. This was a pre-requisite to better understand interaction between a bacterium isolated from a harsh environment and a non natural host.

Materials and methods

Bacterial culture

Sa. algeriensis NRRL B-24137 was used throughout this work. This strain was grown at 30°C on ISP 2 (International *Streptomyces* Project 2) solid medium (pH 7.0) containing per liter of distilled water: 4 g D(+) glucose (Acros organics), 10 g malt extract (Fluka), 4 g yeast extract (Fluka) and 18 g agar (Sigma). 8 days after growing on plate, aerial mycelium + spores of strain NRRL B-24137 was harvested in PBS and concentration was adjusted to 5 x 10^7 CFU.ml^{-1}.

Plant material

Grapevine plants harboring as graft part cv. Cabernet Sauvignon clone 15 and as rootstock 44-53 M (Malègue) were provided by „Pépinières Colombie Vendries (Camparnaud, France). Plants were stored at 4°C in a dark cold chamber for at least 2 weeks before to be treated with cryptonol at 0.05% for 15h at ambient temperature (20-25°C). Plants were then surface sterilized with 1.6% bleach (10 min) and 70% ethanol (30 min) before to be rinced with sterile tap water and planted in 2 times autoclaved soil containing 1/3 perlite, 1/3 potting soil and 1/3 sand. Plants were then allowed to grown in a phytotronic growth chamber

(16h photoperiod, 20-25°C night-day, and 70% relative humidity) and watered with sterile tap water.

Plant inoculation

After 1 month after planting, grapevine plants were delicately separated from their soils. Root systems were immersed in bacterial solution of *Sa. algeriensis* NRRL B-24137 during 3 minutes before that plants be replaced in pots filled with soils (as described before). Plants were then allowed to grow for 10 days before the sampling of plant parts for microscopic analysis.

Probe design for *Saccharothrix* spp. and labelling

To create probes specific to *Saccharothrix* spp., the partial 16S rDNA sequence of *Sa. algeriensis* NRRL B-24137 (Accession: AY054972.2 GI: 134034183) was used. Design of 16S rRNA probes was made by using Stellaris™ FISH Probes software. Specificity of probes created was then checked on NCBI, Silva, Green genes blast (Altschul *et al*. 1997) or Probe Check at microbial-ecology.net (Loy et al. 2007). ΔG, FA, FAm, as well as hybridization efficiency were calculated according to Yilmaz et al. (2004; 2006; 2007) and Tm was calculated by using Tm=64.9 + 41 x ((G + C - 16.4)/length) according to Loy et al. (2007). These parameters were evaluated for different temperatures of hybridization.

Among probes designed one found as specific to *Saccharothrix* spp. was then purchased at Genecust (Luxemburg) with aminomodifier C6 at 5' and 3' position (for FOPE-FISH) before to be labeled with dylight488 fluorochrome (Piercenet) enabling green fluorescence under UV light.

DOPE-FISH microscopy

For visualization of rhizosphere colonization, roots of grapevine 10 days post inoculation with strain NRRL B-24137 were cut in small parts and fixed overnight at 4°C in a paraformaldehyde solution (4% in PBS) in eppendorf tubes, before to be rinsed twice with PBS. Samples were treated with 1 mg/ml lysozyme at 37°C during 15 min, rinced with PBS and were then dehydrated in an ethanol serie (50 to 99.9%; 30 min each step). DOPE-Fluorescence *in situ* hybridization was then carried out according to Compant et al. (2011) by using 15 ng/µl of a probe specific to *Saccharothrix* spp. labeled at both 5' and 3' with dylight 488 fluorochrome. Following DOPE-FISH hybridization at 51°C, post hybridization at 52°C (46/48°C was not used due to the fact that the probe selected has a FAm lower at the

temperature selected of 51°C and also a better hybridization efficiency, Table 1) and rinsed with sterile distillated water (prewarmed at 51°C). Samples were then kept in dark during at least 1 day. Samples were then observed under an epifluorescence microscope (BH2, Olympus, Japan) under UV light and pictures were taken with a camera.

In parallel to the rhizosphere colonization study, endophytism was evaluated for *Sa. algeriensis* NRRL B-24137. For this, root samples were treated as described before except that after the ethanol serie, samples were included in LR white resin according to manufacturer instructions. Embedded tissues were then sliced with a microtome and glass knives and slices of 1-1.5 µm were deposited on microscopic slides previously treated with 70% ethanol. DOPE-Fluorescence *in situ* hybridization was then done by using 15 ng/µl of a probe specific to *Saccharothrix* spp. labeled with dylight 488 fluorochrome as described before. Following DOPE-FISH hybridization, post hybridization and rinsed, slides containing slices were kept in dark during at least 1 day. Slices on slides were then observed under an epifluorescence microscope (BH2, Olympus, Japan) under UV light 1 day after hybridization and pictures were taken with a camera.

Statistical analyses

All experiments have been repeated three independent times with similar results on each time 10 plants. More than 20 slices were used per plant to visualize the colonization process.

Results

Probe specific to four species of *Saccharothrix*

Different probes of 20 and 25nt were designed. However, no probe with this length was exclusively specific to *Saccharothrix*, therefore additional probes of 30nt were created. Among them a probe (Table 1) named Sac135 was designed and checked on probe check, Silva, green genes, and NCBI databases. Data revealed that the probe is specific to four species of *Saccharothrix* including *Saccharothrix algeriensis*. The probe has a % G-C content of 53.3, a position 135 according to the *E. coli* gene numbering, an exp Td (Tm) of 64°C, a ΔG_1: -33.0 kcal/mol, a ΔG_2: 0.1 kcal/mol, a ΔG_3: -14.4 kcal/mol, a $\Delta G_{overall:}$ -18.2 kcal/mol, and a FAm of 47.3 % at 51°C hybridization with 0.9M Na^+ (Table 1). At 46°C used for the majority of FISH, the probe could not be used due to not inefficient hybridization and a high Fam according to Yilmaz et al. (2004; 2006; 2007). This probe was then further used with a

formamide concentration of 20% as hybridization efficiency at 0-20% of formamide was of 1.0000 and decreased then (Table1).

Root hair zone colonization by *Saccharothrix algeriensis* NRRL B-24137

Colonization by strain NRRL B-24137 was evaluated by DOPE-fluorescence *in situ* hybridization (DOPE-FISH) analyzis by using the Sac135 probe. Colonization by strain NRRL B-24137 was firstly evaluated at the root hair zone level. The bacterium was visualized as spores near root hairs (Fig. 1a-b) or in close contact to the surface of root hairs (Fig. 1c). The mycelial form was visualized also as colonizing externally the basis of root hairs (Fig. 1d). Germinated spores were further detected at the root hair zone (Fig. 1e-g) in contact to the surface of root hairs and mycelium was additionally visualized in this root zone, at the basis of the surface of root hairs (Fig. 1i-j). Finally a form corresponding to pack of mycelium was noticed (Fig. 1k) and spores production by mycelial form was visualized at the root hair zone just in close contact to the surface of root hairs (Fig. 1l-m).

Root emergence site colonization by *Saccharothrix algeriensis* NRRL B-24137

10 days post inoculation, the bacterium was also found at root emergence sites as a mycelial form (Fig. 2a-b). No spores, or germinated spores were however visualized. Only this type of actinobacterial form was noticed in all analyzed samples.

Root elongation zone colonization but not root tips by *Saccharothrix algeriensis* NRRL B-24137

Colonization by strain NRRL B-24137 was evaluated not only at the root hair zone and emergence site levels but also at the root elongation zone. The bacterium was visualized as spores (Fig. 3a) and germinated spores (Fig. 3b-c) on the rhizoplane. Visualization of the process of colonization revealed that the bacterium could be also in a filamentous form interacting with some cells of the rhizodermis (Fig. 3d-g). Colonization could be achieved with spores, germinated spores and mycelia form,but also colonization of parts of rhizodermal cells (Fig. 3h) and on the whole outline of some cells on the rhizoplane (Fig. 3i) were noticed.

In contrast to the root hair zone, root emergence site and root elongation zone, no detection of strain NRRL B-24137 was reported on any of the analyzed samples at the root tip level (data not shown).

Endorhizal colonization by *Saccharothrix algeriensis* NRRL B-24137

The strain NRRL B-24137 was easily visualized inside roots in LR white sections and in highest numbers in comparison to the root surfaces. It was visualized as inside endorhiza of grapevine plants 10 day post inoculation, crossing from the rhizoplane (Fig. 4a) to intracellularly to the inside rhizodermis (Fig. 4b). The filamentous form was also visualized between rhizodermis and exodermis (Fig. 4c). Additionally, mycelium of the strain was visualized intercellularly between some cortical cells in the cortex zone (Fig. 4d). A pack of mycelium was also noticed intercellulary (Fig. 4e-f) and some cells corresponding to the strain was further visualized intracellularly in cortical cell layers (Fig. 4g-h).

Discussion

In the present study, a probe was designed for *Saccharothrix* spp. DOPE-FISH technique has been used because of low signal intensity by using single labeled probe for some filamentous microorganisms (Stoecker et al. 2010). This probe and the DOPE-FISH tool have allowed visualizing the early colonization process of *Saccharothrix algeriensis* NRRL B-24137 on and inside roots of grapevine plants. Since the strain was isolated from a harsh environment, it could not be expected before that it can colonize grapevine plants under climatic chamber conditions. However, it was shown that the bacterium can colonize the rhizoplane of the root system of grapevine plants.The strain can colonize the root hair zone, root elongation as well as root emergence site but not root tips. Pathways of colonization have been described for different kinds of bacteria colonizing the surfaces of root systems of their hosts (Hardoim et al. 2008; Compant et al. 2010a) and on the grapevine rhizoplane (Compant et al. 2005; 2008). However and interestingly, these root surfaces were not colonized in the same way by the strain NRRL B-24137. Preferential sites of some bacteria were at the root hair zone corresponding to a rich zone of exudates (Hallmann and Berg 2007). Strain NRRL B-24137 was not visualized inside root hairs, but only at the surfaces of root hairs. It has been recently established that some bacteria could colonize root hair internally (Priesto et al. 2011; Mercado-Blanco and Priesto 2012) but this depends of the strain and plant-microbe interactions.

Strain NRRL B-24137 was not visualized at the root tip, suggesting that there is no colonization of such root parts during the process of colonization. However, we cannot exclude that this may be also an artefact of fixation and ethanol dehydratation.

In this study we show that strain NRRL B-24137 can be endophytic in grapevine roots crossing from the rhizodermis to several cortical cell layers. Several studies have examined colonization process by beneficial bacteria (reviewed in Compant et al. 2010a). Some can be systemic colonizers whereas other could be restricted to root parts. Although we studied early colonization process by NRRL B-24137, we detected only colonization of this strain up to several cortical cell layers but not in the vascular system, suggesting that NRRL B-24137 will be restricted to root internal parts. However, experiments were done only at 10 days post inoculation and it may be possible then that the bacterium could reach vascular system in longer colonizations.

The strain was found more easily inside the endorhiza than on the surfaces of the root system of grapevine plants, suggesting preferential sites of colonization for the strain in grapevine, e.g. endophytic niches, as it has been described for some others beneficial bacteria (Hallmann and Berg 2007).

The strain used in this study is an actinobacterium that could form spores and myceliums (Zitouni 1995; Zitouni et al. 2004). Therefore it was not surprising to see that during the colonization different forms of the strain could be visualized. Different studies have described colonization by actinomycetes (see for instance Coombs and Franco, 2003; Merzaeva and Shirokikh 2006). However this has been never done by DOPE-FISH nor with a Saharan desert soil bacterium, or with grapevine plants and a *Saccharothrix* member.

Although more works are needed to better understand the interaction between *Saccharothrix algeriensis* NRRL B-24137 and grapevine plants, this study shows therefore that the strain, isolated from Saharan soil, could colonize epi- and endophytically roots of grapevine plants as well as forms different kinds of forms during the colonization processes.

This strain is known as inducer of systemic response in grapevine towards *B. cinerea* following the early colonization processes under climatic chamber conditions (unpublished), as well as under high temperature conditions (Muzammil et al. in press). However the beneficial strain and pathogen should be distant during understanding of systemic resistance (van Loon et al. 1998). This study shows that the beneficial strain is present only in root of grapevine plants and not a systemic colonizer during time of experiments (data not shown) and also some few days more; although a systemic colonization could not be excluded in a long term experiment as some rhizospheric strains could colonize endorhiza and spread inside plants to reach vegetative and/or reproductive organs (Compant et al. 2008; 2010a).

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Table 1: Probe and specificity of probe related to *Saccharothrix* spp.

Probe name	Sac135
Target molecule	16S rRNA
Sequence	5' - TAG TTT CCC AGG CTT ATC CCG GAG TAC AGG - 3'
Specifity	*Sa. algeriensis*, *Sa. espanaensis*, *Sa. australiensis*, *Sa. yanglingensis*
Length nt	30
% GC content	53.3
ΔG [kcal/mol]	ΔG_1: -33.0 ΔG_2: 0.1 ΔG_3: -14.4 $\Delta G_{overall:}$ -18.2
Position*	135-165
Tm** [°C]	64°C
MW [g/mol]	9198.02
Formamide %	0-20%
Hyb. effic.***	1.0000
FAm**** %	47.3

Tm** [°C]**:** melting temperature dissociation
Hyb. effic.*:** Hybridation efficiency at 0% of formamide
FAm**** %: melting formamide concentration
ΔG, Formamide %, **Hyb. effic. and FAm** % calculated for 51°C and 0.9M Na^+

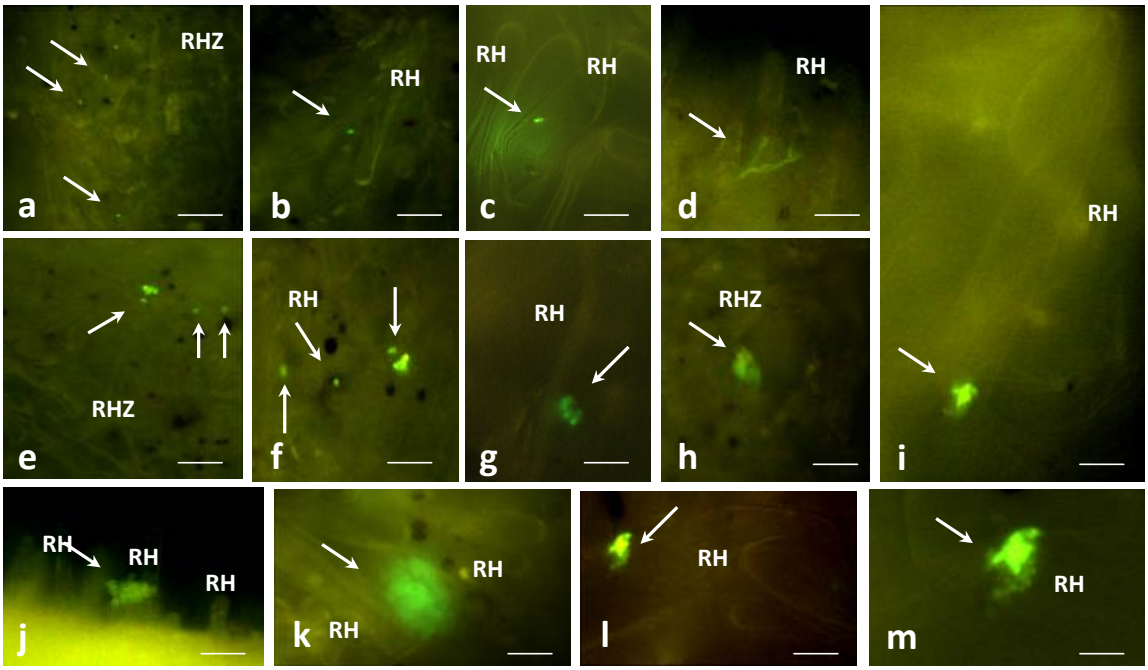

Fig. 1: Visualization of grapevine root colonization by *Saccharothrix algeriensis* NRRL B-24137 by DOPE-FISH microscopy at the root hair level showing (arrows) spores (a-c), mycelial form (d, i-j), germinated spores (e-g), a package of mycelium (k), and spores production by mycelia (l-m). RHZ: Root Hair Zone, RH: Root Hair. Scale bars: a) 30µm, b) 20 µm, c-d) 10µm, e-f) 20 µm, g-h) 10µm, i) 5 µm, j) 10µm, k-l) 20µm and m) 2.5 µm.

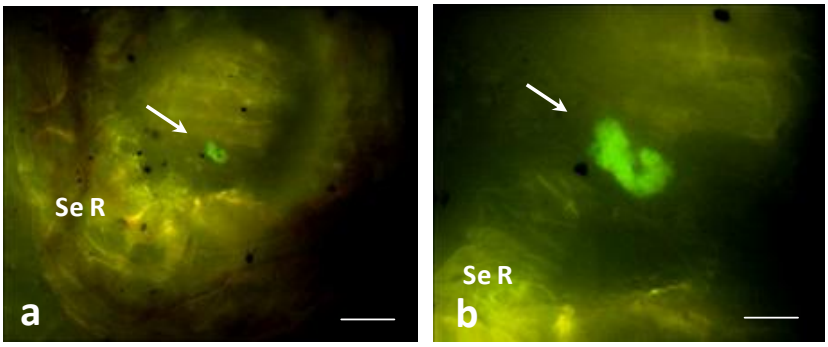

Fig. 2: Visualization of grapevine root colonization by *Saccharothrix algeriensis* NRRL B-24137 by DOPE-FISH microscopy at root emergence sites showing (arrows) mycelial form (a-b). SeR: Secondary Root. Scale bars: a) 50 µm, and b) 15 µm.

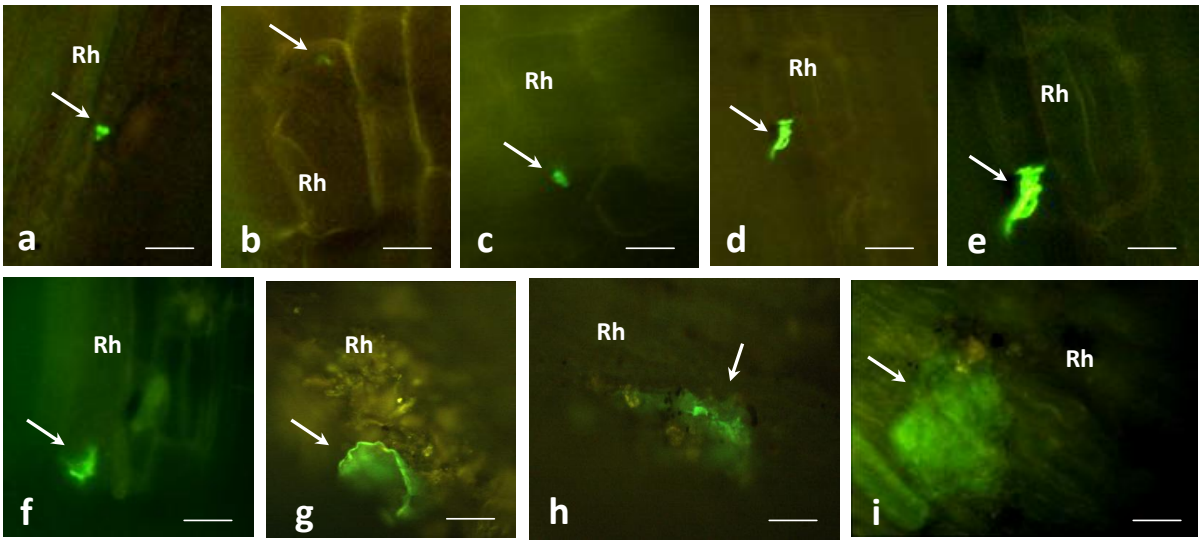

Fig. 3: Visualization of grapevine root colonization by *Saccharothrix algeriensis* NRRL B-24137 by DOPE-FISH microscopy at the root elongation zone showing (arrows) spores (a), germinated spores (b-c), mycelial form (d-i), and parts (h) or complete outline (i) of some rhizodermal cells. Rh: Rhizoplane. Scale bars: a) 5 µm, b-c) 10 µm, d) 20µm, e) 10 µm, f-i) 20 µm.

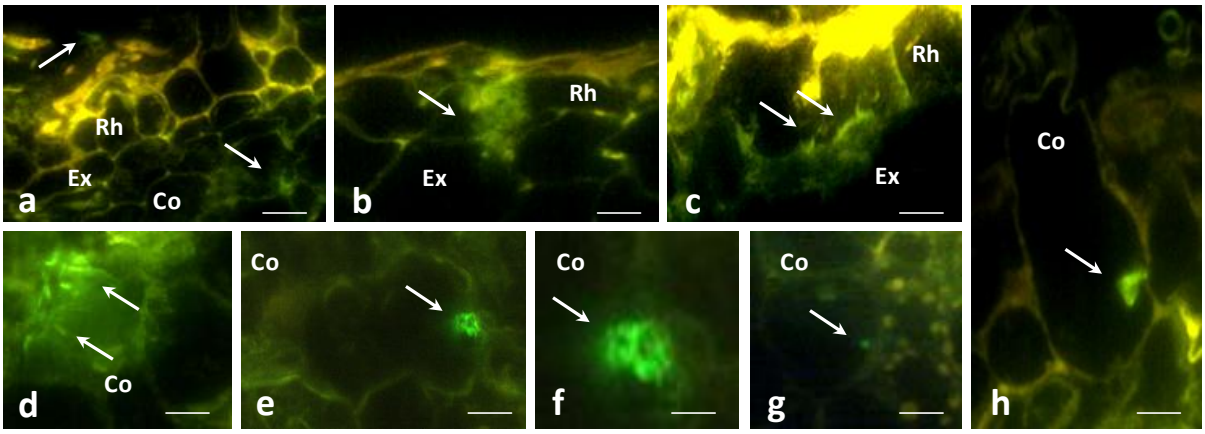

Fig. 4: Visualization of grapevine root colonization by *Saccharothrix algeriensis* NRRL B-24137 by DOPE-FISH microscopy inside the endorhiza showing (arrows) mycelia colonization from rhizodermis (a) to exodermis (b-c), cortex intercellularly (d-f) and intracellularly (g-h). Rh: Rhizoplane, Ex: Exodermis, Co: Cortical cells. Scale bars: a) 50 µm, b) 15 µm, c-e) 10 µm, f) 5 µm, g) 10 µm, h) 7.5 µm.

In this work, colonization of the rhizoplane and inside the root system of grapevine plants by NRRLB-24137 has been demonstrated.

Following this work, it is assumed that the defence mechanisms might be triggered in the plant during bacterial colonization. Moreover, it seemed interesting to see if the plant roots inoculated with the strain NRRL B-24137 result in a protection against infection caused by *Botrytis cinerea.* However, to describe ISR beneficial strain should be absent at the point of pathogen infection (Pieterse et al., 2002; Van Loon and Bakker, 2005). The phenomenon of resistance induced by beneficial bacteria can only be considered if there is indeed a separation in space between rhizobacteria and infectious agent (Van Loon, 1998). To investigate the ISR (or a SAR) as possible resistance towards *B. cinerea* at leaves, the bacteria should not be present at foliar level. In our case, the beneficial bacterium is absent in leaves at 10 days post inoculation as well as 13dpi (data not shown). Therefore, in this time lapse, systemic resistance could be studied. This will be carried out in the next chapter.

Chapter IV

Systemic resistance of grapevine plants towards *Botrytis cinerea* induced by the desert soil bacterium *Saccharothrix algeriensis* NRRL B-24137

Saima Muzammil[1], Clotilde Graillon[1], Florence Mathieu[1], Ahmed Lebrihi[1], and Stéphane Compant[1]

[1] Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Dépt Bioprocédés et Systèmes Microbiens, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1, France

Running title: Protection of grapevine gray mould by *Sa. algeriensis* NRRL B-24137

*Corresponding author: Dr. Stéphane Compant, Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Dept BIOSYM, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1, France. Tel: +33 (0) 6 43 44 00 60. E-mail: scompant@ensat.fr

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Abstract

In this study the potential of *Saccharothrix algeriensis* NRRL B-24137 to protect grapevine plants towards the gray mould agent *Botrytis cinerea* was evaluated. Results showed that the beneficial endophyte could reduce infection of *B. cinerea* strain Bc1 on leaves of cv. Cabernet-Sauvignon following root colonization. Further examination of grapevine gene expressions was evaluated to better understand mechanisms of protection. Priming of *VvGlu1*, *VvChit3* and *VvPGIP* expressions was particularly monitored by semi-quantitative RT-PCR. However results did not lead to a conclusion of a putative priming of these genes for explication of resistance mechanisms.

Keywords: *Saccharothrix algeriensis* NRRL B-24137, desert soil, endophyte, grapevine, *Botrytis cinerea*

Introduction

Beneficial rhizo- and endophytic bacteria can protect various hosts towards pathogenic diseases directly by secretion of allelochemicals but also indirectly by inducing a systemic resistance (Bakker et al., 2007; Lugtenberg and Kamilova, 2009; Zamioudis and Pieterse, 2011). Sources of these microbes are diverses but almost of them have been isolated from crops grown in temperate regions. However some new competent rhizo- and endophytic bacteria enabling plant resistance could be also isolated from harsh environments. It is becoming increasingly evident moreover that cryptic symbiotic microbes from soil and plants growing in harsh environments may represent an enormous untapped genetic reservoir for plant improvement (Barrow et al., 2008). In this study we evaluate the potential of one bacterium isolated from desert soil, *Saccharothrix algeriensis* NRRL B-24137 to protect grapevine plants towards the phytopathogen *Botrytis cinerea* responsible of gray mould disease.

To further evaluate mechanisms involved in a putative resistance induced by the strain NRRL B-24137, priming effect was evaluated. Different bacteria have been shown to induce priming effect leading to pathogen reduction. Priming is known as a potentialization of gene expression once plants are challenged with a phytopathogen or due to an abiotic stresses (Bakker et al., 2007). On grapevine, priming effect has been demonstrated with some rhizo- and endophytic bacteria and *B. cinerea* (Verhagen et al., 2010; 2011). Different gene expressions during priming can be correlated to *Botrytis cinerea* growth reduction. In this

study we wanted to determine if expression of genes coding chitinases, glucanases, and polygalacturonase inhibitor could be primed as this could explain reduction of *B. cinerea* growth.

In this study we evaluate therefore the potential of *Saccharothrix algeriensis* NRRL B-24137 to control infection caused by the gray mould agent as well as try to determine parts of the mechanisms of resistance in case of a putative protection.

Materials and methods

Bacterial and fungal cultures

Sa. algeriensis NRRL B-24137 was used throughout this work. This strain was grown at 30°C on ISP 2 (International *Streptomyces* Project 2) solid medium (pH 7.0) containing per liter of distilled water: 4 g D(+) glucose (Acros organics), 10 g malt extract (Fluka), 4 g yeast extract (Fluka) and 18 g agar (Sigma). 8 days after growing on plate, aerial mycelium + spores of strain NRRL B-24137 was harvested in PBS and concentration was adjusted to 5 x 10^7 CFU.ml^{-1}.

Botrytis cinerea (strain BC1 isolated by S. Compant from grapevine plants in Illmitz, Burgenland, Austria in 2008) was grown on solid and sterilized medium PDA (Potato Dextrose Agar) at ambient temperature (20-25°C). Spores were harvested in a half PDB solution from 9 days old *Botrytis cinerea* grown on plate at a concentration of 6.5x10^5 conidia/mL.

Plant material

Grapevine plants harboring as graft part cv. Cabernet Sauvignon clone 15 and as rootstock 44-53 M (Malègue) were provided by „Pépinières Colombie Vendries' (Camparnaud, France). Plants were treated with cryptonol at 0.05% before to be placed at 4°C in a dark cold chamber for at least 2 weeks. Plants were then surface sterilized with 1.6% bleach (10 min) and 70° ethanol (30 min) before to be rinced with sterile tap water and planted in 2 times autoclaved soil containing 1/3 perlite, 1/3 potting soil and 1/3 sand. Plants were then allowed to grown in a growth phytotronic chamber (16h photoperiod, 20-25^{o}C night-day, and 70% relative humidity) and watered with sterile tap water.

Plant inoculation with *Sa. algeriensis* NRRL B-24137

After 1 month after planting, grapevine plants were delicately separated from their soils. Root systems were immersed in the bacterial solution of *Sa. algeriensis* NRRL B-24137 during 3 minutes whereas control plants were inoculated with PBS. Then plants were replaced in pots filled with soils (as described before). Plants were then allowed to growth for 10 days before to challenge them with the phytopathogen.

Plants challenged with *Botrytis cinerea*

Leaves were infected by a spore solution by inoculating five leaves per plants with 3 µl of spore solution of *B. cinerea* 5 times per leaves or mock inoculated (control). To ensure infection, inoculated plants were kept at 100% relative humidity during all the infection process, in the growth chamber conditions described before (16h photoperiod, 20-25^{o}C night-day, and 70% relative humidity).

Monitoring susceptibility to *B. cinerea*

Susceptibility to *B. cinerea* was evaluated by the percentage of necrotic leaves, 3 days after the infection. Pictures of leaves inoculated or not (mock) with *B. cinerea* were additionally photographed 3 days post inoculation.

Plant RNA extraction and cDNA synthesis

Leaves tissue samples were harvested from plants inoculated or not with NRRL B-24137 and challenged with *B. cinerea* or not (mock) at time 0, 1, 2, and 3 days post fungal inoculation. Total RNA was extracted by using the RNeasy Plant Mini kit (Qiagen) excepted that the lysis solution RLC was replaced by 1ml per sample of a lysis buffer (300mM Tri HCl pH=8.00, 25 mM EDTA pH=8.00, 2M NaCl, 2% CTAB, 2% PVPP, 0.05% spermidine and just prior to use, 2% β-mercaptoethanol). The mRNA were purified by using the Oligo(dT) primers specific to the Poly(A*)* Tail of mRNAs. DNA contamination on extracts was removed with the RNase-free Amplification Grade DNase I kit (Sigma). Agarose gel electrophoresis and spectroscopy were used to confirm RNA integrity and quality before and after DNaseI treatment.

cDNAs were synthesized from 1µL of total RNA using the TITANIUM One-Step RT-PCR Kit from Clonetech (Ozyme, France), according to the manufacturer's instructions. Agarose gel electrophoresis and spectroscopy were used to confirm cDNA integrity and quality.

Plant gene expression

PCRs were conducted in triplicate in a total volume of 50 µl containing: 5 µl of diluted cDNA solution, 1 µl of Taq polymerase (Promega), and 1 µl of each primer (10 µM), 5 µl of 10X PCR buffer + MgCl2 and 1µl of dNTP mix (10 mM each). DNA amplification was performed on a thermal cycler (Mycycler, Biorad, France) with the following parameters: 10 min at 95°C and then 30 cycles of 94 °C for 45s, 60°C for 45s, and 72°C for 2 min, with a final cycle at 72 °C for 7 min. Primers used were for genes *EF1α, VvPGIP, VvGlu1,* and *VvChit3* according to Aziz et al. (2003). The elongation factor *VvEF1* was used as an internal control. Agarose gel stained with ethidium bromide was used to monitor the gene expression profils.

Statistical analysis

Plant resistance was evaluated 3 independent times of 10 plants for each treatment. Student t test was used for statistical analysis. Semi quantitative RT-PCR was used on 2 sets of plants and PCR and semi quantitative RT-PCR were repeated twice.

Results

Saccharothrix algeriensis* NRRL B-24137 induce a systemic resistance towards *Botrytis cinerea

Control plants and bacterized plants have different *Botrytis cinerea* infection rates (Figure 1a). Control plants have a percentage of leaves with infection of 62+/- 17.51 whereas plants previously inoculated with strain NRRL B-24137 have 42+/-23.94 % of infection (significant $P<0.05$; Figure 1b).

Does *Saccharothrix algeriensis* NRRL B-24137 induce priming of gene expression towards *B. cinerea* infection?

Gene expression was monitored to determine putative priming mechanisms. However data show that expressions of *Gluc* and *Chit3* genes are not primed (Figure 2a-b). It is not clear also if *PGIP* gene is also primed or not because at time 0 there is already gene expression (Figure 2c). However this has been done on only 2 sets of plants and this needs further examination.

Discussion

In the present study, we showed that *Sa. algeriensis* NRRL B-24137 can induce a systemic resistance towards the necrotrophic agent *Botrytis cinerea*. The beneficial actinobacterium could be only present at the root level. Indeed strain NRRL B-24137 has not been recovered from leaves 10 and 13 days post inoculation (unpublished results). Therefore a direct interaction can not be performed between the fungal agent and the beneficial bacterium.

Different bacteria such as members of Bacillaceae, Pseudomonodaceae, Enterobacteriaceae as well as as actinobacteria have been demonstrated as inducing a protection on grapevine following inoculation towards the gray mould agent (reviewed in Compant et al., 2011; Compant et al., accepted). However this was never demonstrated with a member of actinosynnemateae family nor with an isolate from desert soil. In this study we show therefore that strain NRRL B-24137 can protect grapevine plants cv. Cabernet-Sauvignon towards *B. cinerea* and we are sure that the bacterium is only present at the root level and therefore protection could not be correlated to a direct interaction between the beneficial bacterium and the necrotrophic agent.

To determine mechanisms involved in protection towards the gray mould agent, putative mechanisms of priming were tested. However data related to gene expression do not lead to conclusion that *VvPGIP*, *VvChit3* and *VvGlu1* are primed. We used semi quantitative RT-PCR and could not determine however well plant gene expressions. However it is rather better to use quantitative RT-PCR to be sure of the gene expression profils. We further did only experiments on 2 sets of plants and this work needs repetition. Therefore more work is needed to better understand how strain NRRL B-24137 could have an impact on grapevine plants leading to a systemic resistance towards *B. cinerea*. Recently Verhagen et al. (2011) showed that grapevine plants inoculated with some rhizospheric and endophytic bacteria can induce protection and that phytoalexins are primed during *B. cinerea* infection. Phytoalexins secretion should be also tested with our model.

Acknowledgements

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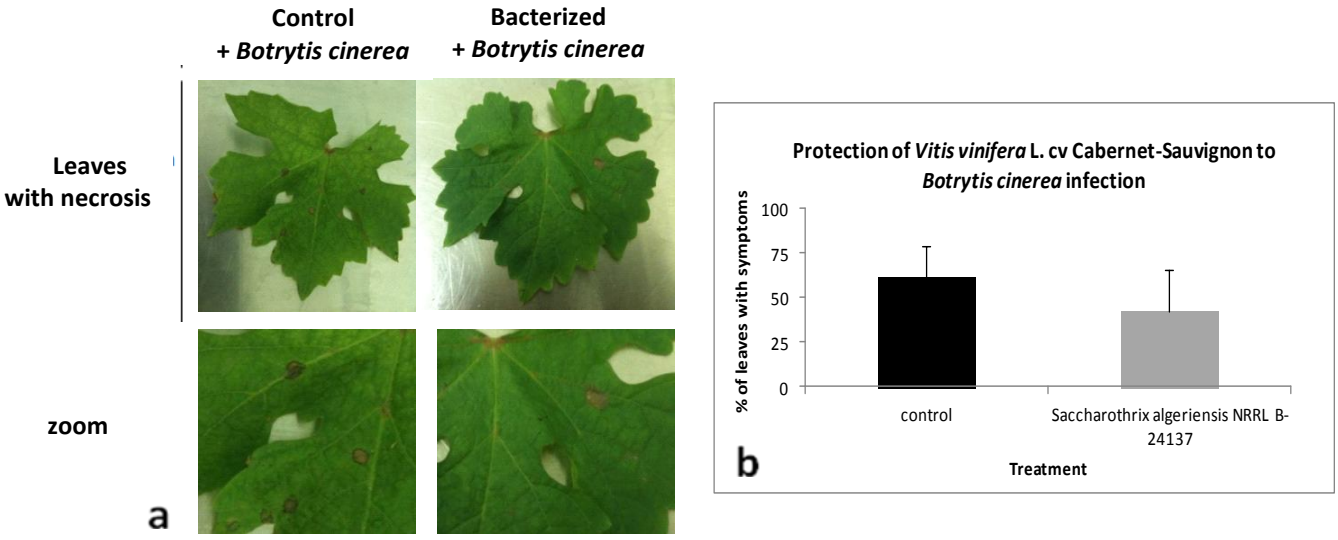

Figure 1: comparison of symptoms due to *Botrytis cinerea* infection on control plants or plants previously inoculated with *Sa. algeriensis* NRRL B-24137 (a) and % of leaves of symptoms (b; $P<0.05$).

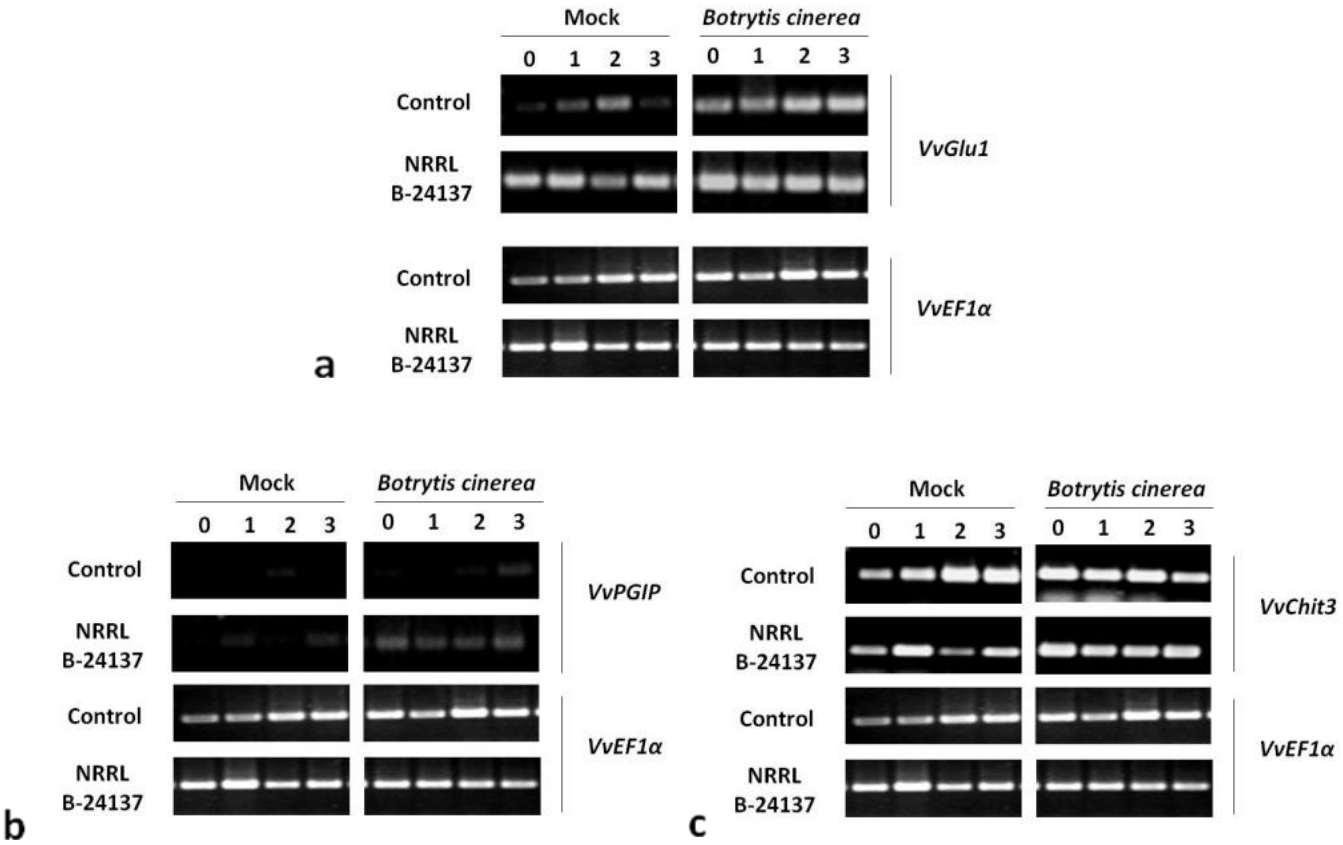

Figure 2: semi quantitative RT-PCR of gene expressions of *VvGlu1* (a), *VvPGIP* (b) and *VvChit3* (c) of leaves inoculated or not with *Botrytis cinerea* and previously inoculated or not with *Sa. algeriensis* NRRL B-24137. The elongation factor *VvEF1* was used as an internal control.

In this chapter, the study clearly demonstrated that *Sa. algeriensis* NRRL B-24137 induces systemic resistance in grapevine plants towards leaves infection caused by *B. cinerea*.

Gene expressions were also monitored to determine priming mechanisms. However results obtained showed that genes *VvGlu1*, *VvChit3* and *VvPGIP* were not primed (although further experiments need to be carried out). We used semi quantitative RT-PCR but this does not allowed us to be sure about gene expression. However, it is better to use quantitative RT-PCR to be sure about the gene expression profiles.

As NRRL B-24137 can induce an ISR towards *B. cinerea,* mechanisms should be undertood. In the next chapter we will determine parts of the mechanisms. However we will not use grapevine plants but the model plant *Arabidopsis thaliana* as it would be easy to determine what kind of mechanisms are involved as well as if it is a SAR or an ISR phenomenon involved.

Chapter V

The desert isolate *Saccharothrix algeriensis* NRRL B-24137 can be endophytic in *Arabidopsis*, induces ISR towards *Botrytis cinerea* as well as allows to determine new mechanisms involved

Saima Muzammil[1], Clotilde Graillon[1], Rayenne Saria[1], Florence Mathieu[1], Ahmed Lebrihi[1], and Stéphane Compant[1]

[1] Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Dépt Bioprocédés et Systèmes Microbiens, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1, France

Running title: Colonization and ISR induced by *Sa. algeriensis* NRRL B-24137 in *Arabidopsis* towards *Botrytis cinerea*

*Corresponding author: Dr. Stéphane Compant, Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Dept BIOSYM, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1, France. Tel: +33 (0) 6 43 44 00 60. E-mail: scompant@ensat.fr

Molecular Plant Microbe Interaction, submitted

Abstract

In this study the desert soil bacterium *Saccharothrix algeriensis* NRRL B-24137 was evaluated for its abilities to colonize *Arabidopsis* seedlings both in the rhizo- and endosphere of plants, to protect *Arabidopsis* towards *Botrytis cinerea* as well as parts of the mechanisms involved. The results showed that strain NRRL B-24137 can colonize the surfaces of roots as well as the endorhiza, but cannot systemically spread inside the plant at early step of colonization. Strain NRRL B-24137 allows also reduction of leaves symptoms caused by *B. cinerea* on *A. thaliana* Columbia plants although the bacterium can not be endophytic inside leaves. Different plant mutants were further screened to evaluate what can be parts of the mechanisms of protection. Especially, known mutants of genes involved in ISR and/or SAR such as *eds4-1, eds5-1, eds8, eds9-1, ein2-1, ein4, ein5-1, jar1-1, aos, coi1-16, NahG, npr1-1, npr1-3, pad1, pad3-1,* and *pad4-1* were assessed. Results show that NRRL B-24137 induces known mechanisms of ISR that are ethylene and jasmonate dependents. Other mutants were additionally screened for positive or lost ISR induced by NRRL B-24137. Data demonstrated that ISR towards *B. cinerea* and induced by NRRL B-24137 requires also functionality of NADPH oxidases, and of UPS1.

Keywords: *Saccharothrix algeriensis* NRRL B-24137, desert soil, endophyte, *Arabidopsis*, mechanisms, ISR

Introduction

Plant growth-promoting bacteria are known as helping their hosts by increasing plant growth *via* conferring nutriments, phytohormones as well as by reducting phytopathogenic infections through direct biocontrol activities and induction of systemic resistance (Bakker et al. 2007; Lugtenberg and Kamilova 2009; Zamioudis and Pieterse 2012). The majority of these bacterial microsymbionts are epiphytics and colonize the rhizosphere (Compant et al. 2005; Lugtenberg and Kamilova 2009). A subset of the rhizosphere microflora can however also enter inside plants and proliferate within as endophytes (Rosenblueth and Martínez-Romero 2006; Hallmann and Berg 2007; Reinhold-Hurek and Hurek 2011). Nowadays, a current gap is however still present regarding if some specific endophytes can induce

systemic resistance towards phytopathogens, as well as what are the mechanisms involved. It is also unknown if some specific soil bacteria responsible of biocontrol activities can be endophytic following early step of colonization (Compant et al. 2005; 2010; Reinhold-Hurek and Hurek 2011). Such interactions have been demonstrated for some bacterial taxa but for some others information rests indeed still unknown. It is however of special interest to examine this kind of interactions, to increase knowledge of how a beneficial bacterium can interact with its host as well as protect it to colonizers responsible of infection.

Almost beneficial rhizo- and/or endophytic bacteria can induce phenomenons of resistance alleviating pathogenic infections that can be correlated to a form of ISR (Induced Systemic Resistance). However in some case SAR (Systemic Acquired Resistance) mechanisms have been in contrary demonstrated for some bacterial strains. Although some authors described the two kinds of resistance SAR and ISR as synonyms, recent researches have shown that they are still different (van Loon 2006). Extensive researches have demonstrated for instance that salicylic acid (SA) plays a key role in local and SAR to pathogenic agents (Durrant and Dong 2004; Gaffney et al. 1993), and that SAR is associated with the expression of the so-called SAR genes (Ward et al. 1991), such as pathogenesis-related (PR) genes like PR-1 and PR-5 (Linthorst 1991). On the contrary in *Arabidopsis* it has been demonstrated that ISR is independent of SA accumulation (Gaffney et al. 1993; Pieterse et al. 1996; Pieterse and Van Loon 1999) and beneficial microbes-mediated ISR is controlled by signaling pathway in which ethylene (ET) and jasmonate (JA) play a key role (Van Loon et al. 1998; van Wees et al. 2008; van der Ent et al. 2009). This was demonstrated for instance with various ET and JA signaling mutants (*ein2, ein4,* and *ein5-1* for ET dependant and *jar1, aos* and *coi1-16* for JA pathway; van der Ent et al. 2009). Such mutants lost their ISR induced by beneficial microbes whereas mutants *NahG* expressing salicylate hydroxylase do not lose the phenomenon of ISR (Pieterse et al. 2002). Various gene products have been additionally correlated to ET/JA and/or SA signaling such as some enhanced disease susceptibility (*eds*) genes compounds that can be required for ISR (*eds4* and *eds8*, respectively ET and JA signaling dependant), whereas others are related to pathogenic interaction involved in SA signaling such as *eds5* (Ton et al. 2002). Node of convergence NPR1 (NON EXPRESSOR OF PR PROTEINS) was also demonstrated as associated with ISR but also with SAR (Pieterse and van Loon 2004). Moreover some phytoalexins were additionally demonstrated as required for ISR and/or SAR and are JA dependant (PAD1) whereas others were demonstrated as SA signaling dependant (PAD3 and PAD4; Glazebrook et al. 2003; Zhou et al. 1998). Therefore some common pathways were additionally described for ISR and SAR.

However it is still unknown if all the non pathogenic bacteria can trigger the same kind of resistance, if the resistance requires SA, JA/ET signaling, NPR1, *eds* gene products and phytoalexins.

Additionally, some early events were described for SAR mechanisms whereas in ISR it is still unknown if they can be involved. Some of them are related for instance to the production and signaling of radical oxygen species (ROS) as demonstrated under pathogenic infections but it still unknown if they can be involved in ISR as for instance the NADPH oxidase RBOHD (respiratory burst oxidase homolog D) and RBOHF (respiratory burst oxidase homolog F; Torres and Dangl 2005). Interestingly recently UPS1 (UNDERINDUCER AFTER PATHOGEN AND STRESS1) was demonstrated as involved in stress signalling following plant perception of pathogen or abiotic stresses (Denby et al. 2005). Mutants Camalexin *ups1* have reduced expression of phosphoribosylanthranilate transferase, a tryptophan biosynthetic enzyme. This mutant can be defective in a wide range of defence responses due to SA and JA/ET pathways signalisation reduced, and has reduced oxygen species (ROS)-mediated gene expression also compromised (Denby et al. 2005). However it is unknown if UPS1 can be required for resistance induced by beneficial microbes and needed to be studied if involved in resistance induced by a beneficial microbe.

In this study, a bacterial strain from desert soil was evaluated for its abilities to colonize *Arabidopsis* plants before to study if the strain can induce ISR or SAR resistance towards one phytopathogen, *Botrytis cinerea.* Different mutants of genes required for ISR and/or SAR were screened and additional mutants with a possibility to be involved in resistance by beneficial microbes were also used. This strain is *Saccharothrix algeriensis* NRRL B-24137, an actinobacterial strain that was isolated from desert soil (Zitouni et al. 2004) but for which there is a current growing interest for biotechnology and agriculture and that may allow to better understand plant/rhizo-endophyte interaction (Compant et al. submitted) and mechanisms of resistance towards phytopathogens.

Results

The soil bacterium *Saccharothrix algeriensis* NRRL B-24137 can be rhizospheric and endophytic in roots of *Arabidopsis thaliana* plants but absent of leaf infection site of the necrotroph *Botrytis cinerea*

Colonization by strain NRRL B-24137 was evaluated by fluorescence *in situ* hybridization (FISH) analysis by using different probes (Table 1). However before to study

colonization by microscopy, colonization study of *Arabidopsis thaliana* with strain NRRL B-24137 was firstly studied by plate counting. Following root inoculation strain NRRL B-24137 was detected in the rhizosphere at the beginning of the experiment (Fig. 1a), as well as inside endorhiza from 2-3 days (Fig. 1b) up to end of the experiment, i.e. 10 days post inoculation. However and although it was detected inside roots, strain NRRL B-24137 was not detected as endophyte in leaves from the beginning to the end of the experiment (Fig. 1b).

To determine where can be the niches of colonization of strain NRRL B-24137 on and inside *Arabidopsis* seedlings and to further prove rhizo- and endophytism by strain NRRL B-24137, FISH method coupled with microscopy was then used. No structure similar to the strain was detected by fluorescent microscopy when FISH was not used. Microscopic analyzis of FISH experiment allowed however to show that strain NRRL B-24137 10 dpi has colonized the rhizoplane in a mycelial form state, especially at root hairs level (Fig. 1c-d) following root inoculation. The bacterial strain was also visualized in the elongation zone (Fig. 1e-f) as well as at emergence site of secondary roots (Fig. 1g-h). Inside endorhiza (Fig. 1i), strain NRRL B-24137 was visualized between or inside rhizodermal cells as a filamentous form (Fig. 1j-k) as well as inter- or intracellularly in the cortex region (Fig. 1l-m) where a parietal apposition was additionally also reported (Fig. 1m). In root xylem vessels, the bacterium was not visualized in the lumen but was found near a xylem element although not inside it (Fig. 1n). In contrary to root, microscopic analysis showed however that inside leaves strain NRRL B-24137 was not visualized (data not shown) as demonstrated by plate counting.

Experiments of colonization were not only done with *Arabidopsis* seedlings and NRRL B-24137 but also when leaves were challenged with *B. cinerea* strain BC1. As for non inoculated pathogenic agent, the bacterium was detected in the rhizosphere as well as inside the endorhiza but not inside leaves of *Arabidopsis* seedlings as demonstrated 3 days after pathogenic infection (Fig. 2).

***Saccharothrix algeriensis* NRRL B-24137 protects *Arabidopsis thaliana* against *Botrytis cinerea* infection**

Following the study of colonization of strain NRRL B-24137 on and inside *Arabidopsis* seedlings in presence or not of *B. cinerea*, the ability of the actinobacterial strain was evaluated for its potential to protect the plants towards the necrotrophic agent *Botrytis cinerea* BC1. The results showed that *Arabidopsis* plant roots inoculated with *Sa. algeriensis* NRRL B-24137 have less leaves with infection or surfaces of necrosis due to *Botrytis cinerea*

BC1 than control plants. Indeed *B. cinerea* infection on WT Col was of 68.18 +/- 13.97 for % of leaves with infection and of 1.12 +/- .0.24 mm^2 of surfaces of necrosis for control plants whereas 30.71 +/-14.47% and 0.33 +/- 0.11 mm2 were recorded for plants with root inoculated with NRRL B-24137 (different with $P<0.05$; Fig. 3a-c).

Protection induced by *Saccharothrix algeriensis* NRRL B-24137 requires EDS4, EDS8, but not EDS5 and EDS9

In order to know more about the mechanisms that can be involved in the systemic resistance induced by NRRL B-24137, different plant mutants were then screened. Among them some "enhanced disease resistance" mutants were firstly evaluated. Data showed that plants mutants "enhanced disease resistance" *eds4-1* lose their resistance towards *B. cinerea* when they have been inoculated with NRRL B-24137 in comparison to wild type plants as well as mutants plants non inoculated with the beneficial strain. This was revealed both with % of leaves of infection with 60,67 +/- 9.45 for *eds4-1* control and 73,33 +/- 12.22 for *eds4-1* inoculated with NRRL B-24137 (not different $p>0.05$) and surfaces of necrosis (in mm^2) of respectively 1.57 +/- 0.25 and 1.85 +/- 0.26 for *eds4-1* control and *eds4-1* NRRLB-24137 inoculated (not different $p>0.05$; Fig. 4a-b).

As for *eds4-1*, *eds8* plant mutants also lose the ISR phenomenon. The results showed 65.11 +/- 10.25 % of leaves with infection and 1.22+/- 0.2 mm^2 of surfaces of necrosis for *esd8* control plants whereas 56.18+/- 7.14 % and 1.43+/-0.25 mm^2 was recorded with *eds8* treated with NRRL B-24137 (not different $p>0.05$; Fig. 4a-b). However for plant mutant *eds5*, plants did not lose the ISR phenomenon (Figure 4a-b) as % of leaves with infection and surfaces of necrosis were not similar for *eds5* non inoculated, and *eds5* mutants plants inoculated with NRRL B-24137 (Fig. 4a-b). Eds9 mutants also did not lose the ISR phenomenon as demonstrated with percentages of leaves with infection as well as surfaces of necrosis (Fig. 4a-b).

Protection induced by *Saccharothrix algeriensis* NRRL B-24137 requires EIN2, EIN4, and EIN5

Plant mutants involved in ethylene signaling were evaluated to determine if genes related to ethylene signalling were required for the resistance induced by NRRL B-24137 allowing reduction of *B. cinerea* infection. The results showed that the plant mutants *ein2, ein4,* and *ein5-1* lost their resistance towards *Botrytis cinerea* when they have been inoculated

with NRRL B-24137 in comparison to wild type plants and mutants plants non inoculated with the beneficial strain (Fig. 4c-d).

Protection induced by *Saccharothrix algeriensis* NRRL B-24137 requires JAR1, AOS, and COI1

To continue to describe putative mechanisms involved in resistance induced by NRRL B-24137 mutants *jar1-1* and *aos (allene oxide synthase)* were used. Results showed that these mutants completely lose the phenomenon of resistance towards *B. cinerea* (Fig. 4e-f).

In the case of mutants normally used to describe SAR mechanisms *via* SA signalling, i.e. mutants *NahG* expressing salicylate hydroxylase, inoculation with NRRL B-24137 of these mutants did not however result in complete lost of protection towards *B. cinerea* in comparison to non inoculated control plants (Fig. 4g-h).

Other mutants were also used in this study. Especially the jasmonate receptor mutant *Coi1-16* was screened. The results showed that the ISR phenomenon induced by NRRL B-24137 towards *B. cinerea* is lost when this mutant was tested (Fig. 5a-b).

Protection induced by *Saccharothrix algeriensis* NRRL B-24137 is NPR1 dependent

Mutants *npr1* normally losing the resistance in both SAR and ISR phenomenons were also used in this study. The results showed that mutants *npr1-1* and *npr1*-3 inoculated with NRRL B-24137 completely lose the phenomenon of resistance towards *B. cinerea* (Fig. 5c-d).

Protection induced by *Saccharothrix algeriensis* NRRL B-24137 requires PAD1 but not PAD3 nor PAD4

Phytoalexin mutants were additionally screened to determine if the gene products can be required for ISR induced by NRRL B-24137. The results showed that mutants *pad1* (JA-dependant) lost the resistance towards *B. cinerea* normally induced by NRRL B-24137 (Fig. 5e-f). However *pad3-1* (SA-dependant) and *pad4-1* (SA-dependant) do not lost the resistance towards *B. cinerea* (Fig. 5e-f).

UPS1 is required for the ISR induced by *Saccharothrix algeriensis* NRRL B-24137

In the case of Camalexin *ups1* mutant, defective in a wide range of defence responses due to SA and JA/ET signalization reduced and ROS-mediated gene expression

compromised, the results showed that this mutant lost the ISR induced by NRRL B-24137 towards *B. cinerea* (Fig. 6a-b).

AtrbohD, AtrobohF are required for the ISR induced by *Saccharothrix algeriensis* NRRL B-24137

To continue to describe putative mechanisms involved in the resistance, we evaluated implication of *AtrbohD* and *F*, involved *via* respiratory burst oxidase in ROS signaling, in our model. The results showed that ISR is lost in the mutants as well as in double mutants *AtrbohDF* (Fig. 6c-d).

Discussion

In this study part of interaction between *Arabidopsis thaliana* plants and a desert soil bacterium was investigated both in terms of colonization and resistance towards the necrotrophic agent *B. cinerea*. We firstly showed that the actinobacterial strain *Saccharothrix algeriensis* NRRL B-24137 can colonize the root surfaces of *Arabidopsis* seedlings as well as establish endophytic subpopulations inside roots of the plants. Several studies have shown the root surface colonization as well as endophytism of some bacterial strains on and inside host plants (Compant et al. 2005; 2010; Roseblueth and Martinez-Romero 2006). However some of these bacteria can colonize systemic plant parts whereas others are only restricted to the root systems (Compant et al. 2005; 2010; Rosenblueth and Martinez-Romero 2006). In this study strain NRRL B-24137 is shown to be restricted at time of experiment to the root level. However although strain NRRL B-24137 can not be present in aerial plant parts during the time of experiments (Fig. 7a), a systemic colonization later on can not be excluded (although this needs to be demonstrated). In fact a short experiment was used to demonstrate colonization by a soil–derivated bacterium before to study a systemic resistance towards *B. cinerea*. To study resistance, the beneficial bacterium needs indeed to be absent of site of infection of the pythopathogen and the beneficial bacterium need to be spatial separated of the site of the phytopathogenic agent infection (van Loon et al. 1998). As the beneficial strain NRRL B-24137 was absent of leaves, this has allowed to determine then its potential to alleviate *B. cinerea* infection.

In this study it is demonstrated that strain NRRL B-24137 confers leaves protection to *B. cinerea*. Several bacteria have been demonstrated as inducers of systemic resistance towards phytopathogens and the model of *Arabidopsis* have been extensively studied (van

Loon and Bakker 2004, 2005). This has been demonstrated with strains of *Pseudomonas* spp., *Bacillus* spp. (Pieterse et al. 1996; Hammerschmidt 1999; Ryu et al. 2004) as well as many others including also actinobacteria (Conn et al. 2008). However it has been never described for an actinomycetaceae member such as for the *Saccharothrix* genus. This bacterium was isolated from desert soil and results showing protection towards *B. cinerea* suggest that a reservoir of new inducers of resistance can be found in extreme environments such as in desert soil.

In this study we further evaluate if an ISR or SAR protection occurred when NRRL B-24137 was inoculated on roots of *Arabidopsis*. This study has evaluated that some genes products related to ET sensitivity, JA signaling are required to the systemic resistance induced by the beneficial endophyte. On the contrary SA signaling is not required as demonstrated with the *NahG* mutant used. All the mutants used in this study have been demonstrated as losing their resistance towards phytopathogens once inoculated with a beneficial bacterium (Kloepper et al. 2004; Pieterse et al. 1996, 1998). With our phytosystem we demonstrate that the resistance corresponds to an ISR not a SAR as described before with almost rhizosphere and endophytic bacteria. However as others beneficial bacteria can trigger SAR mechanisms, this needed to be evaluated with *Sa. algeriensis* NRRL B-24137.

Other mutants of genes that have been demonstrated as required for ISR in the model of Pieterse et al. (2002) such as *eds4* and *eds8* were additionally used in this study and results have shown that the gene products EDS4 and EDS8 but not EDS5 are additionally correlated to the resistance induced by strain NRRL B-24137 towards *B. cinerea*. EDS4 is known as correlated to ET, EDS8 to JA whereas EDS5 is correlated to SA signaling (Ton et al. 2002)

In this study we further evaluate if some gene products not previously reported as involved in ISR can be responsible of the resistance observed towards *B. cinerea*. Especially mutant *ups1* were used. UPS1 has been demonstrated as involved in resistance induced by abiotic stresses (Ferrari et al. 2007) as well as by phytopathogens (Denby et al. 2005) but never by beneficial microbes. In this study we demonstrated that UPS1 integrity is required for the systemic resistance induced by NRRL B-24137, adding therefore to previous models new components of ISR (Fig. 7b). *ups1* appears to encode a regulatory protein required for the expression of different defence genes activated by reactive oxygen species (Denby et al. 2005). As UPS1 has been suggested to act downstream of ROS signaling, we also evaluate if respiratory burst oxidases RBOHD and F involved in ROS signaling can be involved. Results have showed that RBOHD and F are required for the ISR induced by strain NRRL B-24137

towards *B. cinerea* adding also components to our model of ISR induced by NRRL B-24137 (Fig. 7b).

In this study the colonization behaviour of the desert soil bacterium *Saccharothrix algeriensis* NRRL B-24137 on *Arabidopsis* seedlings as well as its abilities to protect plants towards *B. cinerea* and the mechanisms involved were partly characterized. This study shows therefore that even not common bacteria can be used to determine colonization on and inside plants, ISR as well as the mechanisms involved and to find new mechanisms involved in ISR (Fig. 7). However further works are needed to better understand all the interaction formed. Strain NRRL B-24137 secretes a large variety of secondary metabolites having antifungal properties (Lamari et al. 2002). It is possible that these metabolites may also act as molecular determinants of ISR although this needs to be determined.

Materials and methods

Bacterial culture

Sa. algeriensis NRRL B-24137 was used throughout this work. This strain was grown at 30°C on ISP 2 (International *Streptomyces* Project 2) solid medium (pH 7.0) containing per liter of distilled water: 4 g D(+) glucose (Acros organics), 10 g malt extract (Fluka), 4 g yeast extract (Fluka) and 18 g agar (Sigma). 8 days after growing on plate, aerial mycelium of strain NRRL B-24137 was harvested in PBS and concentration was adjusted to 5 x 10^7 CFU.ml^{-1}.

Fungal culture

Botrytis cinerea strain BC1 (isolated by S. Compant from grapevine plants in 2008 in Austria) was grown on PDA (Potato Dextrose Agar) medium at 25°C under light conditions. Spores of *B. cinerea* were harvested from 9 day old culture and adjusted then with a Thoma cell at 6.5 x 10^5 conidia.mL^{-1} in a half PDB solution.

Plant material

Seeds of *Arabidopsis thaliana* accession Columbia (Col, and Col0) and almost all mutants: (*eds4-1, eds5-1, eds8, eds9-1, ein2-1, ein4, ein5-1, jar1-1, col-6(gl-1), aos, npr1-1, npr1-3, pad1, pad3-1, pad4-1, AtrbohD-3, AtrbohF-3, AtrbohDF,* and *ups1-1*) were obtained from the Nottingham *Arabidopsis* stock center. Other mutants *NahG*, and *coi1-16* were kindly provided by Dr. Günter Brader from Austrian Institute of Technology (Tulln, Austria).

Seeds of *Arabidopsis* were surface sterilized with 70% ethanol (5 min), 2.6% NaClO (2 min) before to be rinsed 3 times with sterilized distilled water. Seeds were then placed on plates containing Murashig and Skoog medium (Sigma, France) amended with 2% saccharose and 0.8% agar (pH 5.7). Seedlings were then allowed to grown *in vitro* for 16 days in a growth phytotronic chamber (16h photoperiod, 20-25^{o}C night-day, and 70 % relative humidity) by placing vertically plates.

Induction treatment

Following growth of seedlings, seedlings (aged of 16 days) corresponding to WT or mutants were taken from *in vitro* plates, rinced with sterilized distilled water and roots were dipped in a suspension of NRRL B-24137 or with PBS (control) for 3 min. Then seedlings were planted in plates perforated at their bottom and amended with sterilized soil (1/3 perlite, 1/3 sand, 1/3 potting soil). Seedlings were allowed to grow horizontally for 10 days in a box covered with transparent perforated film. Sterilized tap water was added daily with a syringe.

Bacterial populations on and inside seedlings

At 0, 1, 3, 5, 7 and 10 days post NRRL B-inoculation, roots and leaves of WT were taken for evaluating bacterial populations on and inside plant tissues of WT (Col) seedlings. Similar experiments were done 72 hpi of *B. cinerea*. For root populations, roots were washed with distilled water and then 100 mg of samples were used. For endophytism, root and leaves were washed as described before and then surface sterilized with ethanol 70% during 5 min, bleach 2.5 % during 1 min, and rinsed 3 times with distilled water.

100mg of each plant tissues were ground with a mostar and pestle before to be placed in 1 mL of distilled water. Then the solution was tenfold diluted in PBS and 100µL of dilutions were plated on plate amended with cycloheximide (30 mg/mL). Plates were then allowed to grow for one week before to determine populations related to samples.

FISH microscopy

Plant tissues from seedlings WT (Col) 10 days post inoculation with strain NRRL B-24137 were fixed overnight at 4°C in a paraformaldehyde solution (4% in PBS), before to be rinsed twice with PBS. Samples were then dehydrated in ethanol serie (50 to 99.9%; 30 min each step) before to be embedded in LR white resin. Embedded tissues were then sliced with a microtome and slices of 1-1.5 µm were deposited on microscopic slides. Fluorescence *in*

situ hybridization was carried out according to Compant *et al*. (2011) by using mixture of EUB338, EUB338II, EUB338III coupled with dylight 488 and HGC probes coupled with dylight 533. Slices without FISH experiment were used as control. Slides were observed under an epifluorescence microscope (BH2, Olympus, Japan) and pictures were taken with a camera.

Challenge Inoculation

Five leaves of each *A. thaliana* seedling (WT or mutants) inoculated or not with NRRL B-24137 (10 days post inoculation) were infected with a solution of 9 days old *Botrytis cinerea* by inoculation of 3 µl of 6.5 x 10^5 conidia.mL^{-1}. To ensure infection, inoculated plants were then kept at 100% relative humidity during all the infection process in celled boxes with transparent film under phytotronic chamber conditions as described before.

Disease assessment

Three days after challenge, infection caused by *B. cinerea* was evaluated: i) by the percentage of necrotic leaves with symptoms per plant and ii) by the surface area of necrosis per leaf with the help of image J software.

Statistical analyses

All experiments have been repeated three independent times with similar results on each time 10 plants. Statistical analyses were performed by using student t test (http://studentsttest.com/).

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Table 1: Probes used for fluorescence *in situ* hybridization

Probe names	Sequences 5'- 3'	References
EUBI	GCT GCC TCC CGT AGG AGT	Amman et al., 1990
EUBII	GCA GCC ACC CGT AGG TGT	Daims et al., 1999
EUBIII	GCT GCC ACC CGT AGG TGT	Daims et al., 1999
HGC69a	TAT AGT TAC CAC CGC CGT	Roller et al., 1994

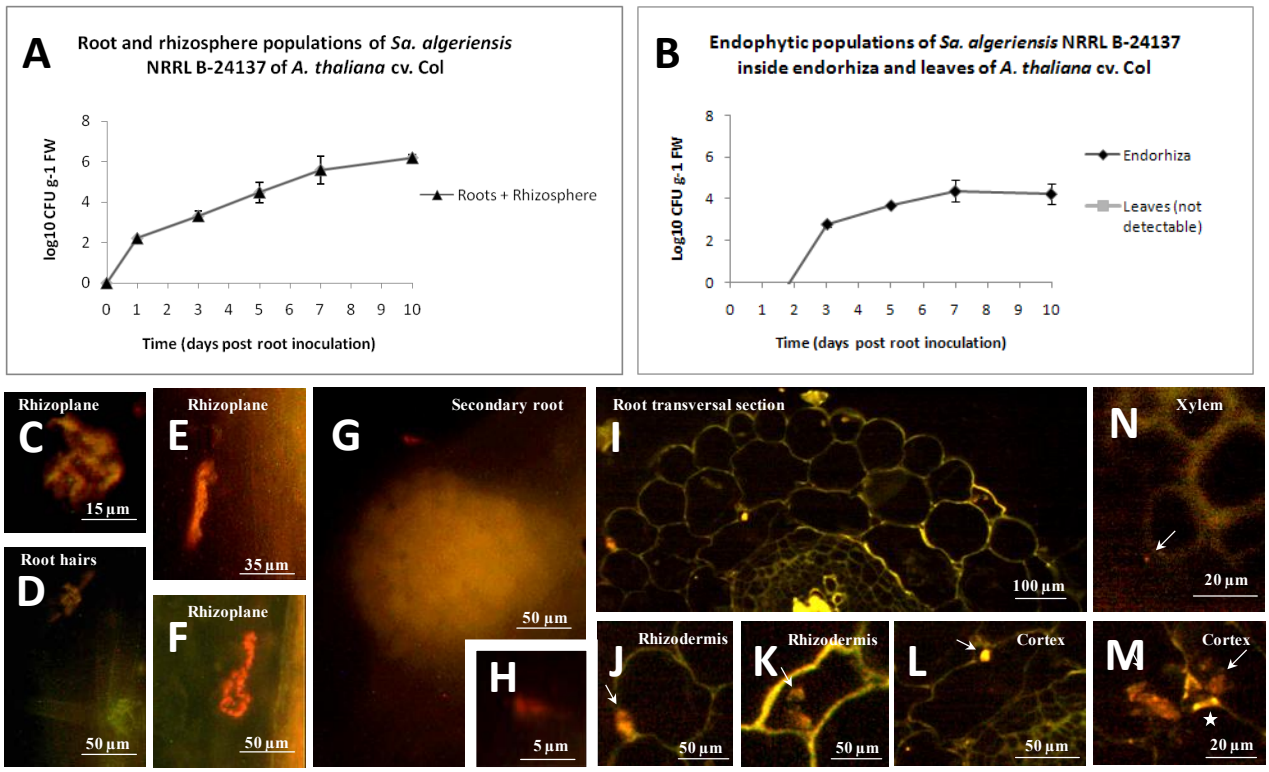

Figure 1: Rhizosphere and endophytic colonization of *Arabidopsis thaliana* cv. Columbia by *Saccharothrix algeriensis* NRRL B-24137 analyzed by CFU and by FISH microscopy showing kinetic of colonization as well as different forms of the strain (sporulated, mycelial and filamentous)

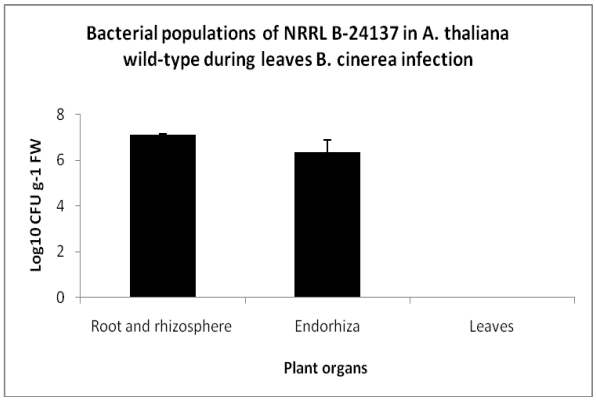

Figure 2: Root, rhizosphere, endorhiza and leaf populations of *Saccharothrix algeriensis* NRRL B-24137 in *Arabidopsis thaliana* cv. Columbia wild-type plants during *Botrytis cinerea* infection (72 hpi).

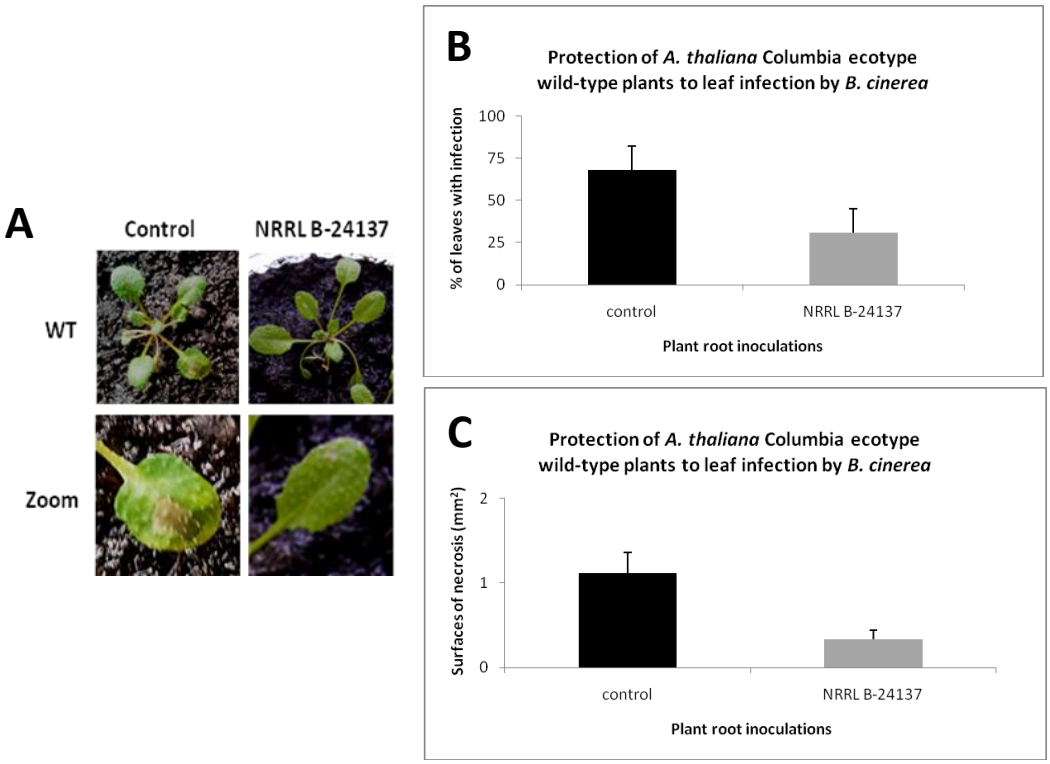

Figure 3: Protection of *Arabidopsis thaliana* cv. Columbia against *Botrytis cinerea* by *Saccharothrix algeriensis* NRRL B-24137

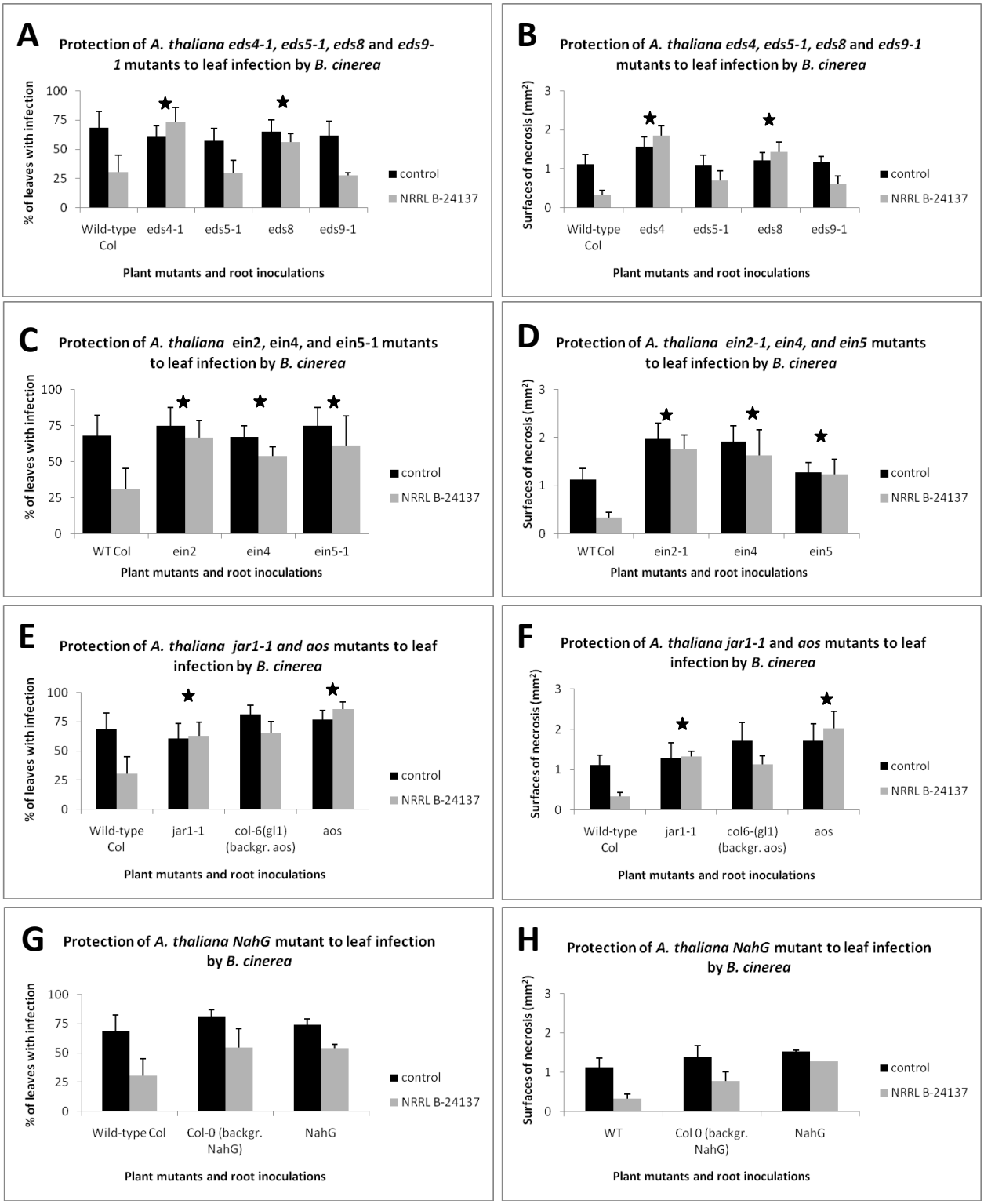

Figure 4: Analysis of *Arabidopsis thaliana* cv. Columbia mutants protection to *Botrytis cinerea* following inoculation of *Saccharothrix algeriensis* NRRL B-24137. * indicates not different (P> 0.05). Wild type Col: Col parental line used by L.Comai, I. Henry and S. Somerville; Col-0: parental line fron NASC.

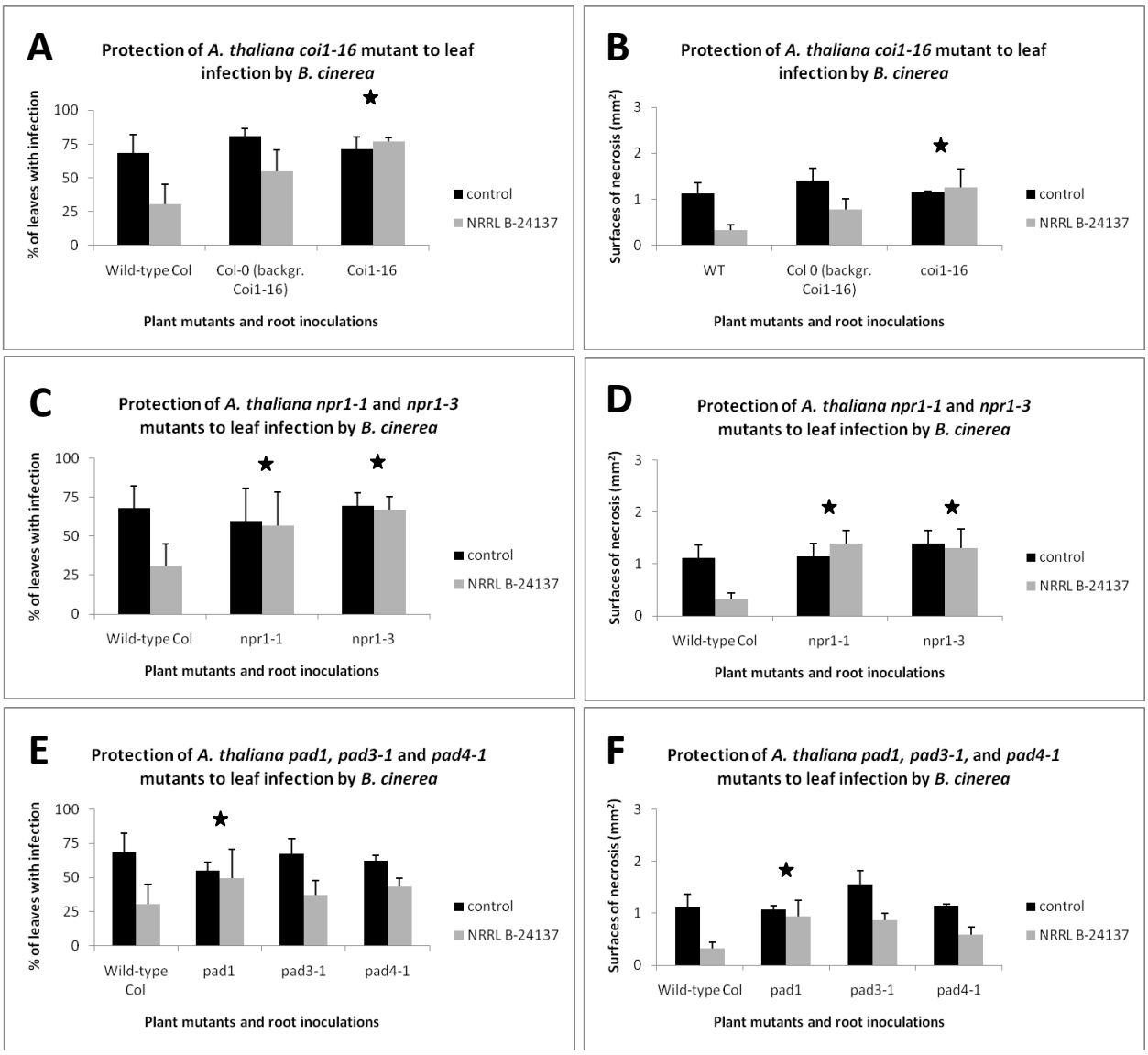

Figure 5: Analysis of *Arabidopsis thaliana* cv. Columbia mutants protection to *Botrytis cinerea* following inoculation of *Saccharothrix algeriensis* NRRL B-24137. * indicates not different (P> 0.05). Wild type Col: Col parental line used by L.Comai, I. Henry and S. Somerville; Col-0: parental line fron NASC.

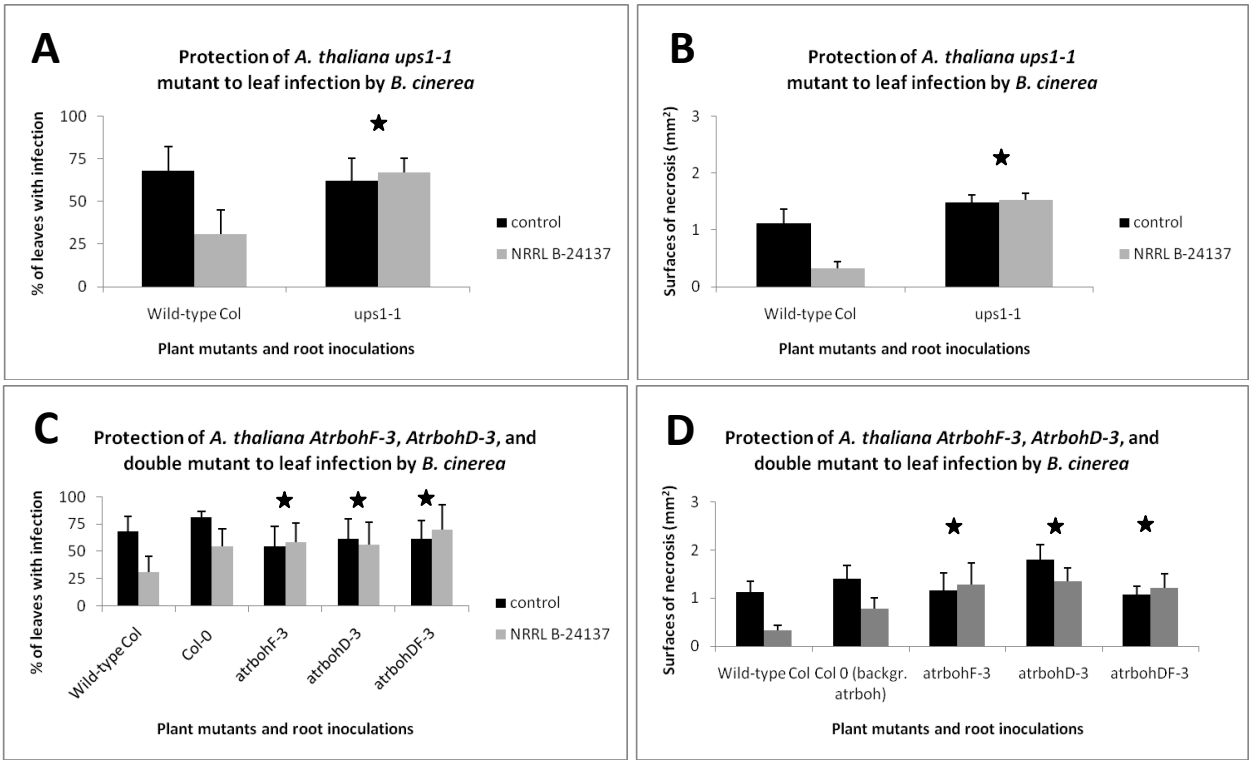

Figure 6: Analysis of some others *Arabidopsis thaliana* cv. Columbia mutants protection to *Botrytis cinerea* following inoculation of *Saccharothrix algeriensis* NRRL B-24137. * indicates not different (P> 0.05). Wild type Col: Col parental line used by L.Comai, I. Henry and S. Somerville; Col-0: parental line fron NASC.

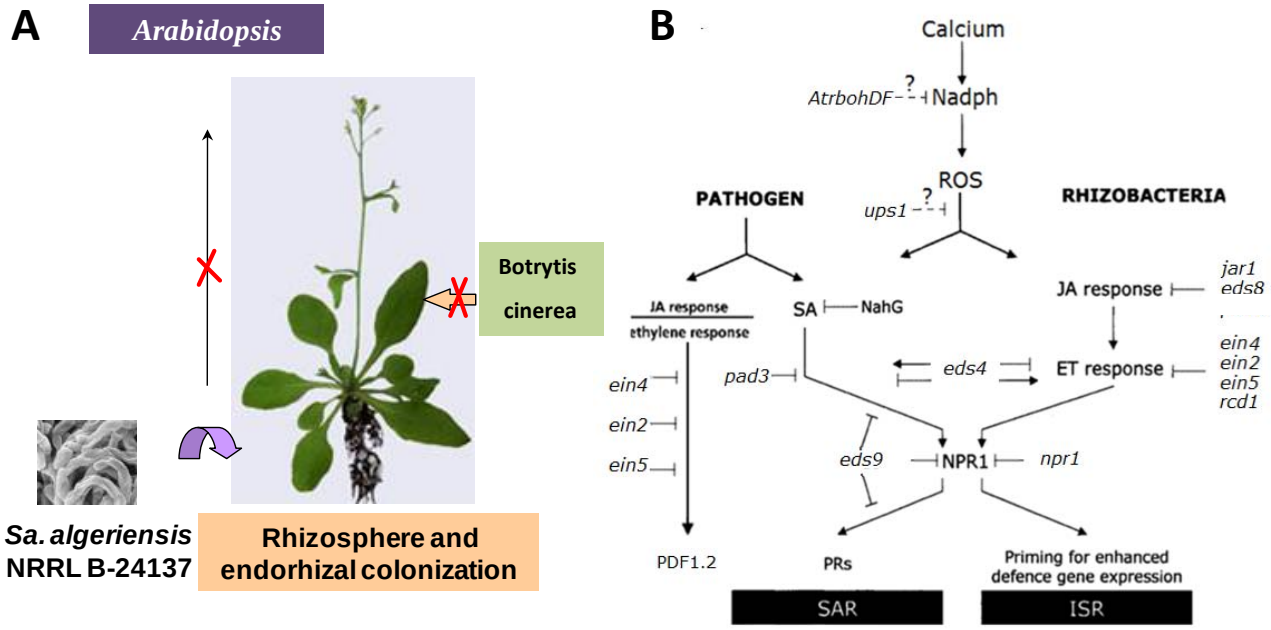

Figure 7: Schematic representation of colonization of *Arabidopsis thaliana* by *Saccharothrix algeriensis* NRRL B-24137 (a) and parts of mechanisms involved in induced systemic resistance towards *Botrytis cinerea* modified from Pieterse et al. 2002 (b).

In this chapter, the study clearly demonstrated that *Sa. algeriensis* NRRL B-24137 can be rhizospheric and endophytic (inside the endorhiza) of *Arabidopsis thaliana* plants and not systemic at the time of the experiment (to study then ISR or SAR phenomenons). Inoculation of the beneficial strain allows also inducing an ISR, not a SAR, allowing to reduce leaves infection caused by *B. cinerea*. Parts of mechanisms have been described and we also suggested new components of the ISR network induced by beneficial microbes.

Chapter VI

Conclusions and Future Prospects

In this thesis, different aspects of the use of *Sa. algeriensis* NRRL B-24137 against B. *cinerea* were studied.

Biocontrol properties towards *B. cinerea* and secondary metabolites secreted by *Sa. algeriensis* NRRL B-24137

Firstly biocontrol of *B. cinerea* by using *Sa. algeriensis* NRRL B-24137 was monitored. The results have shown that the strain can inhibit directly the growth of the fungus on dual culture plates (Figure 12). In this *Sa. algeriensis* and *Botrytis cinerea* interaction, the development of the *Botrytis cinerea* BC1 was indeed prevented around the *Sa. algeriensis* strain. The region around the *Sa. algeriensis* was found to be red/orange colored showing the secretion of some bioactive antifungal and pigmented molecule *by Sa. algeriensis*. After this, thin layer chromatography (TLC) was performed that allowed us to obtain a red color band having strong antifungal properties. The active molecule was detected by bio-autography with the *B. cinerea* BC1 and it was observed that a red-pigment like antibiotic has antifungal activity against *B. cinerea* BC1. HPLC analysis performed allowed us to obtain the metabolite profile. Previously, *Saccharothrix algeriensis* NRRL B-24137 was known to produce bioactive compounds belonging to the dithiolopyrrolone class of antibiotics (Lamari *et al.,* 2002a, b; Zitouni *et al.,* 2004). But the metabolite secreted in our work does not correspond to the dithiolopyrrolones as compared with thiolutin (Retention time = 12.3 min) as shown by Chorin (2009). Chemical characterization of secondary metabolites secreted by NRRL B-24137 and involved in biocontrol *ytis cinerea* is under progress. The bacterial metabolites are currently unde zation by NMR spectroscopic and mass spectrometric investigations.

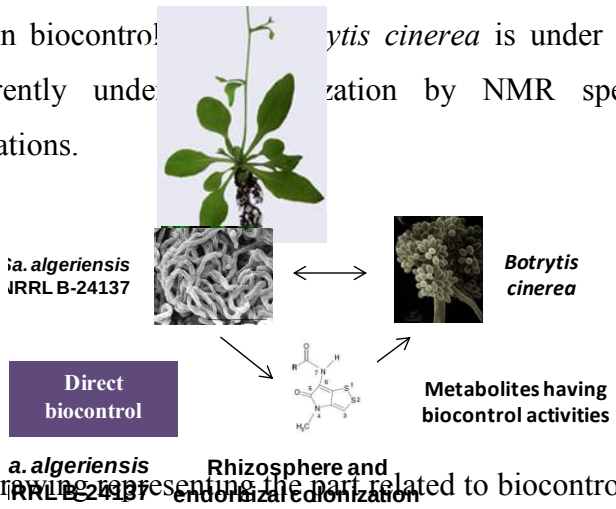

Figure 12: Drawing representing the part related to biocontrol activities of NRRL B-24137 towards *B. cinerea* BC1.

Colonization of grapevine by *Saccharothrix algeriensis* NRRL B-24137

The study of interactions between endophytic PGPR and their host plants is currently a major issue. In this thesis, one of these interactions has been studied between the model plant *Arabidopsis thaliana* and *Saccharothrix algeriensis* NRRL B-24137. Meanwhile, interaction between *Vitis vinifera* L. and *Saccharothrix algeriensis* NRRL B-24137 has also been studied. Some parts of this interaction has been characterized, especially colonization of the plant by the actinomycete and induction of ISR leading to some level of protection against *Botrytis cinerea* BC1 (Figure 13).

Colonization of the grapevine plants by *Sa. algeriensis* NRRL B-24137 was firstly characterized at the rhizosphere level. The colonization process was analyzed with the help of DOPE-FISH technique by creating specific probe for strain NRRL B-24137. Before this study, colonization process for actinomycetes have been described (Coombs and Franco, 2003; Merzaeva and Shirokikh, 2006) but DOPE-FISH technique has been used for the first time for *Saccharothrix* and in general for plant-bacteria interaction. Following plant root inoculation with NRRL B-24137, it has been observed that the strain colonizes the root hair zone, root elongation zone as well as root emergence site. This type of colonization has been already described for *Burkholderia* sp. strain PsJN in grapevine rhizoplane (Compant *et al.,* 2005b). It was found that the strain could form mycelium and spore forms during colonization of the root surfaces, which was not so surprising to observe because the strain used was an actinomycete that form spores as well as mycelium (Zitouni *et al.,* 2004).

The strain was visualized inside endorhiza of grapevine plant roots. So it was clear that bacteria crossed the rhizoplane. Some bacteria were also found intercellularly between cortical cells. So from our study, it became clear that strain NRRL B-24137 can be endophytic in grapevine roots crossing from the rhizodermis to several cortical cell layers. However, we found colonization of *Sa. algeriensis* NRRL B-24137 just up to several cortical cell layers but not in the vascular system. So it was suggested that NRRL B-24137 have been restricted to root internal parts. However, the experiment needs to be performed for more than 10 days post inoculation to confirm this.

Protection and mechanism involved in grapevine with *Sa. algeriensis* NRRL B-24137 towards *B. cinerea* BC1

In this thesis, the induction of defenses by NRRL B-24137 was studied on leaves following root inoculation with the beneficial strain. This allowed us to characterize systemic response induced in plants following bacterial inoculation (Figure 13).

The establishment of induced systemic resistance requires about 10 days after inoculation of a microorganism (Van Loon *et al.,* 1998). As for ISR, the beneficial organism must be absent from the place of infection of the pathogen (Van Loon *et al.,* 1998). We already demonstrated before to study a systemic resistance towards *B. cinerea* BC1 that NRRL B-24137 was not detected in systemic plant parts. As the beneficial strain NRRL B-24137 was absent in leaves, this has allowed to determine then its potential to alleviate *B. cinerea* infection.

After root inoculation at 10dpi, leaves of grapevine were infected with *Botrytis cinerea* BC1. We obtained the results that plants treated with *Sa. algeriensis* NRRL B-24137 at the root level have less percentages of infected leaves as compared to control (non-treated) plants. The possibility of direct antagonism between *Botrytis cinerea* BC1 and *Sa. algeriensis* NRRL B-24137 is excluded because of absence of bacteria in leaves. So, it was assumed that defense responses induced by beneficial bacterium *Sa. algeriensis* in the grapevine plant towards the necrotrophic agent *B. cinerea* was correlated to a systemic resistance.

We also studied the defense genes expression to determine priming mechanisms. But the results obtained showed that genes *VvGlu1*, *VvChit3* and *VvPGIP* were not primed. These genes are found to be expressed during *B. cinerea* infection (Aziz *et al.,* 2004) and could explain a reduction of leaves symptoms due to *Botrytis cinerea* BC1. However this part of the thesis should be improved and further studied. This was only preliminary results and experiments should be again performed to be sure of the results.

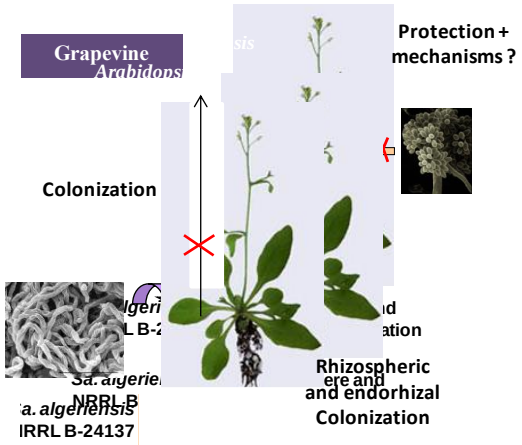

Figure 13: Drawing representing the part related to the results regarding grapevine plants

Colonization of *Arabidopsis thaliana* by *Saccharothrix algeriensis* NRRL B-24137

As with the study of colonization by NRRL B-24137 in grapevine plants, the colonization of the strain was also evaluated with *Arabidopsis thaliana* by fluorescence *in situ* hybridization (FISH) analysis. Following root inoculation with bacteria, NRRL B-24137 was detected in the rhizosphere. We observed that *Saccharothrix algeriensis* NRRL B-24137 can colonize the root surfaces of *Arabidopsis* seedlings as well as can be endophytic inside roots of the plants.

It has been observed that strain NRRL B-24137 colonized the rhizoplane in a mycelial form, especially at root hairs level following root inoculation. These results correspond to our observation with grapevine plants. The bacterial strain was also visualized in the elongation zone as well as at emergence site of secondary roots.

Sa. algeriensis NRRL B-24137 was also visualized between or inside rhizodermal cells as well as inside cortex region. The bacterium was also found near the xylem vessels but not inside it. These results showed similar behavior of colonization of NRRL B-24137 as in grapevine. Some beneficial bacteria can colonize the upper plant parts also whereas others can be restricted to root parts only (Compant *et al.,* 2010a).

The strain NRRL B-24137 was not found in systemic plant parts. Although we studied the colonization process up to 10 dpi, it could be a possibility to detect the NRRL B-24137 in the systemic parts afterwards. But our results allowed us to show that colonization of NRRL B-24137 up to several cortical cell layers but not in the vascular system that allows to suggest that NRRL B-24137 is restricted to the root internal parts (Figure 14).

In *Arabidopsis thaliana*, we also performed the experiments of colonizations when leaves of *Arabidopsis* were challenged with *B. cinerea* but still 3 days after bacteria was only found in rhizosphere not inside the leaves. However this non presence has allowed then to study a putative protection in the systemic plants parts towards *B. cinerea* BC1 that can be due to a systemic resistance phenomenon.

Induced systemic resistance by NRRL B-24137 in the *Arabidopsis thaliana*

In one part of this thesis the impact of inoculation of *Sa. algeriensis* NRRL B-24137 in *Arabidopsis thaliana* leaves towards the infection caused by *B. cinerea* BC1 was analyzed. The results obtained demonstrated that Col (wild type) inoculated plants with bacteria are less infected than untreated plants. The presence of the beneficial bacterium was only visualized at the root level, suggesting that a systemic resistance could be correlated to a reduction of leaves symptoms caused by *B. cinerea* BC1 (Figure 14).

To understand the mechanisms involved in the protection toward *B. cinerea* different mutants related to ET sensitivity, JA signaling and SA signaling have been screened. We observed that gene products related to ET sensitivity *ein2, ein4,* and *ein5-1* and JA signaling *jar1-1 Coi1-16* are required to the systemic resistance induced by the beneficial endophyte. On the other hand SA signaling is not required as demonstrated with the results obtained by the *NahG* mutant used. With these results, it became clear that the resistance induced by NRRL B-24137 corresponds to an ISR.

To understand more about the mechanism involved in the protection toward *B. cinerea* BC1, some "enhanced disease resistance" mutants were evaluated and the results showed that *eds4-1* (related to ET) and *eds8* (related to JA) are involved in ISR but *eds5* (related to SA) and *eds9* did not lose their resistance and found not to be involved in ISR mechanism induced by NRRL B-24137.

Mutants *npr1* (associated both with SAR and ISR) and *aos (allene oxide synthase)* were studied and they showed their involvement in ISR. Phytoalexin mutants were additionally screened. Results showed that mutants *pad1* (JA-dependant) is involved but *pad3-1* (SA-dependant) and *pad4-1* (SA-dependant) are not required for ISR induced by NRRL B-24137.

All the results described before correspond to previous model coorelated to ISR. However in this thesis, some other mechanisms were also described. Camalexin *ups1* mutant,

defective in a wide range of defense responses due to SA and JA/ET signalization reduced and ROS-mediated gene expression compromised also lost the resistance as compared to non inoculated plants. We also evaluated implication of *AtrbohD* and *F*, involved *via* respiratory burst oxidase in ROS signaling and the results showed that they are required for the ISR induced by strain NRRL B-24137 towards *B. cinerea* BC1.

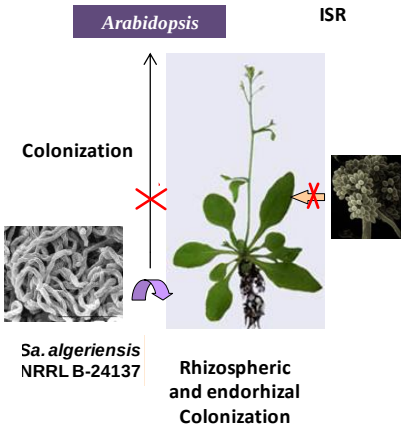

Figure 14: Drawing representing the part related to the results regarding *Arabidopsis thaliana* plants

Future Prospects

As perspectives of this thesis, different points can be considered.

1) The secondary metabolite secreted by NRRL B-24137 and responsible of reduction of *B. cinerea* BC1 should be characterized. This would be helpful for the knowledge of different metabolites secreted by NRRL B-24137.

2) It would be also interesting to determine whether the compounds produced *in vitro,* could be produced during the interaction between the grapevine and *Sa. algeriensis* NRRL B-24137. This analysis would be helpful to better understand if they can act microbial associated molecular pattern (MAMP). Different other MAMPs could be also considered. This could be parts of the future prospects.

3) It would be interesting to study the colonization process of the beneficial strain with grapevine and *Arabidopsis thaliana* plants in a long study to better understand if the strain can be a systemic colonizer or rather restricted to the root parts of the plants.

4) Thorough study of intercellular signaling may also be considered. Our results suggest that JA/ET signaling appears to be involved at the systemic level. Further study of genes induced in grapevine defense in response to *Sa. algerinsis* may also be considered. The expression of defense related genes particulary those encoding PR proteins should be studied. Some of them have been studied during the interaction between the grapevine and micro-organisms (Bezier, 2003, 2007). This is the case for example with *VvChi1b, VvPR10.2*. These genes can be induced particularly in response to *B. cinerea* (Bezier, 2007). However their expressions have not been studied in the case of interaction *Vitis vinifera* L. and NRRL B-24137. It would, therefore, interesting to determine whether these genes are induced in our model study. As the signalling pathway that regulate the expression of these genes are known *e.g.* the expression of *VvPR1* thus depends on the SA pathway and *VvPR4* depends on JA pathway (hamiduzzaman *et al.,* 2005). Monitoring the expression of these genes would confirm the involvement of the signaling pathway in our model.

Other defense genes like *PAL* and *LOX* coding the PAL and LOX could also be examined. In fact, it would be interesting to analyze their expression because PAL depends on SA signaling pathway and LOX on JA signaling pathway.

5) Meantime, other mutants in *Arabidopsis* should be studied to further see the involvement of different other genes in the mechanisms of ISR induced by NRRL B-24137. Monitoring of other genes will provide us the significant information on plant defence that is set up in response to PGPR/endophyte. This will enhance our current knowledge of the interaction between *Vitis vinifera* L./*Arabidopsis thaliana* L. and NRRL B-24137.

6) It would also be interesting to analyze if the induced state of resistance of grapevine plants by NRRL B-24137 also protects grapevine berries from *B. cinerea* infection. During the thesis application of the strain was done in field. We did not present the results in this thesis because it was only carried out during one year. Furthermore, the resultq were not obtained on the Cabernet Sauvignon cultivar. The disease rate of *B. cinerea* was quasi absent in non treated plants leading to difficulty to understand if a protection occurs. However some other prelimary results show the high potentiel to protect grapevine plants cv. Chardonnay towards *B. cinerea* (Figure 15). Interestingly during the field experiment we could not apply the same concentration than the one used during this thesis due to a considerable amount of medium plates of NRRL B-24137 required. We reduced 10 fold the concentration and this seems to be enough for field application (Figure 15).

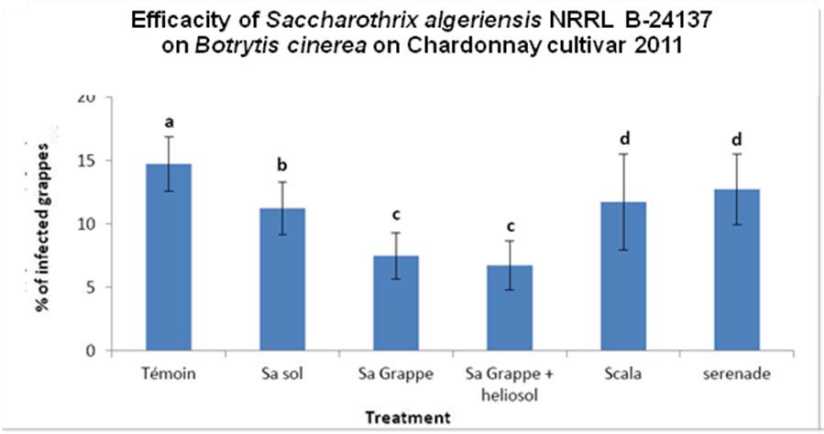

Figure 15: Data results from a field evaluation of NRRL B-24137 following soil application, grape application without and without an adjuvant (Heliosol) on cv. Chardonnay towards *B. cinerea*. Treatments were compared to scala (fungicide) and serenade (containing *Bacillus* sp). 100 plants were evaluated each time and repeated 4 times. Experiments were carried out in Languedoc-Roussillon with Anadiag SA (industrial society of registration).

7) It would also be interesting to analyze if the induced state of resistance of grapevine plants by NRRL B-24137 also protects grapevine berries from *B. cinerea* infection in case of a climate change scenario. Temperature can change and increase. Part of this work was done during the thesis. We did not present the results in this thesis because it was only carried out without more knowledge (mechanisms). However the results show the high potentiel to protect grapevine plants towards *B. cinerea* in case of a high temperature condition and that this study should be continued (the results are presented in the following proceeding publication).

8) It would also be interesting to find if there is a resistance against various phytopathogens. It could be possible that strain NRRL B-24137 or its produced compounds could constitute a novel and non-polluting tools useful for the development of a sustainable biocontrol of *Vitis vinifera* L. pathogenic agents.

Saccharothrix algeriensis NRRL B-24137: a new endophyte with high potential to protect grapevine towards *Botrytis cinerea* in case of high temperature conditions

S. Muzammil[1*], S. Compant[1, 2*‡], Z. Yu[1*], F. Mathieu[1], and A. Lebrihi[1]

[1] *Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Dépt Bioprocédés et Systèmes Microbiens, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1, France.* [2] *Université de Toulouse, Centre de Viticulture-Œnologie de Midi Pyrénées, Avenue de l'Agrobiopôle BP 32607, F-31326 Auzeville-Tolosane, Castanet-Tolosan Cedex 1, France.*

‡Corresponding author. Dr. Stéphane Compant. E-mail: scompant@ensat.fr

*authors contributed equally

KEYWORDS: endophyte, grapevine, protection, *Botrytis cinerea*

Introduction

Plant growth-promoting bacteria are known as stimulating plant growth as well as protecting their hosts to various pathogen diseases (1; 2; 3). This has been demonstrated for instance with various crops and also on grapevine plants with members of Firmicutes, Gamma-proteobacteria, Actinobacteria as well as many others taxa. However there is a current need to research beneficial bacteria that can not only confer pathogen protection but also that can alleviate pathogen stress under high temperature conditions (4). This is correlated to a putative climate change that will occur. Indeed, there is a possibility that climate become more and more warmed (5). In this case pathogens will surely proliferate in a higher level than previously seen (4). There is therefore a current need to evaluate protection of grapevine towards phytopathogen infection and colonization. A rare actinobacterium from the family *Actinosynnemataceae, Saccharothrix algeriensis* NRRL B-24137 that was isolated from desert soil (6) and can be root endophytic on various plants (Muzammil, personal communication) was previously evaluated to protect grapevine towards *Botrytis cinerea* infection. This bacterium can colonize beneficially grapevine plants but not in the systemic plant parts and protect them to gray mould disease by inducing mechanisms of induced systemic resistance (Muzammil et al., in prep). This bacterial strain can also protect various plants to phytopathogens and is of currently of special interest for agronomy. However, it is still unknown if this bacterial strain can protect plants under high temperature conditions. In this study its potential was evaluated under elevated temperature conditions to evaluate if protection against *Botrytis cinerea* can also occur under high temperature conditions.

Materials and Methods

Culture of bacterial and fungal strains

Saccharothrix algeriensis NRRL B-24137 (DSM 44581) was grown on ISP2 medium at 30°C during 8 days before to harvest spores and aerial mycelium in PBS (10mM, ph 7.0) and to adjust concentrations to 5.10^7 UFC/mL.

Botrytis cinerea strain BC1 isolated from infected grapevine plants by S. Compant in 2008 was cultivated on PDA medium at 25°C during 7 days before to harvest spores in ½ PDB and to adjust concentration to $6{,}5.10^5$ spores per ml with a Thoma cell counter.

Preparation of grapevine plants

Grapevine plants harbouring as graft part cv. Cabernet Sauvignon clone 15 and as rootstock 44-53 were provided by "Pépinières Colombie Vendries" (Camparnaud, France). Then plants were treated with cryptonol at 0.05% before to be placed at 4°C and in dark in a cold chamber. Plants were then planted in non sterile potting soil and placed in a glasshouse were temperature was of 35-40°C and 14/10 day-night light. One month after planting plants were inoculated with *Saccharothrix algeriensis* NRRL B-24137.

Inoculation of grapevine plants with *Saccharothrix algeriensis* NRRL B-24137

Before inoculation of grapevine plants with the beneficial bacterium, plants were separated from their soils. Then the root systems were placed in 400 ml of the bacterial solution (or PBS as control) during 3 min. Then plants were placed again in pots filled with potting soil and placed in the glasshouse.

Inoculation of grapevine plants with *Botrytis cinerea*

16 days post bacterial inoculation, 5 leaves of each grapevine plant were inoculated or not with 3µL of *B. cinerea* spore suspension and inoculation was done on 5 different places of the leaves. Then plants were recovered with a plastic bag and celled to maintain high humidity and avoid propagation of *B. cinerea*. Plants were then put again in the glasshouse with the same conditions of temperature and light.

Evaluation of protection

3 days post *B. cinerea* infection, plants were photographed and percentages of leaves of plants with necrosis were evaluated. For surfaces of leaf necrosis, all surfaces of leaves inoculated were analyzed with image J software.

Statistics

Experiments were done 3 independent times with each times 8 plants as control and 8 plants as bacterized with *Saccharothrix algeriensis* NRRL B-24137 and inoculations were done five times on five leaves on each plant. Controls of experiments were done also on plants without *Botrytis cinerea*. Data were analyzed by using student t test.

Results and discussion

Plants inoculated with PBS or *Saccharothrix algeriensis* NRRL B-24137 and without *Botrytis cinerea* did not show any symptoms (data not shown). However with *Botrytis cinerea* infection experiments, results showed that plants bacterized with *Saccharothrix algeriensis* NRRL B-24137 have less symptom of necrosis caused by *Botrytis cinerea* on leaves in comparison to control plants (figure 1a-d). Some leaves do not show however symptoms both in control and bacterized plants incoualted with *Botrytis cinerea*. In case of control plants this can be due to a basal resistance enabling protection to *Botrytis cinerea*. The average of percentages of leaves with symptoms was however of 30.9 % +/- 5.44 for control plants and of 12.5 % +/- 6.61 for bacterized plants (figure 1e). The surfaces of leaf necrosis per point of inoculation were of 64.1 +/- 57.23 mm^2 for control and of 16.48 +/- 22.08 mm^2 for bacterized plants (figure 1f). These data were different with $P<0.05$, demonstrating that plants bacterized with *Saccharothrix algeriensis* NRRL B-24137 are protected towards *Botrytis cinerea*. The beneficial bacterium *Sa. algeriensis* NRRL B-24137 is known as protecting grapevine towards *Botrytis cinerea* at temperature of 25°C. The beneficial strain can also induce defences mechanisms that can explain the protection observed (Muzammil et al., in preparation). However with this study we show that beneficial bacterial strain can also protect grapevine plant to *B. cinerea* even under high temperature. Although the mechanisms responsible for need to be characterized, these results are important for grapevine in case of climate changing conditions. Indeed, if the temperature will increase in the future some products registered to alleviate *B. cinerea* on plants will may be not functionned. With *Sa. algeriensis* NRRL B-24137, protection can however occurs as demonstrated under glasshouse and non sterile conditions. Although we used in this study a high temperature that can however arrived or not in some regions, biocontrol with *Sa. algeriensis* NRRL B-24137 can be used therefore to alleviate *B. cinerea* infection in case of increasing temperature.

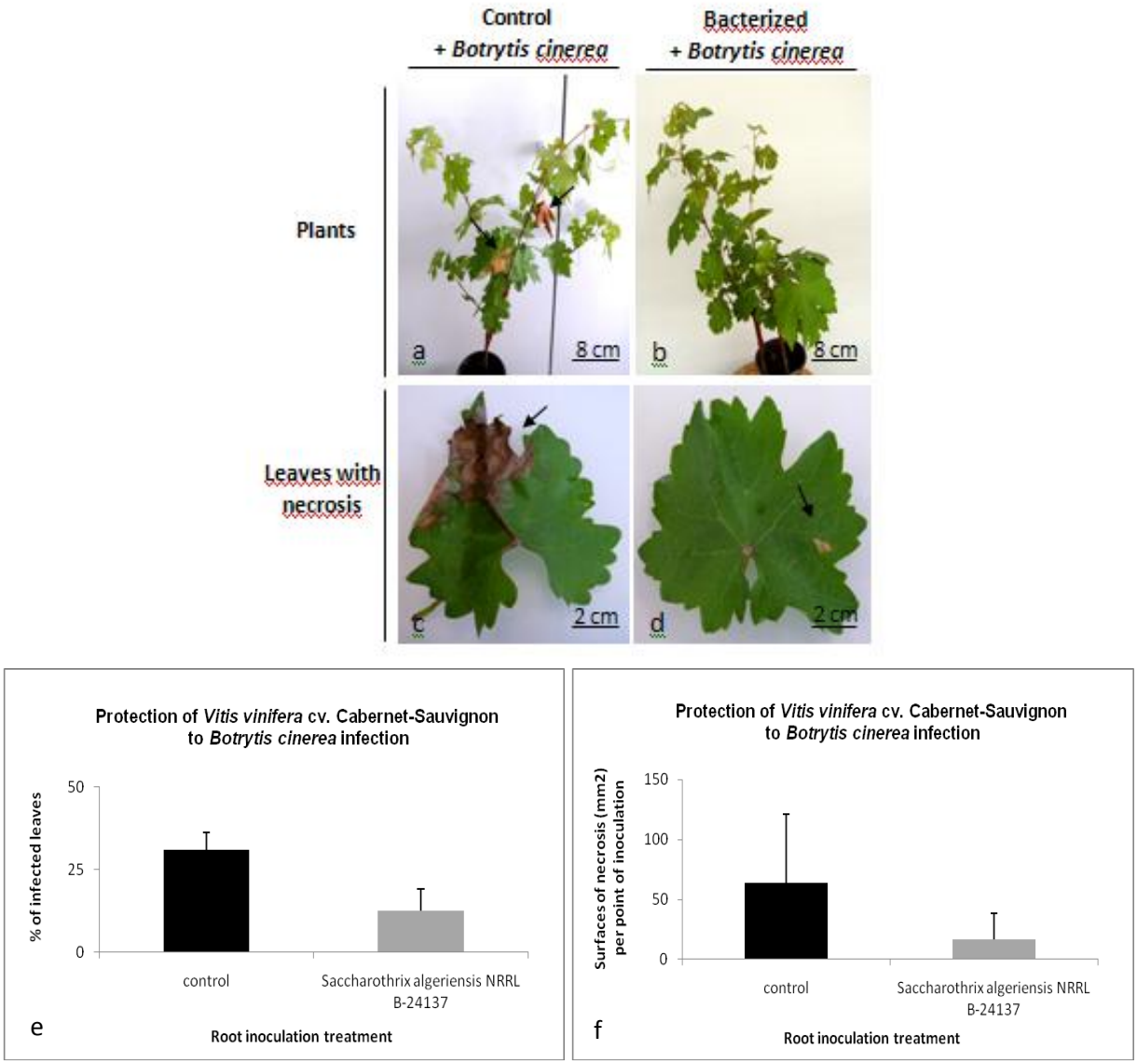

Figure 1: Pictures of *Botrytis cinerea* symptoms on leaves of plants inoculated with PBS or *Saccharothrix algeriensis* NRRL B-24137 and then inoculated with *B. cinerea* BC1 (a-d) and percentage of protection (e) and surfaces of necrosis per point inoculation (f). Arrows indicated on pictures a to d symptoms due to *Botrytis cinerea.* Plants (control of with *Sa. algeriensis* NRRL B-24137) without *Botrytis cinerea* did not show any symptoms (data not show).

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Curriculum Vitae

Saima MUZAMMIL

Université de Toulouse, LGC UMR 5503 (CNRS/INPT/UPS), Département Bioprocédés et Systèmes Microbiens, ENSAT-INP de Toulouse, 1 Avenue de l'Agrobiopôle, B.P. 32607, F-31326 Castanet-Tolosan Cedex 1
Saimamuzammil83@yahoo.com

Personal Information

Name	Saima MUZAMMIL
Date of Birth	September 3, 1983
Marital Status	Married
Postal Address	Saima Muzammil House No. 242, St. No.5, Main Bazar, Nisar Colony, Faisalabad, Pakistan

Educational Qualification

2009 - 2012	PhD, INP-ENSAT, Toulouse
2007 - 2008	M2R, BioSciences Végetales, University of Toulouse,
2005– 2007	MSc (Hons) in plant, University of Agriculture, Faisalabd
2001 – 2005	BSc (Hons) in plant, University of Agriculture, Faisalabd

Research Interest

Microbial ecology, rhizosphere, endophyte, induced systemic resistance

Publications

Compant S.*, Brader G.*, Muzammil S.*, Sessitsch A., Lebrihi A. and Mathieu F. **Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases.** Accepted with minor changes in Biocontrol.*Equivalent work.

Compant S., Muzammil S., Lebrihi A., and Mathieu F. **Visualization of root colonization of grapevine by the Saharan soil isolate *Saccharothrix algeriensis* NRRL B-24137 by using DOPE-FISH microscopy and a probe specific to *Saccharothrix* spp.** Plant and Soil, submitted.

Muzammil S., Graillon C., Saria R., Mathieu F., Lebrihi A., and Compant S. **The soil bacterium *Saccharothrix algeriensis* NRRL B-24137 can be endophytic in *Arabidopsis,* induces ISR towards**

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Oral Communication

Muzammil S., Compant S., Graillon C., Mathieu F., and Lebrihi A. ***Saccharothrix algeriensis* NRRL B-24137: an endophyte with high potential to protect grapevine towards *Botrytis cinerea* and for understanding new mechanisms involved in ISR of plants.** 1st International Symposium "Biocontrol of Grapevine Diseases" Toulouse Castanet-Tolosan, France 2011.

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Muzammil S., Graillon C., Mathieu F., Lebrihi A., and Compant S. ***Saccharothrix algeriensis* NRRL B-24137: a new endophyte enabling protection towards *Botrytis cinerea.*** Current aspects of European endophyte research Reims, France, 2012.

Muzammil S., Graillon C., Mathieu F., Lebrihi A., and Compant S. ***Saccharothrix algeriensis* NRRL B-24137: a new endophyte enabling protection towards *Botrytis cinerea.*** Oeno 2011, Bordeaux, France, 2011.

Muzammil S., Compant S., Graillon C., Mathieu F., and Lebrihi A. **Bioactive metabolites secreted by *Saccharothrix algeriensis* NRRL B-24137: characterization of metabolites involved in biocontrol of *Botryospheria parva* and *Botryospheria obtusa*.** 1st International Symposium "Biocontrol of Grapevine Diseases" Toulouse Castanet-Tolosan, France 2011.

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Muzammil S., Graillon C., Mathieu F., Lebrihi A., and Compant S. (2011) **Preliminary study on the potential of *Saccharothrix algeriensis* NRRL B-24137 to control *Botryospheria parva* and *Botryospheria obtusa*.** In Compant S. and Mathieu F., editors. Recent advances on biocontrol of grapevine diseases: from fundamental knowledge to application in the vineyards, Oenoplurimedia. Submitted

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Compant S., Muzammil S., and Mathieu F. (2011) **DOPE-FISH, a tool to visualize beneficial bacteria in the rhizosphere of grapevine plants following inoculation?** In Compant S. and Mathieu F., editors. Recent advances on biocontrol of grapevine diseases: from fundamental knowledge to application in the vineyards, Oenoplurimedia. Submitted