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FULL RESEARCH PAPER

Biological control of *Botrytis cinerea* by selected grapevine-associated bacteria and stimulation of chitinase and β-1,3 glucanase activities under field conditions

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Abstract In this study, the biocontrol ability of seven grapevine-associated bacteria, previously reported as efficient against *Botrytis cinerea* under in vitro conditions, was evaluated in two vineyard orchards with the susceptible cv. Chardonnay during four consecutive years (2002–2005). It was shown that the severity of disease on grapevine leaves and berries was reduced to different levels, depending on the bacterial strain and inoculation method. Drenching the plant soil with these bacteria revealed a systemic resistance to *B. cinerea*, even without renewal of treatment. Accordingly, this resistance was associated with a stimulation of some plant defense responses such as chitinase and β-1,3-glucanase activities in both leaves and berries. In leaves, chitinase activity increased before veraison (end-July) while β-1,3-glucanase reached its maximum activity at ripening (September). Reverse patterns were observed in berries, with β-1,3-glucanase peaking at full veraison (end-August) and chitinase at a later development stage. Highest activities were observed with *Acinetobacter lwoffii* PTA-113 and *Pseudomonas fluorescens* PTA-CT2 in leaves, and with *A. lwoffii* PTA-113 and *Pantoea agglomerans* PTA-AF1 in berries. These results have demonstrated an induced protection of grapevine against *B. cinerea* by selected bacteria under field conditions, and suggest that induced resistance could be related to a stimulation of plant defense reactions in a successive manner.

Keywords Biocontrol · Grey mould · Induced resistance · Rhizobacteria · *Vitis vinifera*.

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Introduction

Grapevine (*Vitis vinifera*) varieties are sensitive to a great number of fungal pathogens. Among them *Botrytis cinerea*, the causal agent of grey mould provokes heavy damages, yield losses and affects wine quality. The spread of this fungal disease is generally controlled by applications of chemicals. However, because of the emergence of pesticide-resistant pathogen strains (Leroux, Chapeland, Desbrosses, & Gredt, 1999), and in

an effort to reduce the impact of pesticides on the environment, considerable interest has been devoted to sustainable alternative strategies. Grapevine resistance could be improved by genetic engineering, but hybrids and transformed grapevine (Coutos-Thévenot et al., 2001) are forbidden in French vineyards. Thus, the alternative strategies most commonly involved are the activation of plant defense mechanisms using natural elicitor products (Aziz et al., 2003; Elmer & Reglinski, 2006; Trotel-Aziz, Couderchet, Vernet, Aziz, 2006b), or else biological control of pathogens by microorganisms (Barka, Gognies, Nowak, Audran, & Belarbi, 2002; Pieterse, van Wees, Hoffland, van Pelt, & van Loon, 1996; Trotel-Aziz, Aziz, Magnin-Robert, Aït Barka, & Gogniès, 2006a; van Wees et al., 1997; van Loon, Bakker, & Pieterse, 1998; Whipps, 2001).

Several non-phytopathogenic rhizosphere-colonizing or endophytic bacteria have been frequently shown to provide plant protection against various pathogens. Direct antagonistic interactions with the pathogen have been described as a mechanism of biocontrol mediated by rhizobacteria. These antagonistic interactions can involve the production of antibiotic compounds, competition for nutrients, siderophore-mediated competition for iron and production of extracellular enzymes (Dowling & O'Gara, 1994; Iavicoli, Boutet, Buchala, & Métraux, 2003; Maurhofer, Hase, Meuwly, Métraux, & Defago, 1994; Meziane, van der Sluis, van Loon, Höfte, & Bakker, 2005; Whipps, 2001). Beside these direct interactions, some plant growth-promoting bacteria are also able to reduce disease through the stimulation of inducible plant defense mechanisms, which lead to a state of resistance in the whole plant against pathogens. This type of resistance generally called rhizobacteria-induced systemic resistance (ISR) (Pieterse et al., 1996; van Loon et al., 1998) has been demonstrated in different plant species against several pathogens under conditions where the bacteria and the pathogen remained spatially separated (Hoffland, Pieterse, Bik, & van Pelt, 1995; Iavicoli et al., 2003; Meziane et al., 2005; Pieterse et al., 1996). For example, *Pseudomonas fluorescens*, a gram-negative soil bacterium is among the most effective and has been shown to be responsible for the reduction of *Fusarium* wilt in *Arabidopsis thaliana* (Pieterse et al., 1996; van Wees et al., 1997), radish (Leeman et al., 1995; Raaijmakers et al., 1995) and tomato (Duijff, Gianinazzi-Pearson, & Lemanceau, 1997), and of *Peronospora parasitica* in *Arabidopsis thaliana* (Iavicoli et al., 2003). Another ubiquitous gram-positive rhizobacterium, *Bacillus subtilis* can also protect plants against fungal pathogen attack (Asaka & Shoda, 1996; Baker, Stavely, & Mock, 1985; Chanway, 2002; Dowling & O'Gara, 1994; Schmidt, Agostini, Killham, & Mullins, 2004).

The mechanisms involved in bacteria-mediated ISR appear to vary among bacterial strains, plant species and pathogens. In some cases, ISR induced by non-pathogenic rhizobacteria is characterized by a systemic accumulation of pathogenesis-related (PR) proteins (Bargabus, Zidack, Sherwood, & Jacobsen, 2003; Maurhofer et al., 1994; Tjamos, Flemetakis, Paplomatas, & Katinakis, 2005), which is mainly associated with systemic acquired resistance (SAR) induced by pathogens (van Loon & van Strien, 1999). Rhizobacterial ISR is also associated with defense mechanisms independent of PR protein expression (Hoffland et al., 1995; Pieterse et al., 1996, 1998; van Wees et al., 1997).

In grapevine, various inducible defense responses have been characterized during interaction with pathogenic fungi including the synthesis of PR proteins in both leaves and berries in response to *B. cinerea* (Bézier, Lambert, & Baillieul, 2002; Derckel, Audran, Haye, Lambert, & Legendre, 1998; Robert et al., 2002) and *Plasmopara viticola* (Busam, Kassemeyer, & Matern, 1997; Robert et al., 2002), and the accumulation of phytoalexins in challenged berries with *B. cinerea* (Montero et al., 2003). Similar responses have also been activated by biotic and abiotic elicitors (Aziz et al., 2003; Bonomelli et al., 2004; Elmer & Reglinski, 2006; Trotel-Aziz et al., 2006b), leading to an increased resistance to *B. cinerea* and *P. viticola*. PR proteins, such as chitinases and β-1,3-glucanases have received particular attention (Aziz, Heyraud, & Lambert, 2004; Bonomelli et al., 2004; Busam et al., 1997; Derckel et al., 1998; Robert et al., 2002), because they are thought to contribute to the state of resistance attained and are known to inhibit

mycelial growth of a wide range of fungal pathogens (Derckel et al., 1998; Mauch, Mauch-Mani, & Boler, 1988; van Loon & van Strien, 1999).

Induced resistance by bacteria has not yet been reported for the biological control of fungal diseases in vineyards. A *Burkholderia* sp. originally isolated from onion was shown to control *B. cinerea* on in vitro grapevine grown-plantlets (Barka et al., 2002). This effect has been 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 also shown to be effective against various pathogenic fungi (http://www.agraquest.com). Moreover, microorganisms isolated from the rhizosphere or from tissues of a specific plant may be better adapted to that plant and may therefore provide better control of diseases than organisms originally isolated from other plant species (Cook, 1993). Such plant-associated microorganisms are considered to be closely associated with the plant or plant part as well as the particular environmental conditions in which they must function.

Recently, we isolated seven new non-phytopathogenic bacterial strains from vineyards that are capable of controlling *B. cinerea* in detached leaves from in vitro-grown plantlets (Trotel-Aziz et al., 2006a). In the present study, we sought to evaluate the effectiveness of these selected bacteria in controlling *B. cinerea* and inducing resistance reactions in grapevine plants under field conditions. Experiments were conducted during four growing seasons (from 2002 to 2005) in two vineyard orchards located in Champagne area (France) (Table 1), where bacteria have been isolated. In the first orchard, we compared the biocontrol efficiency of the selected bacteria in leaves, by using three methods of inoculation: syringe infiltration, foliar spray and soil drenching. In the second orchard, selected bacteria were applied by only soil drenching, and responses of grapevine plants were evaluated by monitoring protection assays against *B. cinerea* and specific activities of chitinase and β-1,3-glucanase (as markers of induced resistance) in both leaves and berries.

Materials and methods

Bacterial cultures

Bacteria used in this study were originally isolated from the rhizosphere of field-grown grapevines in Champagne area, Marne, France. They were selected from several microbial strains based on their efficiency as biocontrol agents of *B. cinerea* in excised leaves from in vitro-grown grapevine plantlets (Trotel-Aziz et al., 2006a), and identified as two strains of *Acinetobacter lwoffii* (PTA-113 and PTA-152), one *Bacillus subtilis* (PTA-271), two *Pantoea agglomerans* (PTA-AF1 and PTA-AF2) and two *Pseudomonas fluorescens* (PTA-268 and PTA-CT2). Bacteria were grown in the dark on Luria-Bertani (LB) liquid medium (Sigma) at 24°C with continuous shaking (150 rpm) for 24 h before they used in all the trials. Cell concentrations were determined with spectrophotometric readings at 450 and 650 nm, and adjusted to 1×10^8 CFU ml^{-1}.

Fungal pathogen

Highly virulent *Botrytis cinerea* strain 630 (Derckel et al., 1999) (kind gift of Y. Brygoo, INRA, Versailles, France) was cultured and maintained on potato dextrose agar (PDA, Sigma) in 200 ml Erlenmeyer flasks at 22°C with a 16/8 h photoperiod (cool-white neon tubes (3,000 l×). Conidia were harvested from 14- to 20-day-old cultures and collected by rubbing with 10 ml of sterile distilled water (SDW), filtered on sterile glass beads to remove mycelia, and the concentration determined using the Thoma slide and photonic microscope, adjusted to 1×10^5 conidia ml^{-1}.

Field experiments

Grapevine plants (*Vitis vinifera* cv. Chardonnay, on 41B rootstock) 10 years old at the beginning of the experiments were treated in field trials of research orchards from 2002 to 2005 in two sites, Cernay (CR) and Nogent (NL) at the Champagne area (France) (Table 1). Vine spacing was 1.05 apart within the row and 1.20 m between rows in both vineyards. Chemicals needed for the control of downy and powdery mildew, as well as canopy

Table 1 Methods of bacterial treatments, disease assessment and defense reactions performed in two vineyard orchards in 2002, 2003, 2004 or 2005

		Vineyard CR				Vineyard NL			
		2002	2003	2004	2005	2002	2003	2004	2005
Application methods	Infiltration	+	+	+	–	–	–	–	–
	Foliar spray	+	+	+	–	–	–	–	–
	Soil drenching	+	+	+	–	+	+	+	–
Disease assessment	Leaves	+	–	+	+	–	–	+	+
	Berries	–	–	–	–	+	–	+	+
Defense reactions	Leaves	–	–	–	–	–	+	+	–
	Berries	–	–	–	–	–	+	+	–

Seven selected bacteria were applied through three methods (infiltration, spray, soil drench) in 2002, 2003 and 2004 in CR orchard. In NL orchard these bacteria were applied only by soil drench in 2002, 2003 and 2004. Bacteria were not reapplied in 2005 in both orchards. Disease was evaluated in leaves (from both vineyards) and berries (in NL orchard) from control and inoculated plants. Chitinase and β-1,3 glucanase activities were determined in leaves and berries of plants cultivated in NL orchard. +, applied or determined; –, non-applied or non-determined.

management were carried out for all trials, but no botryticides were used during growing seasons.

During 2002 and 2004 seasons, there were three modes of bacterial application in the first vineyard (infiltration into buds, foliar spray and soil drench), and in the second one plants were treated only by soil drench with the same bacteria (Table 1). Treatments consisted of non-bacterized controls, syringe infiltration of 10 ml bacterial suspension per plant at budburst (10 buds, with 1 ml per bud), foliar spray by using a misting bottle with 100 ml of each bacterial suspension per plant at the stage of 3–4 unfolded leaves and a soil drench using a graduated cylinder with 200 ml and 150 ml bacterial suspension per plant in CR and NL vineyards, respectively by flooding the first 10 cm of the soil in contact and around the rootstock. Control plants were either treated or not treated with water. The experimental design was a randomized complete block with 5–12 plants per plot and three replications. Both leaves from the top of shoots (30 per treatment) and berries (10 clusters per treatment) without visible symptoms were harvested at different dates during growing each season and stored at –80°C for the analysis of defense reactions. Bacterial treatments were not reapplied in 2005.

Challenge inoculation and disease assessment

Young fully expanded leaves were excised from the top of shoots (30 leaves per treatment) 2 months after application of bacteria, immediately placed into moistened plastic bags and taken to the laboratory. Leaves were then rinsed with SDW and placed on wet absorbing paper in Petri dishes. One needle-prick wound was applied on the middle of the abaxial surface of each leaf, and the fresh wounds were covered with 10 µl drops of a conidial suspension of *B. cinerea* (1×10^5 conidia ml^{-1}). The Petri dishes were then placed at 22°C with a photoperiod of 16 h light. Disease development on leaves was measured as the average diameter of lesions formed 7 days post-challenge with *B. cinerea*.

For grapevine berries, disease severity was evaluated at full ripening in vineyards by measuring the % of naturally infected berries per cluster based on actual berry count. This was determined by measuring actual sporulating berries in each trial on 100 clusters of inoculated plants, and 100 clusters of control plants in 2002, 2004 and 2005, from both sides of the rows in 2004 and 2005.

Chitinase and β-1,3-glucanase activity

Grapevine chitinases and β-1,3-glucanases were extracted by grinding frozen leaves (500 mg FW) and de-seeded berries (1 g FW), respectively into 2 ml of 50 mM and 100 mM sodium acetate buffer, pH 5.0 containing 1 mM dithiothreitol and 0.2% (w/v) phenylmethylsulfonyl fluoride at 0°C. The crude extracts were centrifuged at

10,000 *g* for 5 min at 4°C and supernatants were used for enzymatic activity assays.

Chitinase and *β*-1,3-glucanase activities were assayed according to the method of Wirth and Wolf, (1992) with carboxymethyl-chitin-remazol brilliant violet and carboxymethyl-curdlan-remazol brilliant blue, as respective substrates (Loewe Biochemica, Germany). The reactions were started by mixing 100 µl of specific substrate (2 mg ml^{-1}), 100 µl of 200 mM sodium acetate buffer, pH 5.0 and 50–200 µl of diluted enzymatic extract in 50 mM sodium acetate buffer. The mixtures were incubated at 37°C for various times (1–20 min) and the reactions were stopped by the addition of 400 µl of cold 1 M HCl and immediately kept on ice. The undigested substrate was precipitated by centrifugation (4°C, 10,000 *g* for 10 min) and the absorbance of the supernatant was recorded at 550 nm for chitinase and at 600 nm for *β*-1,3-glucanase activity. Calibration curves were established by specific substrates.

Data analysis

The effects of selected bacteria on disease development, evaluated on 30 leaves per treatment and 100 clusters per treatment, were performed by using analysis of variance (ANOVA), and Duncan's multiple range test ($P \leq 0.05$) was used for post-hoc comparison of means. Statistica software (Statsoft Inc., Tulsa, USA) was used for statistical data analysis. Defense reactions were determined on 10 leaves and 10 berries (from three to four clusters per treatment) all in triplicates. Data are means ± standard errors.

Results

Protection of grapevine leaves against *B. cinerea* depends on bacterial strain and inoculation method

The selected bacteria were used to evaluate their protective ability in two vineyards. In 2002 and 2004 seasons, leaves from control and inoculated plants were collected and artificially challenged in the laboratory with *B. cinerea* after 2 months of bacterial inoculations. No measured effect of bacteria on disease development was done during the 2003 growing season. In 2002 and 2004 the control leaves displayed severe disease symptoms seven days post-infection with *B. cinerea* (Fig. 1). This generally contrasted with leaves of plants previously treated with bacteria, which showed significantly smaller lesions ($P \leq 0.05$), with some interactions among bacterial strains and inoculation methods for each strain ($P \leq 0.05$).

With the infiltration method, the necrosis on the leaves from plants treated with *A. lwoffii* PTA-113 and *A. lwoffi* PTA-152 was reduced by about 24–28% in 2002 (Fig. 1a). With *P. agglomerans* PTA-AF1 disease was reduced by about 20%, while the other strains had no significant effect in the same year. However, in 2004, *A. lwoffii* PTA-152, *P. agglomerans* PTA-AF1, and *P. fluorescens* PTA-268 and PTA-CT2 strains were effective in reducing leaf necrosis by 20–50% compared to the control from non-inoculated plants (Fig. 1d). PTA-113, PTA-271 and PTA-AF2 had no significant effect under field conditions. *Acinetobacter lwoffii* strains and *P. agglomerans* PTA-AF1 provided the greatest reduction of *B. cinerea* symptoms in 2002 and 2004. Spray-applied bacteria had no significant effect on necrotic lesions in 2002 (Fig. 1b). However, in 2004, the spray with *P. agglomerans* PTA-AF1 and *P. fluorescens* PTA-CT2 resulted in a significant disease reduction (39–43%), while *A. lwoffii* PTA-113 and PTA-152, *B. subtilis* PTA-271, *P. agglomerans* PTA-AF2 and *P. fluorescens* PTA-268 had only a moderate protective effect (Fig. 1e). When applied by soil drenching the two *A. lwoffii* PTA-113 and PTA-152, *P. agglomerans* PTA-AF1 and PTA-AF2 as well as *P. fluorescens* PTA-268 resulted in a significant disease reduction (20–37.5%) in 2002 (Fig. 1c). Similarly, *A. lwoffii* PTA-113 caused a great reduction of grey mould symptoms in 2004 (Fig. 1f), which was equivalent to that caused by *P. agglomerans* PTA-AF1 and PTA-AF2, and *P. fluorescens* PTA-268 and CT2 (28–50%). With the soil drench method, *A. lwoffii* PTA-152 and *B. subtilis* PTA-271 were less effective. Control efficacy achieved by *P. agglomerans* PTA-AF1 was, in most cases, higher than that achieved with the other isolates considering the three methods of inoculation. Furthermore, of the three methods, soil drenching

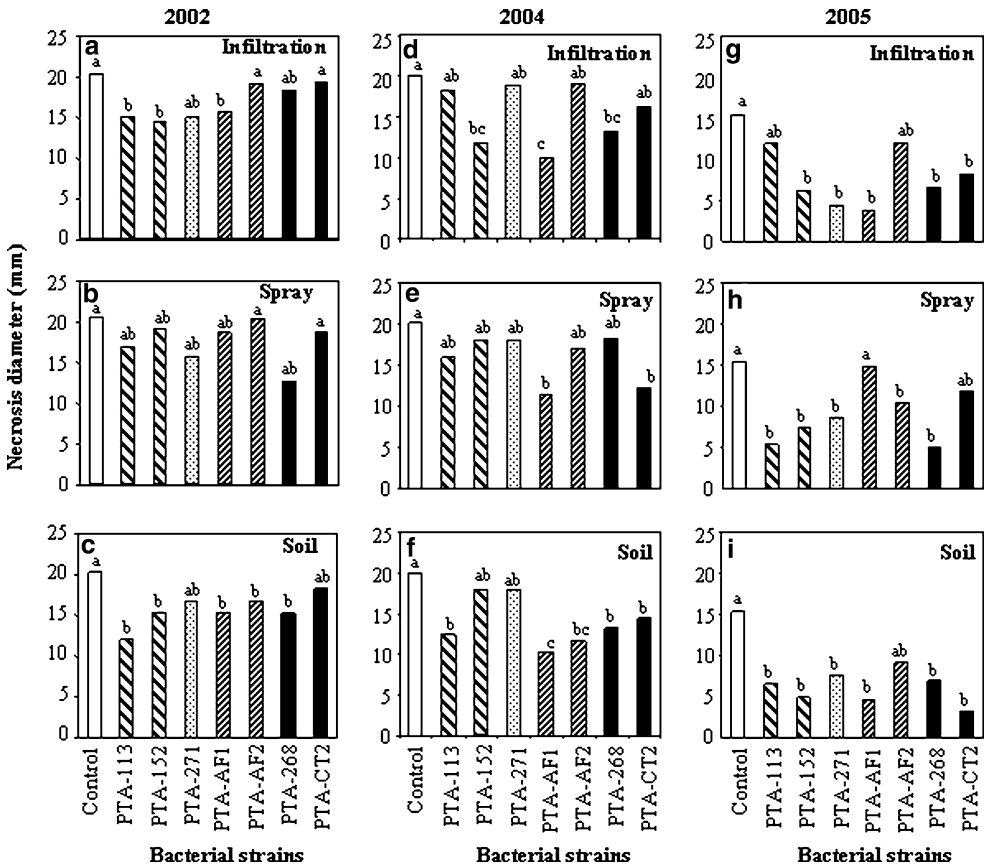

Fig. 1 Protection of grapevine leaves against *B. cinerea* by different bacterial strains and relationship with inoculation method. Each bacterium was applied at 1×10^8 CFU ml^{-1} in vineyard (CR trials) at the beginning of 2002, 2003 and 2004 growing seasons, by infiltration (**a**, **d**) into buds at burst stage (10 ml per plant), spray (**b**, **e**) on 3–4 young unfolded leaves (100 ml per plant) or soil (**c**, **f**) drenching (200 ml per plant). Treatments consisted of different strains of *A. lwoffii* (PTA-113 and PTA-152), *P. fluorescens* (PTA-268 and PTA-CT2), *P. agglomerans* (PTA-AF1 and TA-AF2), *B. subtilis* (PTA-271), and control (without bacteria). After 2 months, expanded young leaves were detached and challenged with *B. cinerea*. Bacteria were not reapplied in 2005 (**g**–**i**), but disease was evaluated in the same trials as above. Data are means of lesion diameter from 30 leaves per treatment recorded 7 days post-challenge. Columns headed by the same letter are not significantly different ($P < 0.05$)

provided the greatest efficiency for most of the bacteria.

A similar tendency was observed during 2005 growing season even without renewal of bacterial inoculation (Fig. 1g–i). Leaves from plants previously inoculated by infiltration with *B. subtilis* PTA-271 and *P. agglomerans* PTA-AF1 showed a strong reduction of disease symptoms (72–75%). *Acinetobacter lwoffii* PTA-152 and both *P. fluorescens* PTA-268 and PTA-CT2 strains slightly reduced disease (Fig. 1g), whereas *A. lwoffii* PTA-113 and *P. agglomerans* PTA-AF2 did not show any significant effect. Leaves from sprayed plants with *A. lwoffii* PTA-113 and *P. fluorescens* PTA-268 showed a reduction of necrosis of 65–68% (Fig. 1h). Importantly, a strong reduction of leaf disease was also obtained following soil drench (Fig. 1i), especially with *A. lwoffii* PTA-152, *P. agglomerans* PTA-AF1 and *P. fluorescens* PTA-CT2, reaching values of about 67–78% when compared to the control.

Assessment of induced systemic resistance by selected bacteria

To assess a systemic effect of inoculation on grapevine plants, protection assays were monitored in both leaves and berries after drenching

the plant soil with each bacterium in a second vineyard (NL trials). In non-inoculated plants, disease symptoms caused by *B. cinerea* on the leaves were characterized by large necrotic lesions, whereas they were much less severe in inoculated plants in the 2004 season (Fig. 2a). *Acinetobacter lwoffii* (PTA-113 and PTA-152) and *P. fluorescens* (PTA-268 and PTA-CT2) strains induced a significant reduction of disease by about 45–70%. *Pantoea agglomerans* PTA-AF1 and AF2 also led to a significant disease reduction, while *B. subtilis* PTA-271 remained less efficient (Fig. 2a). However, reduction of leaf disease in the 2005 season (Fig. 2b) was moderately effective in the same trials. The greatest level of disease reduction in grapevine leaves was observed in trials previously treated with *P. agglomerans* PTA-AF1 and *P. fluorescens* PTA-268 and PTA-CT2.

Mature clusters were also analyzed in the same trials from treated and untreated plants at ripening. Disease severity was determined as the % of naturally infected berries per cluster. In control plants, a mean of 16 and 44.7% of infected berries within the cluster was the most frequent in 2002 and 2004, respectively (Table 2). No disease development was observed during the 2003 growing season in the Champagne trials, due to unfavourable drought weather and non-development of *B. cinerea* (data not shown). In 2004, disease in control plants was 35% infection on the east and 54.5% on the west side of the grapevine rows. Clusters from inoculated plants showed a strong heterogeneity under natural contamination. Only *A. lwoffii* PTA-152 and *P. agglomerans* PTA-AF1 treatments resulted in a strong disease reduction in 2002 (by 42–50%). In 2004, *P. agglomerans* PTA-AF1-treated plants also exhibited a significant reduction of infection (26% protection) (Table 2). PTA-AF1 seemed to be more efficient on the east side with a protection of 40%, versus 12.5% on the west side. PTA-AF2 also resulted in a slight protection of berries only on the west side. The other inoculated plants showed % infected berries similar or greater than control values.

In the 2005 growing season, the intensity of *B. cinerea* contamination was equivalent to that recorded in 2002, but less important than that observed in 2004. Clusters from control plants in 2005 showed a similar degree of infection between east and west sides (13.3% infected berries per cluster) (Table 2). Under these conditions, most of the plants previously treated with bacteria showed a consistent reduction in disease, especially on the east side (42–64% protection). PTA-113 and PTA-AF2-treated plants showed the highest protection against *B.*

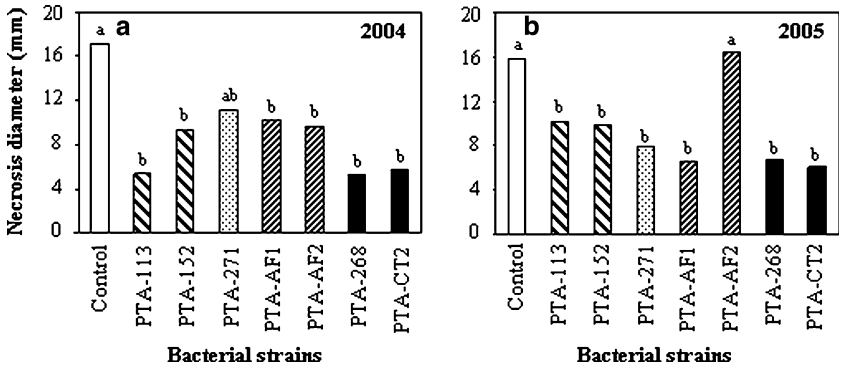

Fig. 2 Protection of grapevine leaves against *B. cinerea* infection in bacteria-treated plants by soil drenching. Treatments consisted of different strains of *A. lwoffii* (PTA-113 and PTA-152), *P. fluorescens* (PTA-268 and PTA-CT2), *P. agglomerans* (PTA-AF1 and TA-AF2), *B. subtilis* (PTA-271), and control (without bacteria). Each bacterium was applied at 1×10^8 CFU ml^{-1} (150 ml per plant) in vineyard (NL trials) at the beginning of 2004 growing season (**a**). After 2 months, expanded young leaves were detached and challenged with *B. cinerea*. Bacteria were not reapplied in 2005 (**b**), but disease was evaluated in the same trials. Data are means of lesion diameter from 30 leaves per treatment recorded 7 days post-challenge. Columns headed by the same letter are not significantly different ($P < 0.05$)

Table 2 Reduction of grey mould disease caused by *B. cinerea* in grapevine berries after application of bacteria in two vineyard trials

Selected bacteria	Strain designation	% infected berries per cluster						
		2002	2004			2005		
		Total	Total	East side	West side	Total	East side	West side
	Control	16.0 a	44.7 a	34.9 a	54.5 a	13.4 a	13.3 a	13.5 a
A. lwoffii	PTA-113	10.8 ab	46.9 a	35.6 a	58.3 a	4.8 b	4.6 b	5.1 b
A. lwoffii	PTA-152	8.0 b	48.6 a	41.1 a	56.0 a	7.9 b	4.6 b	11.2 ab
B. subtilis	PTA-271	10.3 ab	47.2 a	34.7 a	59.8 a	9.8 ab	7.7 b	11.9 ab
P. agglomerans	PTA-AF1	9.2b	34.0 b	20.3 b	47.7 b	7.5 b	5.3 b	9.8 ab
P. agglomerans	PTA-AF2	12.8 ab	37.2 ab	33.9 ab	40.2 b	5.1 b	5.3 b	4.9 b
P. fluorescens	PTA-268	13.3 a	54.7 a	39.4 a	70.0 a	14.5 a	10.6 ab	18.4 a
P. fluorescens	PTA-CT2	10.7 ab	58.4 a	48.8 a	68.0 a	8.5 ab	3.7 b	13.4 a

Treatments consisted of two strains of *A. lwoffii* (PTA-113 and PTA-152), two *P. fluorescens* (PTA-268 and PTA-CT2), two *P. agglomerans* (PTA-AF1 and TA-AF2), one *B. subtilis* (PTA-271), and control (without bacteria). Each bacterium was applied in vineyard NL trials by soil drenching (150 ml plant^{-1}) at the beginning of the 2002 and 2004 growing seasons. Bacterial treatments were not reapplied in 2005, but disease severity was measured in the same trials, at full ripening. Disease assessments were based on % of infected berries per cluster, determined on 100 clusters per treatment, consisting of 50 on east and 50 on west side of the rows. Values followed by the same letters within rows are not significantly different ($P < 0.05$, one-way analysis of variance).

cinerea (62–64%), PTA-152 and PTA-AF1 reduced disease symptoms by about 41–44%, whereas *B. subtilis* and *P. fluorescens* strains led to low protection.

Chitinase and β-1,3-glucanase activities in grapevine leaves and berries

To provide insights into defense reactions associated with the induction of resistance in grapevine, chitinase and β-1,3 glucanase activities were monitored in both leaves and berries of control and inoculated plants by soil drenching, during the 2003 and 2004 seasons. In most cases, the activity of both PR proteins was enhanced following the treatment with selected bacteria. In 2003, bacteria were moderately effective and led to a slight induction of chitinase and β-1,3-glucanase activities when compared to the control (data not shown). Experiments during 2004 showed a greater increase of chitinase activity in leaves before veraison (at July), reaching maximum values within 2 months post-inoculation with bacteria (Fig. 3). Thereafter, chitinase activity declined progressively for both control and inoculated plants until the end of the growing season. Higher chitinase activity was achieved with *P. fluorescens* PTA-CT2 (5-fold increase) followed by PTA-268, *A. lwoffii* PTA-113 and *B. subtilis* PTA-271. The other bacteria also stimulated leaf chitinase activity but to a lesser extent (Fig. 3).

β-1,3-glucanase activity (Fig. 4) increased in leaves of all inoculated plants, with a short early peak before veraison and strongest activity 6 weeks later. This PR protein activity was highest after veraison in response to *A. lwoffii* (PTA-113, PTA-152), *P. fluorescens* PTA-CT2, *P. agglomerans* PTA-AF1 and *B. subtilis* (PTA-271). A significant increase of β-1,3-glucanase activity was also observed in PTA-268 and PTA-AF2-treated plants. No apparent increase in chitinase and β-1,3-glucanase activities above the basal level was observed in leaves earlier than 2 months following the soil drench with bacteria.

In berries, chitinase and β-1,3-glucanase activities (Figs. 5 and 6) showed inverse patterns throughout grapevine development in control and inoculated plants. The soil drenching with most bacteria was effective in inducing both PR proteins in berries. In 2003, the level of chitinase activity was very low before veraison, but a detectable increase was observed with PTA-271, PTA-AF1, PTA-268 and PTA-CT2 (data not shown). Furthermore, at the same stage, β-1,3-glucanase activity was important in control berries and enhanced only in plants treated with

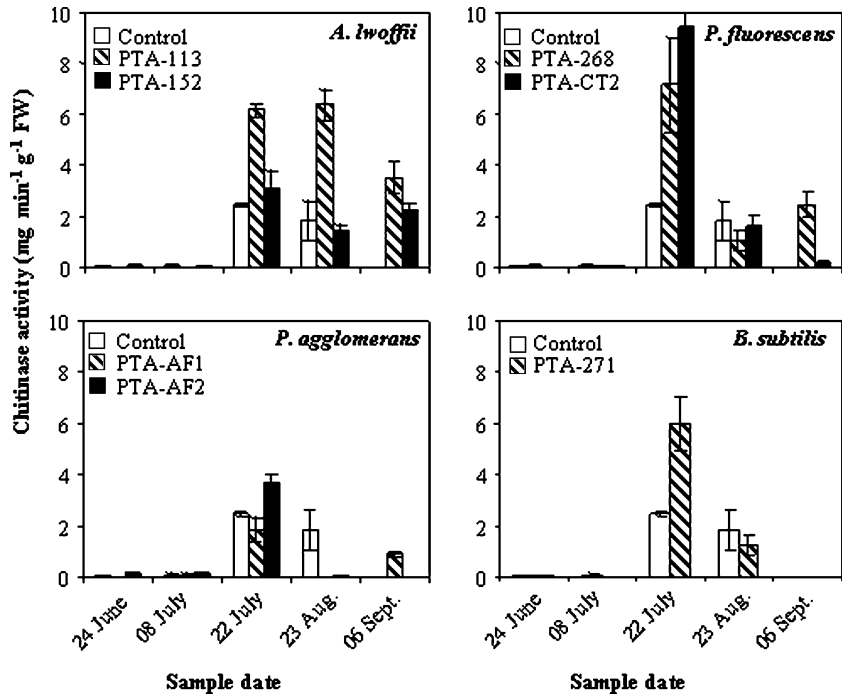

Fig. 3 Chitinase activity in leaves of grapevine plants treated with native bacteria by soil drenching. Each bacterium was applied at 1×10^8 CFU ml^{-1} (150 ml per plant) in vineyard (NL trials) at the beginning of 2004 growing season. Treatments consisted of two strains of *A. lwoffii* (PTA-113 and PTA-152), two *P. fluorescens* (PTA-268 and PTA-CT2), two *P. agglomerans* (PTA-AF1 and TA-AF2), one *B. subtilis* (PTA-271), and control (without bacteria). Chitinase activity was determined at different dates in 2004. Data are means from three independent measurements, each consisting of three replicates. Bars indicate standard errors

PTA-152, PTA-AF1 and PTA-268. With large samplings in 2004, similar trends of chitinase (Fig. 5) and β-1,3-glucanase (Fig. 6) activities were observed in grapevine berries. Chitinase activity increased gradually in both control and inoculated plants starting from veraison until berry ripening (Fig. 5). In berries of inoculated plants (except those treated with PTA-AF2), chitinase activity was two- to five-fold above that observed in the control plants. Inversely, β-1,3-glucanase activity (Fig. 6) was important in berries of control plants before veraison and decreased progressively, reaching a lower value at ripening. Soil drenching with bacteria resulted in a transient increase of β-1,3-glucanase activity with a maximum level at veraison. Highest activity was achieved with *A. lwoffii* PTA-113 and *P. fluorescens* PTA-CT2, followed by *B. subtilis* PTA-271 (Fig. 6). PTA-152, PTA-268 and *P. agglomerans*-treated plants showed only low or no effects.

Discussion

Exploitation of grapevine-associated bacteria as biological control agents could result in the establishment of efficient pathogen management and in a reduction of fungicide use in vineyards. In this context, seven bacteria from the rhizosphere and tissues of healthy grapevines (cv. Chardonnay) showing efficient biocontrol against *B. cinerea* under in vitro conditions, were recently characterised (Trotel-Aziz et al., 2006a). Here we evaluated the effectiveness of these bacteria in inducing resistance of grapevine against grey mould in two vineyard orchards where the bacteria originated. The results with these bacteria show that the biocontrol of *B. cinerea* on grapevine in in vitro experiments can also be obtained under field conditions. Experiments carried out in the first vineyard, using artificially-challenged leaves, showed that the efficiency of biocontrol depends on the bacterial strain and the method of

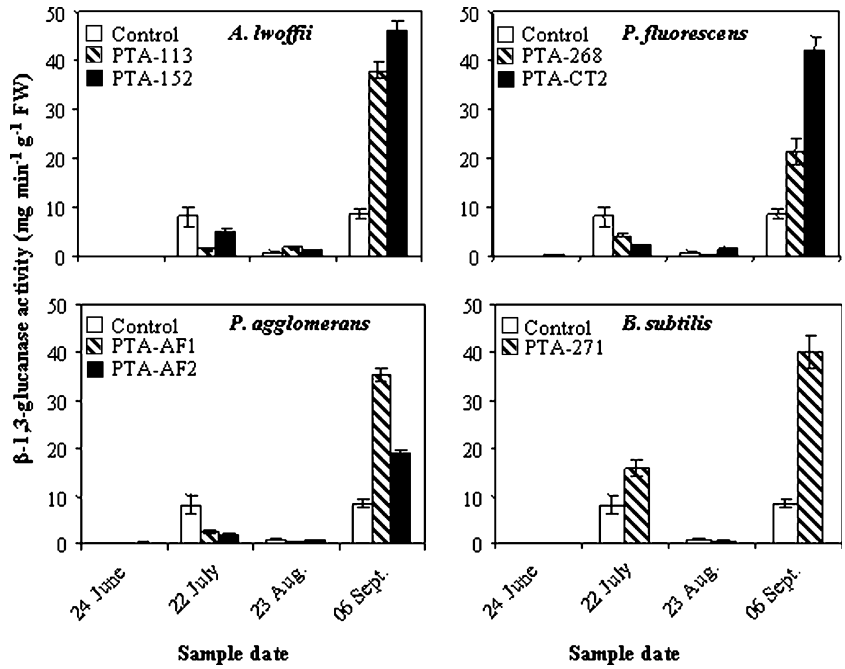

Fig. 4 β-1,3-Glucanase activity in leaves of grapevine plants treated with native bacteria by soil drenching. Each bacterium was applied at 1×10^8 CFU ml^{-1} (150 ml per plant) in vineyard (NL trials) at the beginning of 2004 growing season. Treatments consisted of two strains of *A. lwoffii* (PTA-113 and PTA-152), two *P. fluorescens* (PTA-268 and PTA-CT2), two *P. agglomerans* (PTA-AF1 and TA-AF2), one *B. subtilis* (PTA-271), and control (without bacteria). β-1,3-Glucanase activity was determined at different dates in 2004. Data are means from three independent measurements, each consisting of three replicates. Bars indicate standard errors

inoculation (i.e. infiltration, spray or soil drench). Moreover, biocontrol efficacy of *P. fluorescens* and *P. agglomerans* PTA-AF1 was, in most cases, higher than that achieved with *A. lwoffii* and *B. subtilis* strains, whatever the inoculation method. Nevertheless, applied by soil drench, *A. lwoffii* PTA-113 provides also a consistent protection of leaves against *B. cinerea*. The application method can effectively influence the distribution and efficacy of biocontrol agents (Mazzola, Stahlman, & Leach, 1995).

Inoculated plants by soil drench in the second vineyard orchards showed a significant disease reduction in 2004, in both leaf necrosis and the % of naturally infected berries. This suggests that native bacteria could induce systemic resistance in grapevine, probably through their spread and colonisation of tissues. The greatest level of disease reduction in leaves was obtained with *A. lwoffii* (PTA-113 and PTA-152), *P. fluorescens* (PTA-268 and PTA-CT2) and *P. agglomerans* PTA-AF1. In addition, some heterogeneity exists in the protective effect of bacteria in grape berries. A significant disease reduction was observed with PTA-AF1 only in clusters of the east side of rows, characterized by a natural low *B. cinerea* infection. This could be explained by a difference in microclimate, which could influence fungal growth (Holz, Gütschow, Coertze, & Calitz, 2003). Disease pressure also affects the biocontrol potential of the bacteria since the highest protection (42–64%) observed in 2002 and 2005 was correlated to a lower % of infected berries (13.3–16%). This agrees with other reports showing that biocontrol of some pathogenic fungi is less effective in the presence of high disease pressure (Landa, Navas-Cortés, Hervas, & Jiménez-Diaz, 2001; Schmidt et al., 2004). Nevertheless, it is noteworthy that *Pantoea agglomerans* strains were significantly efficient in controlling *B. cinerea* in both leaves and berries over three years with different fungal pressures. *P. agglomerans* has been described as an efficient epiphytic and rhizospheric biocontrol agent in

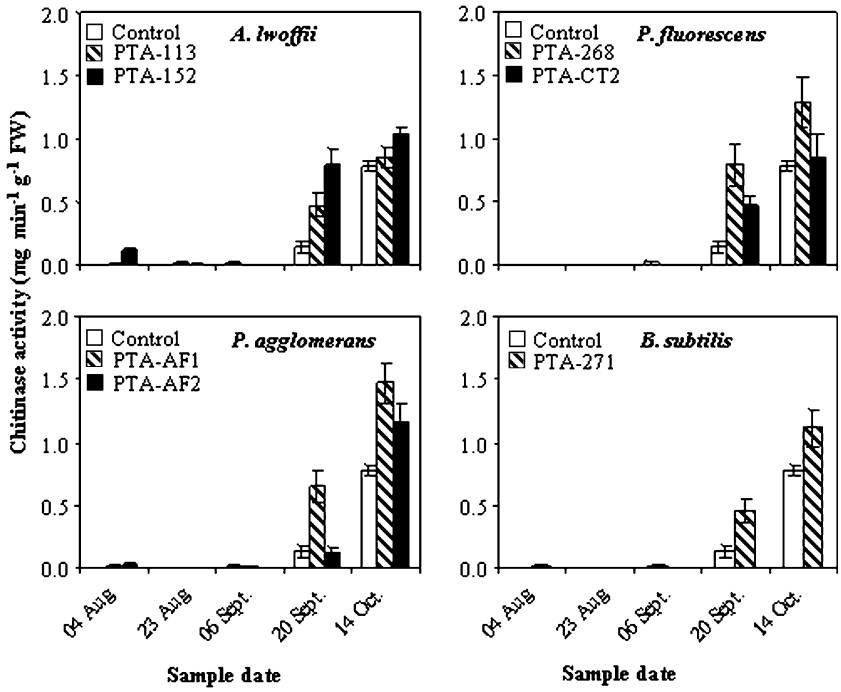

Fig. 5 Chitinase activity in berries of grapevine plants treated with native bacteria by soil drenching. Each bacterium was applied at 1×10^8 CFU ml^{-1} (150 ml per plant) in vineyard (NL trials) at the beginning of 2004 growing season. Treatments consisted of two strains of *A. lwoffii* (PTA-113 and PTA-152), two *P. fluorescens* (PTA-268 and PTA-CT2), two *P. agglomerans* (PTA-AF1 and TA-AF2), one *B. subtilis* (PTA-271), and control (without bacteria). Chitinase activity was determined at different dates in 2004. Data are means from three independent measurements, each consisting of three replicates. Bars indicate standard errors

different plants (Amellal, Burtin, Bartoli, & Heulin, 1998; Wright, Zumoff, Schneider, & Beer, 2001; Stockwell, Johnson, Sugar, & Loper, 2002).

A consistent efficiency regardless of grey mould reduction was also reported in 2005 in the same trials without repeating the bacterial inoculation. The protective effect against *B. cinerea* was very evident in the field trials previously treated with *P. agglomerans* PTA-AF1 and *P. fluorescens* PTA-268 and PTA-CT2 in the leaves, and with both strains of *A. lwoffii* and *P. agglomerans* in berries. These beneficial effects could be related to the origin and source of selected bacteria (Trotel-Aziz et al., 2006a) and therefore to their adaptation to environmental factors, which could contribute to the maintenance of bacteria at a sufficient level in the rhizosphere or in plant tissues during the growing season.

Induction of systemic resistance (ISR) is one of the mechanisms by which selected strains of non-pathogenic *Pseudomonas* spp. can reduce diseases in several plant species (Iavicoli et al., 2003; Leeman et al., 1995; Maurhofer et al., 1994; Pieterse et al., 1996; van Loon et al., 1998; van Peer, Niemann, & Schippers, 1991). In this study, selected bacterial strains also induced ISR in grapevine plants to different degrees. These differences might result from the different origins of the bacteria and / or their concentration in the rhizosphere or in colonized tissues during plant growth (Raaijmakers et al., 1995), even if the initial concentration of bacteria applied was similar for all strains. The biocontrol of *B. cinerea* seems here to originate at least from the ability of the selected bacteria to stimulate defense responses in grapevine plants. Indeed, bacterial treatments enhanced successive activities of chitinase and β-1,3-glucanase in leaves and berries. Highest chitinase activity was globally achieved with both strains of *P. fluorescens* and PTA-113, while β-1,3-glucanase was strongly activated by *A. lwoffii* strains, PTA-CT2 and PTA-AF1. Chitinases and

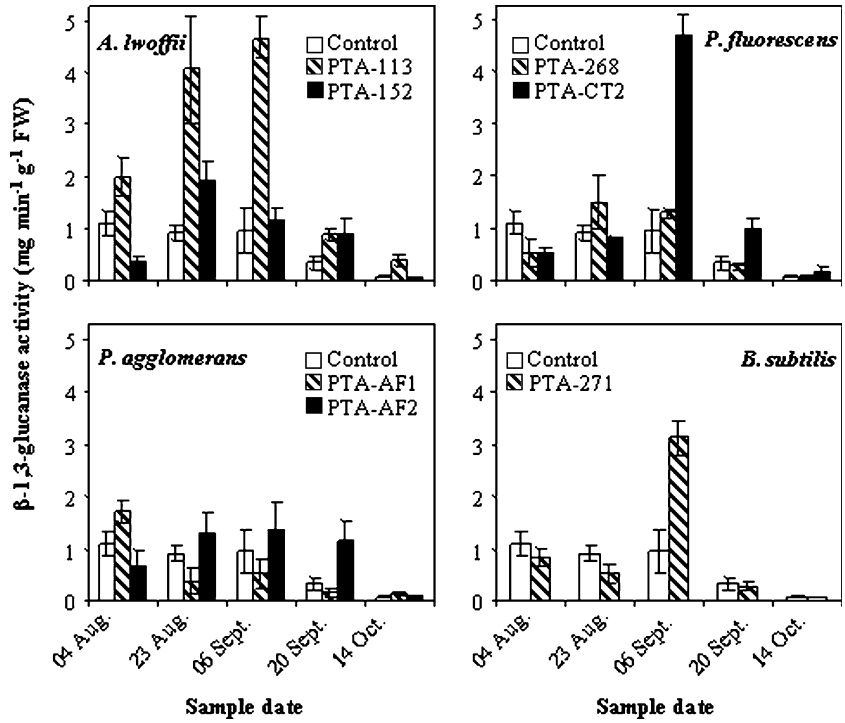

Fig. 6 β-1,3-Glucanase activity in berries of grapevine plants treated with native bacteria by soil drenching. Each bacterium was applied at 1×10^8 CFU ml^{-1} (150 ml per plant) in vineyard (NL trials) at the beginning of 2004 growing season. Treatments consisted of two strains of *A. lwoffii* (PTA-113 and PTA-152), two *P. fluorescens* (PTA-268 and PTA-CT2), two *P. agglomerans* (PTA-AF1 and TA-AF2), one *B. subtilis* (PTA-271), and control (without bacteria). β-1,3-Glucanase activity was determined at different dates in 2004. Data are means from three independent measurements, each consisting of three replicates. Bars indicate standard errors

β-1,3-glucanases are PR proteins strongly induced in grapevine following fungal challenge or treatment with activators of systemic acquired resistance (Busam et al., 1997; Derckel et al., 1998; Robert et al., 2002), as well as in response to biotic and abiotic elicitors (Aziz et al., 2003; Bonomelli et al., 2004; Trotel-Aziz et al., 2006b). The activity of both PR proteins is often associated with disease resistance of different grapevine cultivars to fungal pathogens (Aziz et al., 2004; Bonomelli et al., 2004; Busam et al., 1997; Giannakis, Bucheli, Skene, Robinson, & Scott, 1998). They are well-documented to exert synergistic antifungal effects when both enzymes are present, and might play an important role in the amplification of defense reactions through release of chitin and β-1,3-glucans from the pathogen and host cell walls (Mauch et al., 1988; Derckel et al., 1998; van Loon & van Strien, 1999).

Although a direct antifungal effect was not reported in this study, results from in vitro experiments have revealed that among the seven selected bacteria, only *P. agglomerans* PTA-AF1 and *P. fluorescens* PTA-CT2 displayed antagonistic activity against *B. cinerea* (Trotel-Aziz et al., 2006a). The same bacteria can also induce plant defense reactions as shown here by a stimulation of PR protein activities. It is likely that induced resistance caused by the bacterial strains to *B. cinerea* could result from the stimulation of the plant natural defense mechanisms and from a direct antifungal effect. Indeed, several strains of *P. fluorescens* (Dowling & O'Gara, 1994; Iavicoli et al., 2003; Maurhofer et al., 1994; Meziane et al., 2005), *P. agglomerans* (Amellal et al., 1998; Stockwell et al., 2002; Wright et al., 2001) and *Bacillus subtilis* (Asaka & Shoda, 1996; Chanway, 2002; Schmidt et al., 2004) display biocontrol activity against fungal pathogens by producing different antibiotics, siderophores and hydrolytic enzymes. In this study, even if bacterial strains were inoculated by soil drench

and therefore considered to be spatially separated from *B. cinerea*, we cannot exclude the possibility of direct interaction between bacteria and the pathogen in plant tissues. These secreted compounds can also contribute to induced systemic resistance in plants (Iavicoli et al., 2003; Meziane et al., 2005; Stockwell et al., 2002; van Wees et al., 1997). Specific accumulation of phytoalexins, another important defense mechanism, has also been observed in leaves and berries of grapevine plants inoculated with bacteria (B. Verhagen et al. *unpublished data*). Therefore the protective effect could result either from the induction of plant defense reactions or from an antifungal activity of some factors produced by the bacteria and transported through the plant. Nevertheless, for most of the bacteria, induced resistance responses in grapevine seem to be the primary determinants of biocontrol of *B. cinerea* rather than the mechanisms of antibiosis or competition for nutrients.

In conclusion, our data show that selected grapevine-associated bacteria significantly reduce disease caused by *B. cinerea* and induce specific activity of chitinase and β-1,3 glucanase in leaves and berries. Furthermore, induced resistance is dependent on bacterial strain and the method of inoculation. Soil drenches of *A. lwoffii* PTA-113, *P. agglomerans* PTA-AF1 and *P. fluorescens* PTA-CT2 provided the most consistent induction of grapevine chitinase and β-1,3 glucanase activity and resistance against *B. cinerea*. We also show that reduction of grey mould disease was maintained during the next growing season without renewal of bacterial applications. Further research is now required to evaluate the stability and durability of grapevine protection under field conditions, and to determine the mechanisms by which these bacteria induce plant resistance. Results from this research would be potentially useful for fungal disease management programmes by exploiting beneficial plant-associated bacteria and plant defense mechanisms.

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