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Characterization of new bacterial biocontrol agents *Acinetobacter*, *Bacillus*, *Pantoea* and *Pseudomonas* spp. mediating grapevine resistance against *Botrytis cinerea*

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Abstract

A collection of 282 bacterial isolates from the rhizosphere and different organs of healthy field-grown grapevine plants was obtained and screened for their ability to protect grapevine leaves against *Botrytis cinerea*, the causal agent of gray mold. Twenty-six strains effectively controlled *B. cinerea* infections on leaves. After phenotypic and molecular analysis, seven strains were identified as *Pseudomonas fluorescens* PTA-268 and PTA-CT2, *Bacillus subtilis* PTA-271, *Pantoea agglomerans* PTA-AF1 and PTA-AF2, and *Acinetobacter lwoffii* PTA-113 and PTA-152. In vitro antifungal experiments showed that from these seven strains, only PTA-AF1 and PTA-CT2 exhibited a direct antagonism against *B. cinerea*. Furthermore, the biocontrol activity of the seven bacteria was associated with differential induction of defense-related responses lipoxygenase, phenylalanine ammonia-lyase and chitinase in grapevine leaves. Our results show that the selected bacteria can efficiently protect grapevine leaves against gray mold disease through an induction of plant resistance and in some cases by an additional antagonistic activity.

Keywords: Biocontrol; Grapevine; Gray mold; Induced resistance; Rhizobacteria

1. Introduction

Grapevine (*Vitis vinifera* L.) is highly vulnerable to several fungal diseases, among them gray mold caused by *Botrytis cinerea* Pers.; Fr., which leads to serious damage in French vineyards, particularly in the regions where the climate is cool and humid. This fungus infects flowers, setting fruits, mature fruits, and leaves. Currently, gray mold is controlled before harvest by preventive fungicides. However, because of the increasing worldwide concern about pesticide use due to environmental problems and pathogens developing resistance, alternative plant protection strategies are becoming increasingly attractive. This has promoted the consideration of biological disease control and induction of plant resistance strategies by using either non-pathogenic plant-associated microorganisms (Van Loon et al., 1998; Bargabus et al., 2003; Tjamos et al., 2005) or components derived from microorganisms and plants (Aziz et al., 2003; Nürnberger et al., 2004).

A great number of reports indicated that certain bacterial strains are beneficial for the growth of plants; these are called plant growth-promoting rhizobacteria (PGPR). Colonization of roots with PGPR can also induce resistance in parts of the plant that are spatially separated from the inducing microorganism (Maurhofer et al., 1994; Van Loon et al., 1998). An important trait of these bacteria is their ability to maintain a stable relationship with the associated plant species (Smith and Goodman, 1999; Miethling et al., 2000). Consequently, the plant material can have a significant influence on the composition of the microflora obtained, as well as on the probability of finding isolates with biocontrol activities. Microorganisms isolated from the rhizosphere or from tissues of a specific plant are non-exotic, thereby presenting no risk of proliferation of a new microorganism in the environment. Furthermore, they may be better adapted to that plant and therefore provide better control of dis-

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eases than organisms originally isolated from other plant species (Handelsman and Stabb, 1996).

Biocontrol bacteria may protect plants against pathogens by direct antagonistic interactions between the biocontrol agent and the pathogen, as well as by induction of the host resistance. The biocontrol depends on a wide variety of traits, such as the production by the biocontrol strain of various antibiotic compounds, iron chelators and exoenzymes such as proteases, lipases, chitinases, and glucanases (Leong, 1986; Maurhofer et al., 1994; Chin-A-Woeng et al., 1998; Dunlap et al., 1998; Raaijmakers and Weller, 1998; Trejo-Estrada et al., 1998); as well as competitive root colonization (Chin-A-Woeng et al., 2000; Lugtenberg et al., 2001), and induced resistance in the host plant (Baker et al., 1985).

In recent years, considerable attention has been focused on induced resistance as an important phenomenon that occurs when plants develop enhanced defensive capacity upon appropriate elicitation. Induced defense reactions can be restricted to the tissues close to the site of elicitation or can be expressed systemically throughout the tissue or the whole plant. Bacteria-induced systemic resistance (ISR) has been demonstrated in a variety of plant species against a broad spectrum of pathogens (Hammerschmidt and Kuc, 1995; Van Loon et al., 1998; Magnin-Robert et al., 2007). In some cases, ISR is associated with the expression of some defense genes such as those encoding for pathogenesis-related (PR) proteins (e.g. chitinase) and also phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX) pathways (Maurhofer et al., 1994; Van Loon and Van Strien, 1999). LOX is required for the synthesis of the precursors of jasmonates, compounds that may act as the signal factor in plant defense responses (Creelman and Mullet, 1997; Pieterse et al., 1998). PAL is a key enzyme concerned with the synthesis of salicylic acid and phenolic compounds which were proposed to reduce incidence of plant disease through antifungal activity and stimulation of plant defense responses (Lee et al., 1995; Reymond and Farmer, 1998; Shadle et al., 2003). The relative importance of all these mechanisms differs considerably among strains of biocontrol bacteria (Neiendam-Nielson et al., 1998; Van Loon et al., 1998).

In grapevine, much of research reported on the use of the fungi *Trichoderma* spp. and *Gliocladium* spp. to control gray mold (Elmer and Reglinski, 2006). Nevertheless, a possible control of this disease by a *Burkholderia* sp. originally isolated from onion has been reported and attributed to a systemic spread of the bacterium into the aerial parts of the plant (Compant et al., 2005). Recently, a commercial biofungicide Serenade, which contains a *Bacillus subtilis* strain (QST 713), was reported to be effective against various pathogenic fungi (http://www.agraquest.com).

Our goals were to: (1) screen, identify, and characterize non-pathogenic bacteria isolated from the rhizosphere and tissues of healthy grapevine plants for their effectiveness to control gray mold on grapevine leaves caused by a highly virulent *B. cinerea* isolate and (2) quantify elicitation of some defense-related responses in grapevine leaves by selected bacterial strains.

2. Materials and methods

2.1. Isolation of bacterial strains

Bacteria were isolated during the growing season from the rhizosphere, roots, leaves and stems of healthy grapevine plants (*V. vinifera* L., cv Chardonnay) from a vineyard located in the Champagne area (Marne, France). Leaves, root and stem sections were surface disinfected (20 s with 70% ethanol, and then for 10 min in a 2% sodium hypochlorite solution for root and stem sections or for 20 s in a 2% sodium hypochlorite solution for leaves) and severely washed with sterile aqueous NaCl (0.85%). Each sample was dissected aseptically into small segments and macerated in the 0.85% NaCl solution. The rhizospheric soil samples were directly suspended in the sterile NaCl solution. Tissue and soil extracts were then serially diluted and plated in triplicate onto King's B-agar, glycerol–arginine–agar and Luria–Bertani-agar (LB-agar) media to recover bacteria present in the plant tissues and soil. Bacteria were grown on plates at 30 °C for 24–72 h. Colonies were then counted and isolated on LB-agar, cultured in LB at 30 °C for 24 h, and stored in sterile 20% glycerol solution at −80 °C.

2.2. Plant material

Grapevine plantlets (*V. vinifera* L. cv Chardonnay, clone 7535) grown in vitro from nodal explants on modified Murashige and Skoog (1962) medium (half concentration of macro-elements and glutamine at 200 mg/l), supplemented with 20 g/l sucrose, and 7 g/l agar. Plants were grown in 25-mm test tubes under white fluorescent lamps (60 μmol/m^2/s), 16/8 h photoperiod, and 25 °C day/night temperature.

2.3. Fungal pathogen

A virulent *B. cinerea* (strain 630), isolated from a vineyard in the Marne Valley (France) was a gift of Dr. Y. Brygoo (INRA, Versailles, France). It was cultured in Petri dishes on potato dextrose agar (PDA) medium (Sigma, St Quentin Fallavier, France) at 22 °C for 14 days. Conidial suspension was obtained by flooding the fungal culture with sterile distilled water, rubbing the mycelium and filtering through a sterile nylon gaze (mesh of 200 μm). The conidial suspension was adjusted with sterile distilled water to 2.5×10^5 conidia/ml.

2.4. Effectiveness of bacteria to control gray mold on grapevine leaves

Grapevine leaves excised from in vitro-grown plantlets (10-week-old) were floated with abaxial side down on the buffer surface (2 mM MES pH 5.9, containing 0.5 mM $CaCl_2$ and 0.5 mM K_2SO_4), in the presence of each bacterial isolate at 1×10^7 CFU/ml. Control consisted of leaves incubated on buffer alone. After 20 h, the leaves were rinsed with sterile distilled water, patted dry and placed in Petri dishes, the adaxial side facing a wet absorbing paper. One needle-prick wound was applied to each leaf, and the fresh wounds were covered with 5-μl drops

of the *B. cinerea* conidial suspension (2.5×10^5 conidia/ml). Disease development was measured as the average diameter of lesions formed 7 days after inoculation with *B. cinerea*. Percent protection was defined as reduction in lesion diameter relative to the control. For each isolate (282 bacteria) experiment was done with 10 leaves excised from four to five plants. Each experiment was repeated three times for the 26 isolates listed in Table 1. Statistical significance of the results was evaluated with Statistica Software (Statsoft Inc., Tulsa, USA) by using analysis of variance (ANOVA), and Duncan's multiple range test ($P \leq 0.05$) was used for post-hoc comparison of means. Isolates that showed high protection of grapevine leaves against *B. cinerea* were chosen for further examinations.

2.5. Phenotypic characterization of bacterial isolates

Colonies of bacterial isolates were characterized for their colour, form, elevation, margin, diameter, surface, opacity, and texture. Motility, morphology, and size were also evaluated by phase-contrast microscopy. The Gram reaction was performed by using a 3% KOH test, the crystal violet method and spreading on McConkey agar. Discs impregnated with dimethyl-*p*-phenylenediamine (API-bioMérieux, Marcy l'Etoile, France) were used to test for the presence of cytochrome oxidase. The catalase activity was measured by O_2 production after adding a drop of 1.5% H_2O_2 to a young colony growing on nutrient agar. All strains were then characterized by the Analytical Profile Index (API) micro-methods standardized for a rapid identification of non-fastidious Gram-negative and Gram-positive bacteria (bioMérieux, Marcy l'Etoile, France). The production of acid metabolites from 49 carbohydrates was tested with API 50CHB strips for Gram-positive bacteria (Logan and Berkeley, 1984). Tests for proteolysis of gelatin, activities of different enzymes (nitrate reductase, galactosidase, urease, and tryptophanase), H_2S formation, production of acetoin, and citrate utilization were carried out with API 20E strips for Gram-negative bacteria (Smith et al., 1972). The API 20NE test kit was used to identify glucose-non-fermenting Gram-negative non-Enterobacteriaceae. The tests were done in accordance with the recommendations of API-bioMérieux (Marcy l'Etoile, France). The results were interpreted with the API database of the Api-Lab Plus software (version 3.3.3; bioMérieux, Marcy l'Etoile, France).

2.6. PCR amplification and sequencing of rrs gene encoding 16S rRNA

A set of seven strains, according to their origin and phenotypic identification, were further identified by sequencing of *rrs* gene of 16S rRNA at the Pasteur Institute (CIMB, Paris, France). DNA was extracted and purified with the IsoQuick Nucleic Acid Extraction kit (ORCA Research, Bothell, WA) for Gram-negative strains, and with the Wizard Genomic DNA Purification Kit (PROMEGA, Madison, WI, USA) for *Bacillus* sp. The entire 1.5 Kb 16S rRNA gene was amplified by PCR, using the universal primers 8F and 1510R (Edwards et al., 1989; Janvier and Grimont, 1995). The amplified product was sequenced by Genome Express (Melan, France) with three primers from the conserved regions of *Escherichia coli* 16S rRNA (Brosius et al., 1978).

The sequences of selected strains were compared and aligned with those of the other strains obtained from the data deposited in GenBank using BLAST (NCBI) and module MegAlign. Phylogenetic trees were generated using the software Neighbor-joining (Saitou and Nei, 1987). Neighbor-joining trees were constructed using PAUP version 4.0b10 with Jukes–Cantor distance correction (http://paup.csit.fsu.edu/). Trees were drawn using TreeView version 1.5 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Robustness was tested by bootstrap analysis with 1.000 replicates.

2.7. Antagonistic activity

In vitro assays were performed to determine the effects of selected protective bacteria on mycelial growth of *B. cinerea*. Petri plates (9 cm diameter) containing PDA medium (Sigma, St Quentin Fallavier, France) were inoculated in the middle with a 5-μl bacterial suspension (1×10^8 CFU/ml). A 100 μl conidial suspension of *B. cinerea* (strain 630, 2.5×10^5 conidia/ml) was placed surrounding each bacterial inoculum. The plates were incubated at 22 °C for 7 days. Each bacterial strain was tested in triplicate plates and experiments were conducted twice. Bacteria that secrete an effective antifungal substance may prevent the growth of *B. cinerea* mycelium and result in an inhibition zone around the bacterial colony.

2.8. Determination of plant defense reactions

Ten grapevine leaves in triplicate were excised from four to five in vitro-grown 10-week-old plantlets and floated under continuous light with abaxial side down on the MES buffer surface containing 1×10^7 CFU/ml of the selected bacterial strains. Control consisted of leaves incubated on buffer alone. At different incubation times leaves were rinsed with distilled water, fixed in liquid nitrogen and stored at −80 °C until analysis.

LOX was extracted from grapevine leaves (250 mg FW) in 1 ml of sodium phosphate buffer 50 mM, pH 6.5, containing 0.25% (v/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (Sigma, St Quentin Fallavier, France) on ice. The homogenate was centrifuged at 20,000 × *g* for 30 min at 4 °C. The supernatant was used as the crude enzyme extract. LOX activity was assayed by measuring absorption at 234 nm arising from the formation of conjugated double bonds during the reaction according to the method of Axelrod et al. (1981). The reaction mixture (1 ml) containing the enzyme extract (25 μl), 100 μl of 10 mM linoleic acid, and 50 mM sodium phosphate (pH 6.0) was incubated at 25 °C for 1, 2.5, and 5 min. The same procedure was used for the blank reaction in which phosphate buffer (pH 6.5) replaced the enzyme extract. The hydroperoxides formed during enzymatic reaction were quantified by using a molar extinction coefficient of 25,000/M/cm.

PAL was extracted from leaves (250 mg FW) ground in 1 ml of potassium phosphate buffer 100 mM, pH 8.0, containing β-mercaptoethanol (1.4 mM) on ice. The homogenate was

Table 1
Protection of grapevine leaves against *Botrytis cinerea* by selected bacteria isolated from the rhizospheric soil, roots, leaves and stems of healthy plants, and phenotypic characteristics of the 26 strains of bacteria with biocontrol ability

Strain designation	Source	Protective effect		Phenotypic characteristics							Antagonistic activity
		Necrotic lesion diameter (mm)	Corresponding protection (%)	Gram reaction	Motility	Oxidase	Catalase	API 20E **	API 20NE **	API 50CHB **	
Control		15.0 ± 5.0	0 ± 0 [a]								
L3	Leaves	2.8 ± 0.8	82 ± 5 [d]	+	+	+	+			*Bacillus cereus*	−
S4	Soil	4.3 ± 1.2	71 ± 8 [c]	−	−	−	+	*Ewingella americana* (80.8%)*			+
S43	Soil	2.0 ± 0.0	87 ± 0 [d]	+	+	+	+			*Bacillus cereus*	+
St48	Stems	3.2 ± 1.0	78 ± 7 [c]	−	−	−	+	*Pantoea* sp. (99.9%)		*Pantoea* sp.	−
S69	Soil	4.0 ± 1.5	73 ± 10 [c]	−	−	−	+	*Pantoea* sp. (99.9%)		*Pantoea* sp.	−
PTA-113	**Roots**	**2.0 ± 0.0**	**87 ± 0[d]**	−	**+**	−	**+**	***Acinetobacter* sp.**	***Acinetobacter* sp.**		−
S115	Soil	2.0 ± 0.0	87 ± 0 [d]	+	+	+	+			*Bacillus cereus*	+
St137	Stems	2.8 ± 0.8	82 ± 5 [d]	−	+	+	+	Non-fermenter species (32%)	*Pseudomonas fluorescens* (99.8%)		+
PTA-152	**Roots**	**2.0 ± 0.0**	**87 ± 0[d]**	−	**+**	−	**+**	***Acinetobacter* sp.**	***Acinetobacter* sp.**		−
R183	Roots	2.0 ± 0.0	87 ± 0 [d]	+	+	+	+			*Bacillus subtilis*	−
S201	Soil	2.8 ± 0.8	82 ± 5 [d]	+	+	−	+			*Bacillus subtilis*	+
S217b	Soil	4.3 ± 1.2	71 ± 8 [c]	+	+	+	+			*Bacillus cereus*	+
S265	Soil	2.8 ± 0.8	82 ± 5 [d]	+	+	+	+			*Bacillus cereus*	+
PTA-268	**Soil**	**2.8 ± 0.8**	**82 ± 5[d]**	−	**+**	**+**	**+**	**Non-fermenter sp (32%)**	***Pseudomonas fluorescens* (99.8%)**		−
S270	Soil	2.0 ± 0.0	87 ± 0 [d]	−	+	+	+	Non-fermenter species (32%)	*Pseudomonas fluorescens* (99.8%)		−
PTA-271	**Soil**	**4.3 ± 0.9**	**71 ± 6[c]**	**+**	**+**	**+**	**+**			***Bacillus subtilis***	−
PTA-AF1	**Leaves**	**5.8 ± 0.8**	**61 ± 5[b]**	−	**+**	−	**+**	***Pantoea* sp. (61.9%)**		***Pantoea* sp.**	**+**
PTA-AF2	**Leaves**	**5.5 ± 1.0**	**63 ± 7[b]**	−	**+**	−	**+**	***Pantoea* sp. (99.9%)**		***Pantoea* sp.**	−
StAT1b	Stems	5.8 ± 1.5	61 ± 10 [b]	−	+	−	+	*Pantoea* sp. (99.9%)		*Pantoea* sp.	−
PTA-CT2	**Stems**	**2.0 ± 0.0**	**87 ± 0[d]**	−	**+**	**+**	**+**	**Non-fermenter species (32%)**	***Pseudomonas fluorescens* (99.9%)**		**+**
StCT2b	Stems	3.0 ± 0.5	80 ± 3 [d]	−	+	+	+	Non-fermenter species (32%)	*Pseudomonas fluorescens* (99.9%)		−
LEF1b	Leaves	2.8 ± 0.8	82 ± 5 [d]	−	−	−	−	*Pantoea* sp. (99.9%)		*Pantoea* sp.	−
LEF2b	Leaves	5.0 ± 1.3	67 ± 9 [b]	−	−	−	+	*Pantoea* sp. (99.9%)		*Pantoea* sp.	−
StET1b	Stems	2.3 ± 0.3	85 ± 2 [d]	−	−	−	+	*Pantoea* sp. (99.9%)		*Pantoea* sp.	−
StET2b	Stems	2.8 ± 0.8	82 ± 5 [d]	−	+	−	+	*Pantoea* sp. (99.9%)		*Pantoea* sp.	−
StET3b	Stems	2.3 ± 0.3	85 ± 2 [d]	−	−	−	+	*Pantoea* sp. (99.9%)		*Pantoea* sp.	−

Percent protection corresponds to the reduction in lesion diameter relative to the control. Values followed by the same letters are not significantly different ($P \leq 0.05$). * The numbers in parentheses are percentages of certainty. ** Analytical Profile Index (API), micro-methods for the identification of non-fastidious Gram-negative (20E and 20NE) and Gram-positive bacteria (50CHB). The seven strains subjected to molecular identification and used for further investigations are in bold.

centrifuged at 15,000 × *g* for 15 min at 4 °C, and the resulting supernatant was used as the crude enzyme extract. PAL activity was assayed by measuring the formation of cinnamic acid at 290 nm according to the method of Tanaka et al. (1974). The reaction mixture (0.8 ml) containing the enzyme extract (150 μl), 40 mM phenylalanine (200 μl), and 0.1 M Tris–HCl (pH 8.8), was incubated at 37 °C for 10, 20, and 30 min. The reaction was stopped by adding 200 μl of trichloroacetic acid (25%). The assay mixture was centrifuged at 10,000 × *g* for 15 min (4 °C) and cinnamic acid formed during enzymatic reaction was quantified by using a molar extinction coefficient of 17.4/mM/cm.

For the chitinase assay, leaves (250 mg FW) were ground in sodium acetate buffer 50 mM, pH 5.0, containing 1 mM dithiothreitol and 0.2% (w/v) phenylmethanesulfonyl fluoride on ice. The mixture was centrifuged at 10,000 × *g* for 5 min at 4 °C, and the resulting supernatant was used as the crude enzyme extract. Chitinase activity was determined according to the procedure described by Wirth and Wolf (1992) using carboxymethyl/chitin/remazol brilliant violet 5R (Loewe Biochemica, Germany) as a substrate. The reaction mixture (0.4 ml) containing 50 μl of 10-fold diluted extract, 100 μl of substrate (2 mg/ml), and 50 mM sodium acetate buffer (pH 5.0), was incubated at 37 °C for 1, 2, and 5 min. The reaction was stopped by adding 0.4 ml of cold 0.3 M HCl. The assay mixture was cooled on ice for at least 10 min and centrifuged at 10,000 × *g* for 10 min (4 °C) and absorbance of supernatant was quantified by spectrophotometer at 550 nm.

3. Results

3.1. Isolation and screening of bacterial strains

To select populations of potential biocontrol bacteria associated with grapevine plants, rhizospheric soil and tissues of healthy plants were collected and bacteria were counted. Significant differences in population sizes were observed between rhizospheric and endophytic communities. A majority of the microorganisms (1×10^5 to 2.2×10^7 CFU/g soil) was obtained from rhizospheric soil, while 8.5×10^4 CFU/g were recovered from roots, 2.0×10^4 CFU/g from stems, and 1×10^2 CFU/g from leaves. Based on abundance and phenotypic criteria, a total of 282 bacteria was isolated as representative of the different populations, consisting of 68% from rhizospheric soil, 16% from roots, 11% from stems, and 5% from leaves.

The 282 isolates were screened for their effectiveness to control *B. cinerea* using detached leaves from in vitro-grown grapevine plantlets inoculated after 20 h-long incubation with bacteria. Overall, 26 isolates (9.2%) effectively reduced disease development on leaves compared to the non-bacterized control (Table 1). In most cases, the reductions in diameter of necrotic lesions varied from 61 to 87%. Ten of 26 selected strains with effectiveness exceeding 61 ± 5% were isolated from soil from rhizosphere; 8 strains were isolated from stems, 5 strains from leaves, and 3 strains were isolated from roots of grapevine plants.

3.2. Identification of selected bacteria

The extensive phenotypic characterization was carried out with the 26 effective strains as a prerequisite to the standardized API strip systems that enable bacteria pre-identification (Table 1). The API 20E and API 20NE systems indicated that 20% of the isolates belonged to the genus *Pseudomonas* with at least 99.8% certainty of identification for the *Pseudomonas fluorescens* (Table 1). Two strains were also pre-identified as belonging to the genus *Acinetobacter*. Using API 50CHB system, we further identified two genera yielding a high similarity with *Bacillus* and *Pantoea*. This pre-identification revealed that all the isolates with biocontrol ability belonged to five different established genera, and four of them were found in the plant tissues. Although *Pseudomonas* and *Bacillus* species are mostly rhizospheric and endophytic bacteria, *Acinetobacter* seemed to be endophytic like most of the *Pantoea*.

Comparison with GenBank database (EMBL) of the 16S rRNA gene sequences of seven of these isolates, chosen according to their source, phenotypic characteristics, and biocontrol efficiency (accession numbers AM293675 to AM293681), indicated that the *Acinetobacter* (PTA-113 and PTA-152) and *Pantoea* (PTA-AF1 and PTA-AF2) sequences corresponded to that of distinct *Acinetobacter lwoffii* and *Pantoea agglomerans*. Similarly, the 16S sequence of the *Bacillus* (PTA-271) showed up to 99% homology to that of *B. subtilis*, while the *Pseudomonas* (PTA-268 and PTA-CT2) 16S rRNA sequences exhibited a strong homology to the 16S rRNA genes of *P. fluorescens* HhSoUsc group and *P. fluorescens* S2 group, respectively. The corresponding positions of these identified bacteria in their phylogenetic trees further indicated that they are new bacterial strains (Fig. 1).

3.3. Antagonistic activity of selected bacteria

Using an antifungal activity plate assay, it was shown that some of the selected bacteria were able to inhibit *B. cinerea* growth in dual culture. Among the 26 isolates previously selected, 9 strains (34.6%) showed an antifungal effect (Table 1). Among seven bacteria identified by sequencing of *rrs* gene, only *P. agglomerans* PTA-AF1 originating from leaves and *P. fluorescens* PTA-CT2 from stems, showed an apparent antagonistic effect against *B. cinerea* (Table 1).

3.4. Induction of defense-related reactions in grapevine leaves

As shown in Fig. 3, LOX activity was induced in detached grapevine leaves following incubation with the bacteria, the maximal induction was reached after 6–8 h of incubation. Compared to the control, LOX activity appeared almost 2–4-fold higher in bacterized leaves, depending on the bacterial genus. Maximum LOX activity was observed after incubation with the two *A. lwoffii* strains PTA-113 and PTA-152 (3–4-fold increase) (Fig. 2A), followed by the *P. fluorescens* strains PTA-268 and PTA-CT2 (2–3-

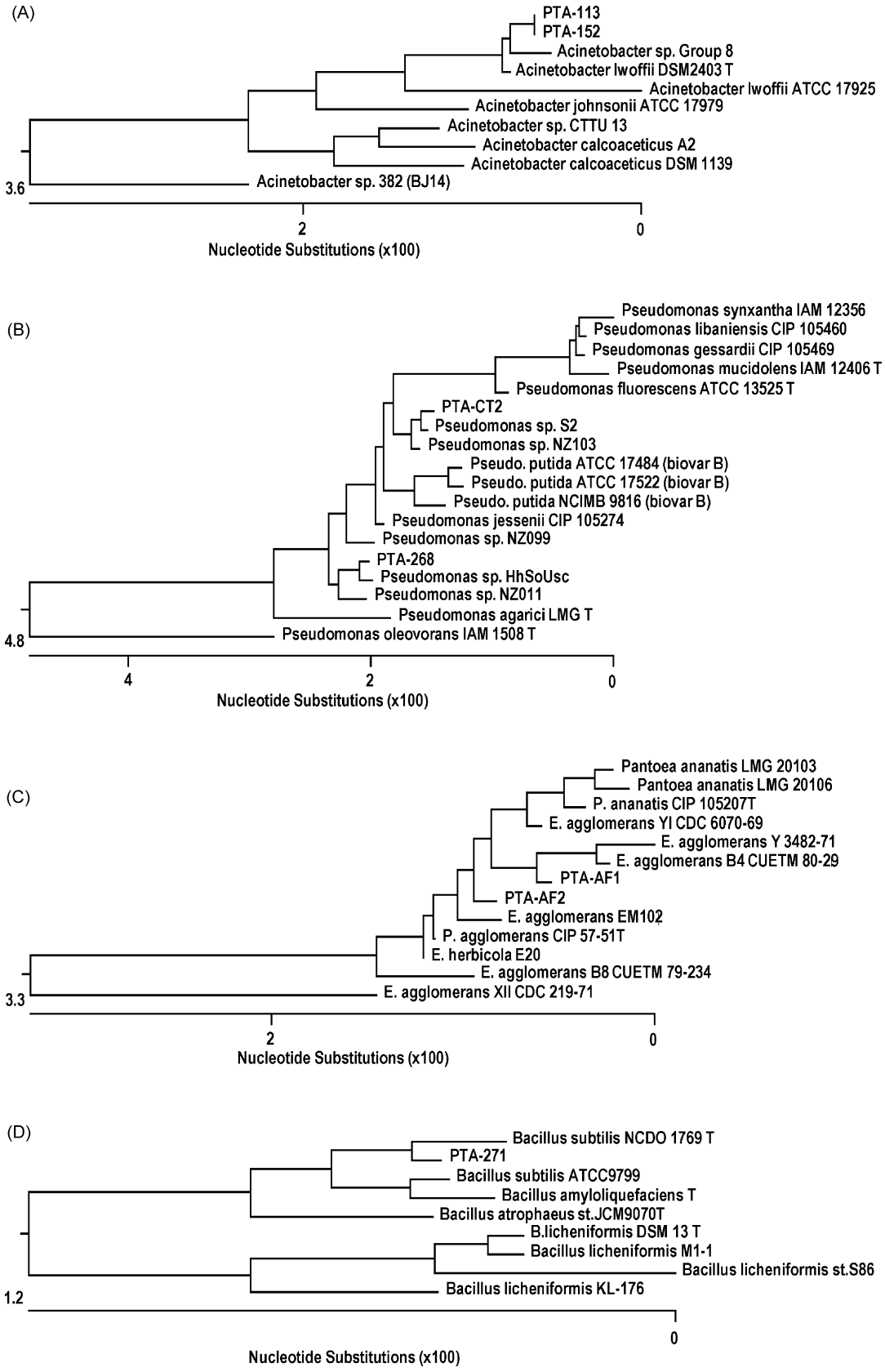

Fig. 1. Phylogenetic trees expressing the relationships of identified bacterial strains to taxonomically similar microorganisms based on the 16S rRNA gene sequences. The unrooted trees were constructed using the Neighbor program. The positions of the identified strains are based on the best match for genus and species. (A) *Acinetobacter* sp., (B) *Pseudomonas* sp., (C) *Entero*/*Pantoea* sp., (D) *Bacillus* sp. Values along branches indicate bootstrap percentages. Strains PTA-113, PTA-152, PTA-268, PTA-CT2, PTA-AF1, PTA-AF2, and PTA-271 were used in this study; the remainders are database reference strains.

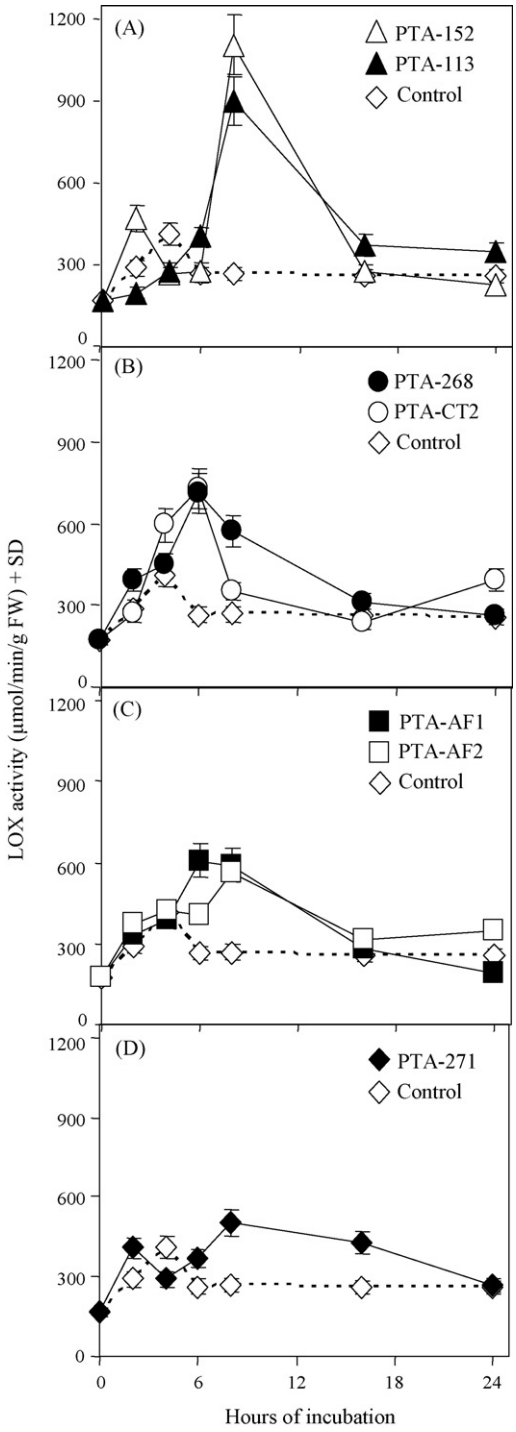

Fig. 2. Time course of lipoxygenase (LOX) activity in grapevine leaves incubated in bacterial suspension of strains of *Acinetobacter lwoffii* (A) PTA-113 and PTA-152, *Pseudomonas fluorescens* (B) PTA-268 and PTA-CT2, *Pantoea agglomerans* (C) PTA-AF1 and PTA-AF2, and *Bacillus subtilis* (D) PTA-271, or buffer control. Each bacterial strain was applied at 1×10^7 CFU/ml at 0 time. Data are means of three repeated experiments. Bars represent standard deviations (S.D.).

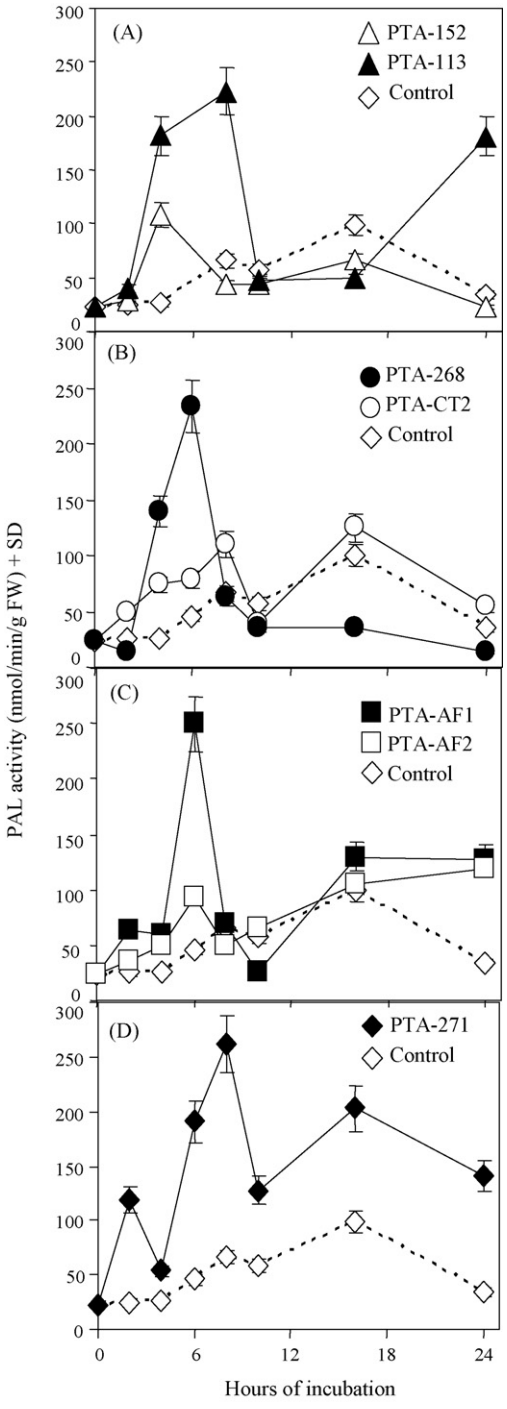

Fig. 3. Time course of phenylalanine ammonia-lyase (PAL) activity in grapevine leaves incubated in bacterial suspension of strains of *Acinetobacter lwoffii* (A) PTA-113 and PTA-152, *Pseudomonas fluorescens* (B) PTA-268 and PTA-CT2, *Pantoea agglomerans* (C) PTA-AF1 and PTA-AF2, and *Bacillus subtilis* (D) PTA-271, or buffer control. Each bacterial strain was applied at 1×10^7 CFU/ml at 0 time. Data are means of three repeated experiments. Bars represent standard deviations (S.D.).

fold increase) (Fig. 2B) and the *P. agglomerans* PTA-AF1 and PTA-AF2 (2–2.5-fold increase) (Fig. 2C). *B. subtilis* PTA-271 also induced LOX activity, but to a lower extent (Fig. 2D).

PAL activity was also induced in bacterized leaves after 2–4 h of incubation (Fig. 3). This response appeared in most cases biphasic, with a maximum PAL activity after 6–8 h, followed by a second peak of lower magnitude after 16 h. Stimulation of PAL activity was also dependent on the bacterial strain. Compared

to the non-bacterized control, the PAL activity after 6–8 h was approximately five times higher in leaves treated with *P. agglomerans* PTA-AF1 (Fig. 3C) and *B. subtilis* PTA-271 (Fig. 3D), and four times higher with *A. lwoffii* PTA-113 (Fig. 3A) and *P. fluorescens* PTA-268 (Fig. 3B). This first activation of PAL was weaker in response to the other bacterial strains. The second peak of PAL activity seemed highest in grapevine leaves treated with *B. subtilis* PTA-271.

Grapevine leaves treated with buffer alone exhibited only low chitinase activity and no significant change during the assay (Fig. 4). In contrast, a rapid increase in chitinase activity was observed in bacterized leaves. Maximum activity was reached 8 h after treatment with the majority of the selected bacteria. After 8 h, chitinase activity decreased progressively, but remained higher than the control throughout the incubation time. The strains *A. lwoffii* PTA-152 (Fig. 4A) and *P. fluorescens* PTA-268 (Fig. 4B) induced 5-fold increase in chitinase activity in grapevine leaves followed by 3–4-fold increase by *A. lwoffii* PTA-113 (Fig. 4A) and *B. subtilis* PTA-271 (Fig. 4D). The induction of chitinase by both *P. agglomerans* strains (PTA-AF1 and PTA-AF2) and *P. fluorescens* PTA-CT2 was not as evident.

3.5. Relationship between biocontrol activity and grapevine defense reactions

Linear regressions were constructed by plotting protection of grapevine leaves by selected bacteria against the average reading of enzyme activity at all incubation times (over a duration of 24 h for LOX and PAL, and 48 h for chitinase). The protection against *B. cinerea* was highly correlated ($R^2 = 0.8917$) with LOX activity (Fig. 5A). This correlation was low ($R^2 = 0.0696$) with PAL response (Fig. 5B) and moderate ($R^2 = 0.6361$) with chitinase activity (Fig. 5C).

4. Discussion

In this study, 282 bacterial strains have been isolated from the rhizosphere and tissues of healthy grapevines located in an area naturally affected by *B. cinerea*. Twenty-six isolates with high biocontrol activity against *B. cinerea* were selected by using detached leaves from in vitro-grown plantlets. Sequencing of 16S rRNA provided consistent results in identifying a set of seven bacterial strains. They have emerged as distinct strains belonging to *P. fluorescens* (designed PTA-268 and PTA-CT2), *A. lwoffii* (PTA-152 and PTA-113), *P. agglomerans* (PTA-AF1 and PTA-AF2) and *B. subtilis* (PTA-271). The phenotypic characteristics and 16S rRNA homology provided the necessary information for phylogenetic position of the different strains, indicating that they form new and distinct bacterial strains for biocontrol purpose. To our knowledge, this study is the first to identify and describe bacteria from the rhizosphere and tissues of grapevine that confer an efficient protection against *B. cinerea*.

Several bacterial strains belonging to the *P. fluorescens* and *Bacillus sp.* displaying biocontrol activities against fungal pathogens have already been isolated from various herbaceous plants (Bargabus et al., 2003; Van den Broek et al., 2003; Cazorla et al., 2006). In this study, *P. fluorescens* PTA-268 and *B. subtilis* PTA-271 were isolated from the rhizosphere, while *P. fluorescens* PTA-CT2 was isolated from grapevine stems. This indicated that *P. fluorescens* could be rhizospheric and endophytic as reported for a *P. fluorescens* strain (FPT9601-T5) originally isolated from tomato rhizosphere and also identified

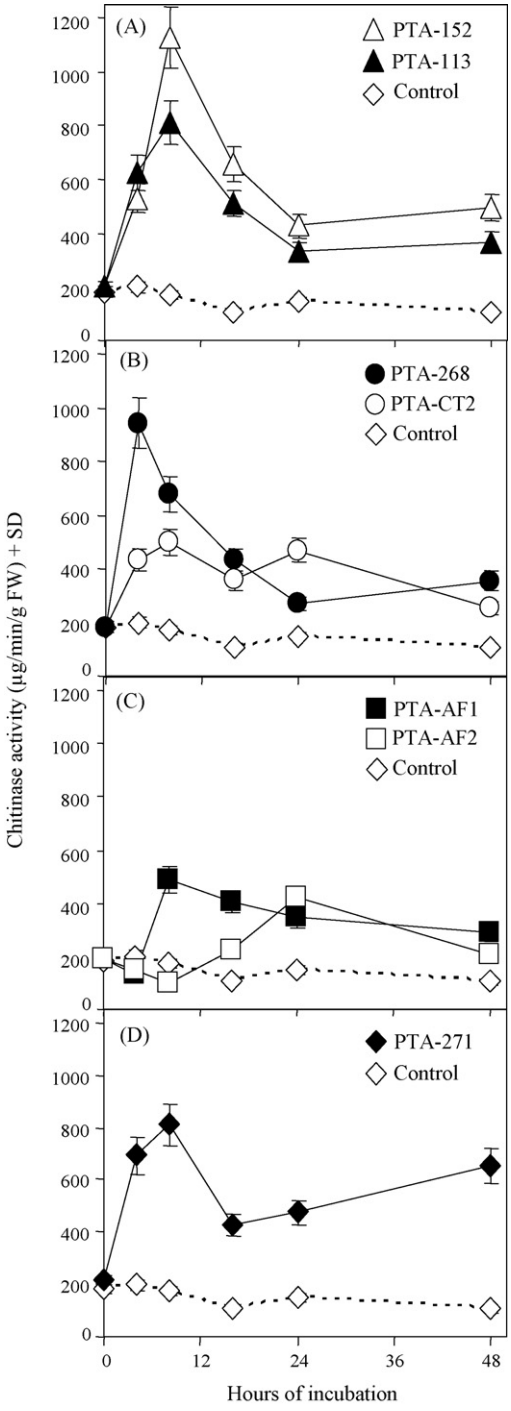

Fig. 4. Time course of chitinase activity in grapevine leaves incubated in bacterial suspension of strains of *Acinetobacter lwoffii* (A) PTA-113 and PTA-152, *Pseudomonas fluorescens* (B) PTA-268 and PTA-CT2, *Pantoea agglomerans* (C) PTA-AF1 and PTA-AF2, and *Bacillus subtilis* (D) PTA-271, or buffer control. Each bacterial strain was applied at 1×10^7 CFU/ml at 0 time. Data are means of three repeated experiments. Bars represent standard deviations (S.D.).

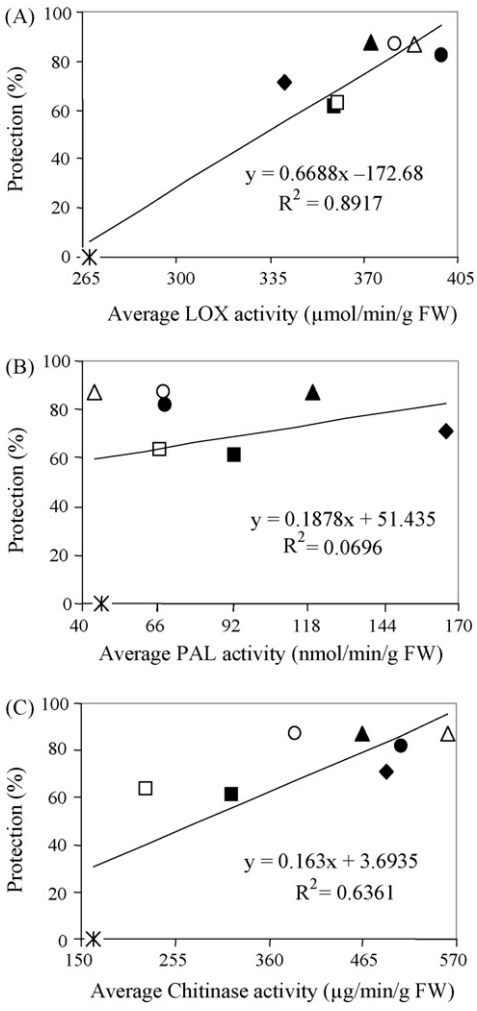

Fig. 5. Relationship between the biocontrol activity of selected bacteria and the activation of grapevine defense markers. Percent protection was defined as reduction in lesion diameter relative to the control. Activity of lipoxygenase (LOX, A), phenylalanine ammonia-lyase (PAL, B), and chitinase (C) at all incubation times were averaged over 24 h (A and B) or 48 h (C) of incubation in bacterial suspension of strains of *Acinetobacter lwoffii* (PTA-113, solid triangle and PTA-152, open triangle), *Pseudomonas fluorescens* (PTA-268, solid circle and PTA-CT2, open circle), *Pantoea agglomerans* (PTA-AF1, solid square and PTA-AF2, open square) and *Bacillus subtilis* (PTA-271, solid diamond) or buffer (asterisk). Data are means of three independent experiments.

as endophytic in *Arabidopsis* (Wang et al., 2005). *B. subtilis* is considered ubiquitous in soil and can protect against fungal pathogens (Asaka and Shoda, 1996; Emmert and Handelsman, 1999). Other beneficial bacteria such as *P. agglomerans* were reported to be efficient epiphytic biocontrol agents (Nunes et al., 2002; Sabaratnam and Beattie, 2003). In our study, both *P. agglomerans* strains PTA-AF1 and PTA-AF2 were isolated from the leaves of grapevine plants. *P. agglomerans* can also colonize roots and the rhizosphere of wheat plants (Amellal et al., 1998). However, little is known about *Acinetobacter* as a biocontrol agent. A previous work reported that the soil bacterium *Acinetobacter* (strain BD413) could colonize plants by their roots and multiply extensively in the pathogen-infected tissues (Kay et al., 2002). In grapevine, the two *A. lwoffii* PTA-113 and PTA-152 bacteria have been isolated from the roots, as such they are endorhizospheric strains, but we cannot exclude their presence in the rhizosphere and other plant organs. This is in agreement with considerations that most bacterial endophytes are the product of a colonizing process initiated in the rhizosphere (Van Peer et al., 1990; McInroy and Klopper, 1995; Sturz et al., 2000), or in the phyllosphere (Hallman et al., 1997).

Mechanisms contributing to disease control by microbial agents include direct antagonistic mechanisms and/or induced resistance in the host plant (Handelsman and Stabb, 1996; Van Loon et al., 1998). In this study, results from the in vitro antifungal experiments revealed that among the seven identified bacteria, only *P. agglomerans* PTA-AF1 and *P. fluorescens* PTA-CT2 displayed important zones of inhibition, which remained constant over time. This indicates the production by these two strains of antifungal metabolites active against *B. cinerea*. Although these antifungal compounds were not further characterized in this study, it is well known that different antibiotics, siderophores and some hydrolytic enzymes such as proteases are produced by several strains of the species *P. fluorescens* (Maurhofer et al., 1992; Iavicoli et al., 2003) and *P. agglomerans* (Kearns and Mahanty, 1998; Wright et al., 2001). Antibiotics or siderophores produced by non-pathogenic rhizobacteria contributed to biological control of various pathogens (Dowling and O'Gara, 1994; Kearns and Mahanty, 1998; Raaijmakers and Weller, 1998; Stockwell et al., 2002).

Selected bacteria-induced grapevine leaf resistance against *B. cinerea*. This resistance was expressed locally on detached leaves pretreated with each bacterium. An induced systemic resistance was also observed when the bacteria were applied to the roots of grapevine plantlets before pathogen challenge (Magnin-Robert et al., 2007). This resistance was clearly associated with early induction of grapevine defense reactions, i.e., stimulation of LOX, PAL and chitinase activities. LOX is required for the synthesis of antifungal oxylipins, such as jasmonic acid that may act as signal factor reinforcing plant defense responses (Creelman and Mullet, 1997; Pieterse et al., 1998). On the other hand, PAL is a key enzyme concerned with the synthesis of salicylic acid and phenolic compounds (Lee et al., 1995; Shadle et al., 2003), both being reported to be associated with plant resistance towards pathogens (Reymond and Farmer, 1998; Aziz et al., 2003, 2006). In bacterized leaves, LOX activity was 2–4-fold higher than the control, depending on the bacterial genus. In our work, *A. lwoffii* strains were more effective in inducing LOX activity than the other genera. Maximum activity of PAL was achieved with *P. agglomerans* PTA-AF1 and *B. subtilis* PTA-271. *A. lwoffii* PTA-113 and *P. fluorescens* PTA-268 also induced a high activity of PAL, while the other strains were less effective. These results suggest that the bacterial strains might induce resistance via distinct signaling pathways. It has already been shown that a non-pathogenic *Pseudomonas* strain could induce LOX pathway, but not PAL activity and reduced disease caused by *B. cinerea* in bean (Ongena et al., 2004). Inversely, induced resistance by *P. fluorescens* in *A. thaliana* was independent on salicylic acid accumulation, but requires jasmonic acid and ethylene pathways (Pieterse et al., 1998). However, another report (Ahn et al., 2002) indicated that

a *Bacillus* sp. induced systemic resistance through both salicylic acid- and jasmonic acid-dependent pathways. Such differential responses might be dependent on the nature of the signal compounds produced by each bacterial strain during interaction with the plant. In our case, grapevine leaf protection appeared better correlated with the stimulation of LOX activity, suggesting a major role of induced resistance through the jasmonic acid dependent pathway.

Bacterial treatment also resulted in an increase in chitinase activity, as a marker of resistance induction (Van Loon and Van Strien, 1999). Chitinase is one of the PR proteins, which constitute a major defense response associated with disease resistance of different grapevine cultivars to fungal pathogens (Derckel et al., 1998; Giannakis et al., 1998; Aziz et al., 2003). Based on their hydrolytic activity some grapevine chitinases exhibit antifungal properties (Salzman et al., 1998). Induction of defense reactions, including chitinase, has also been observed in grapevine leaves and cells following application of salicylic acid or various oligosaccharide elicitors (Derckel et al., 1998; Aziz et al., 2003, 2004, 2006; Trotel-Aziz et al., 2006). Only limited studies attempted to associate non-pathogenic bacteria-inducing plant resistance with PR protein expression. Nevertheless, Bargabus et al. (2003) have reported an induction of chitinase and β-1,3-glucanase activities in sugar beet by an epiphytic bacterium, *Bacillus mycoides*. Similarly, transcriptomic analysis revealed that resistance induced by the rhizobacterium *Pseudomonas thivervalensis* in *A. thaliana* was associated with the expression of various plant defense proteins, including endochitinase (Cartieaux et al., 2003). In this study, grapevine leaf protection appeared only moderately correlated with the stimulation of chitinase activity. Similarly, experiments conducted on *A. thaliana* with *P. fluorescens* indicated that PR protein accumulation is not necessarily associated with induction of plant resistance (Pieterse et al., 1998). This indicates that plant responses to a bacterium may vary according to bacterial strain.

In conclusion, our study demonstrated that some grapevine-associated non-pathogenic bacteria belonging to *A. lwoffii*, *P. fluorescens*, *P. agglomerans* and *B. subtilis* species, are identified as prospective new biocontrol agents against *B. cinerea*. Several bacteria exhibited dual antifungal mechanism through direct antagonism and through inducing the plant defense reactions, while most bacteria-induced plant defense reactions only. This study opens the way to new detailed investigations concerning induced resistance in grapevine by bacteria and their antifungal properties, as well as the determinants of each mechanism.

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