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Differential induction of grapevine resistance and defense reactions against *Botrytis cinerea* by bacterial mixtures in vineyards

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Abstract Previous experiments showed that seven bacteria identified as *Acinetobacter lwoffii* (PTA-113 and PTA-152), *Bacillus subtilis* (PTA-271), *Pantoea agglomerans* (PTA-AF1 and PTA-AF2) and *Pseudomonas fluorescens* (PTA-268 and PTA-CT2) induced systemic resistance in grapevine against *Botrytis cinerea*. Based on these findings, we investigated biocontrol capacity of different mixtures under vineyard conditions over three consecutive years. Treatments with bacterial mixtures were shown to induce systemic resistance against *B. cinerea* on year 2. Efficacy and duration of such a disease control seemed to be reinforced on year three without renewal of bacterial treatments. Accordingly, the effectiveness of induced resistance varied with mixture type of bacteria and was accompanied by a stimulation of chitinase and β-1,3 glucanase activities in both leaves and berries. Interestingly, treatments with mixtures containing both selected strains of *P. agglomerans* (PTA-AF1 + PTA-AF2) appeared as the most effective in triggering systemically the plant defense reactions and reducing the symptoms of grey mould disease.

Keywords Biocontrol · Grey mould · Induced systemic resistance · Bacterial mixtures · *Vitis vinifera* L.

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Introduction

Plant disease control faces considerable challenges in recent years. There are the continuing problems of phytopathogen adaptation leading to fungicide resistance and breakdown in the effectiveness of plant resistance as well the increasing public concern related to the environmental effect of pesticide use. The use of plant growth promoting bacteria as an alternative is a promising approach that can be incorporated into integrated disease management (Rosslenbroich and Stuebler 2000). Several non-phytopathogenic bacteria, rhizospheric or endophytic, were frequently reported to protect plants against various phytopathogens (van Loon et al. 1998; van Wees et al. 1997; Magnin-Robert et al. 2007; Trotel-Aziz et al. 2008; Compant et al. 2010; Verhagen et al. 2011).

Direct interactions with the phytopathogen have already been described as a mechanism of biocontrol with rhizobacteria. Such antagonism can require production of antibiotic compounds, competition for nutrients, siderophore-mediated competition for iron, and/or production of extracellular enzymes (Maurhöfer et al. 1994; Iavicoli et al. 2003; Meziane et al. 2005). Beside direct interactions, some rhizobacteria are also able to reduce disease by inducing or priming plant defense mechanisms, which lead to a state of resistance in the whole plant against phytopathogens (Conrath et al. 2002; Verhagen et al. 2010). This kind of resistance, generally called induced systemic resistance (ISR), has been demonstrated with different plant species against several phytopathogens when bacteria and phytopathogen remained spatially separated (Hoffland et al. 1995; Pieterse et al. 1996; van Loon et al. 1998; Iavicoli et al. 2003; Meziane et al. 2005). In grapevine, some selected bacteria belonging to *Acinetobacter lwoffii*, *Bacillus subtilis*, *Pantoea agglomerans* and *Pseudomonas* spp. were previously shown to induce local and systemic resistance against *Botrytis cinerea* (Magnin-Robert et al. 2007; Trotel-Aziz et al. 2008; Verhagen et al. 2011).

Mechanisms by which bacteria could mediate ISR vary according to bacterial isolates, plant species and phytopathogens. In some cases, bacteria-mediated ISR is characterized by a systemic accumulation of pathogenesis-related (PR) proteins (Maurhöfer et al. 1994; Tjamos et al. 2005) with enhanced chitinase and β-1,3-glucanase activities (Magnin-Robert et al. 2007; Trotel-Aziz et al. 2008). In other cases, ISR seems to be independent on PR protein expression, but dependent on jasmonate and ethylene signallings. ISR has recently been described as a result of bacteria-mediated priming of rapid molecular and cellular defense responses, including transcription of defense-related genes, callose deposition, and accumulation of phytoalexins (Verhagen et al. 2004, 2010; Ahn et al. 2007).

Most experiments for biocontrol of plant disease have used single biocontrol agents against a phytopathogen (Leeman et al. 1995; Asaka and Shoda 1996; van Wees et al. 1997; Magnin-Robert et al. 2007). However, in several cases especially in practical agriculture, the performance of single biocontrol agents remained inconsistent against plant phytopathogens. To maximize the efficacy of biological control, it was possible to develop bacterial mixtures with high biocontrol activity (Raupach and Kloepper 1998). This strategy could concern mixtures of organisms with differential plant colonization patterns or mixtures of different mechanisms of disease suppression or mixtures of taxonomically different organisms. It is likely that application of a mixture of introduced biocontrol agents would more closely mimic the natural situation and might enhance the efficacy of biocontrol (Duffy and Weller 1995). In such combinations, compatibility between micro-organisms might appear as a prerequisite for effective disease suppression (De Boer et al. 1999; Raaijmakers et al. 1995; Anderson et al. 2004). However, there are also reports of combinations of some antagonistic biological control agents that result in an improved disease suppression compared to the related single isolates (De Boer et al. 1999).

Recently, we characterized seven bacteria originating from vineyard that can individually induce systemic resistance of grapevine plants against *B. cinerea* (Magnin-Robert et al. 2007; Trotel-Aziz et al. 2008). These bacteria also induce some defense reactions in grapevine leaves and berries (Magnin-Robert et al. 2007; Trotel-Aziz et al. 2008; Verhagen et al. 2011). In the present study, we investigated the efficiency of different combinations of selected bacteria to control *B. cinerea* through the induction of grapevine resistance under vineyard conditions. Investigations were conducted over three seasons and in all possible combinations applied by drenching soil of grapevine plants. Some mechanisms by which these mixtures may protect grapevine against *B. cinerea* were also assessed through measurement of chitinase and β-1,3-glucanase activities in both leaves and berries.

Materials and methods

Bacteria and culture conditions

Bacteria isolated by Trotel-Aziz et al. (2008) from the rhizosphere and tissues of field-grown grapevines (Champagne area, Marne, France) were two strains of *A. lwoffii* (PTA-113 and PTA-152), a *B. subtilis* (PTA-271), two *P. agglomerans* (PTA-AF1 and PTA-AF2) and two *Pseudomonas fluorescens* strains (PTA-268 and PTA-CT2). Treatments consisting of mixtures of genera, listed in Table 1, are named "AL" for *A. lwoffii* (PTA-113 + PTA-152), "Bs" for *B. subtilis*

Table 1 Identity and source of the bacterial isolates used in this study (Trotel-Aziz et al. 2008)

Code	Strain	Species	Origin
Al1	PTA-113	*A. lwoffii*	Grapevine roots
Al2	PTA-152	*A. lwoffii*	Grapevine roots
Bs	PTA-271	*B. subtilis*	Vineyard rhizosphere
Pa1	PTA-AF1	*P. agglomerans*	Grapevine leaves
Pa2	PTA-AF2	*P. agglomerans*	Grapevine leaves
Pf1	PTA-268	*P. fluorescens*	Grapevine stem
Pf2	PTA-CT2	*P. fluorescens*	Grapevine stem

(PTA-271), "PA" for *P. agglomerans* (PTA-AF1 + PTA-AF2), and "PF" for *P. fluorescens* (PTA-268 + PAT-CT2). Each bacterium was grown separately in the Luria–Bertani (LB) liquid medium (Sigma) at 24 °C with continuous shaking (150 rpm) for 24 h before use. Cell concentrations of bacteria were adjusted to 1×10^8 CFU ml^{-1} before the mixtures were made for applications in the vineyard. Previous results regarding their possible combinations have shown that these bacteria are all compatible with each other on LB-agar medium (B. Verhagen, personal communication).

Plant material and field experiments

Grapevine plants (*Vitis vinifera* cv Chardonnay, on 41B rootstock) planted in 1993 in a research vineyard located in Nogent l'Abbesse in the Champagne area (49.255°N latitude and 4.156°E longitude, Marne, France) were treated from 2003 to 2005. Vine spacing was 1.05 m within row and 1.20 m between rows. Treatments consisted of non-bacterized controls and a soil drench using 150 ml bacterial suspension per plant by flooding the first 10 cm of the soil in contact and around the rootstock at 2003 and 2004 growing seasons. Bacterial isolates were applied individually or in mixtures of genera as indicated above. Control plants were treated either with water or with LB. 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 (100 clusters per treatment) without visible symptoms were harvested at different dates during growing seasons and stored at −80 °C before analysis of defense reactions. No bacterial treatments were conducted in 2005, but analysis of resistance against *B. cinerea* was performed.

Fungal phytopathogen

A virulent *B. cinerea* strain 630 (gift of Y. Brygoo, INRA, Versailles, France) was cultured in Erlenmeyer flasks on potato dextrose agar medium (PDA, Sigma, St. Louis, USA) 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 sterile nylon gauze. The conidial suspension was adjusted with sterile distilled water to 1×10^5 conidia ml^{-1}.

Infection assays of leaves with fungal pathogen

Fully expanded young leaves excised from the top of shoots of field-grown grapevine plants (30 leaves per treatment) two months after bacterial application, were immediately placed into moistened plastic bags and taken to the laboratory. Leaves were then rinsed with sterile distilled water, patted dry and their adaxial side placed facing a wet absorbing paper on Petri dishes. One needle-prick wound was applied on the middle of the abaxial side of each leaf, and the fresh wounds were covered with 10-µl drops of the 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 of light. Disease development was measured as the average diameter of lesions formed seven days post-inoculation with *B. cinerea* in comparison with the control (leaves excised from control plants).

Contamination of grape berries by *B. cinerea* in vineyards

At full ripening, the grey mould development was estimated in mature clusters from several grapevines in vineyard as the percentage of naturally infected berries (sporulating berries) per cluster of bacteria-treated plants in comparison with control (berries from non-treated plants). Each counting was performed with 100 clusters per treatment.

Chitinase and β-1,3-glucanase activities

Chitinase and β-1,3-glucanase activities were chosen as markers of plant defenses in grapevine. Both enzymes were extracted on ice by grinding frozen leaves (500 mg fresh weight -FW-) or deseeded berries (1 g FW) with 2 ml of sodium acetate buffer 50 or

100 mM, respectively, pH 5.0, containing 1 mM dithiothreitol and 0.02 % (w/v) phenylmethylsulfonyl fluoride. After centrifugation at 10,000*g* for 5 min at 4 °C, the resulting supernatants were directly used as the crude enzyme extracts.

Chitinase and β-1,3-glucanase activities were determined according to the procedure described in Magnin-Robert et al. (2007) using CarboxyMethyl/chitin/Remazol Brillant Violet and CarboxyMethyl/curdlan/Remazol Brillant Blue (Loewe Biochemica, Germany) as respective substrates. Briefly, the reaction mixture (0.4 ml) containing 100 µl of the enzyme substrate (2 mg ml^{-1}) with 50–200 µl of the diluted enzymatic extract in 50 mM sodium acetate buffer (pH 5.0), was incubated at 37 °C for 1–20 min. The reactions were stopped by adding 400 µl of cold 1 M HCl and immediately kept on ice for at least 10 min. Non-hydrolyzed substrates were precipitated by centrifugation at 10,000*g* for 10 min at 4 °C, and the absorbance of the supernatants was recorded at 550 and 600 nm, respectively for quantification of the hydrolyzed chitin and of the hydrolyzed glucan, using the related substrates as standards.

Statistical 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 ten leaves and ten berries (from three to four clusters per treatment) all in triplicates. Data are means ± SE.

Results

Chitinase activity in grapevine leaves and berries after bacterial treatments

Chitinase activity was measured in July and/or September 2003 and during the whole 2004 growing season after bacterial treatments on May 20th, 2003 and May 20th, 2004. Treatments were moderately effective in 2003 except for *P. fluorescens* Pf1 and Pf2 which led only to a slight induction of chitinase activity in grapevine leaves when compared to the control (Table 1s, supplemental data). However, chitinase activity was transiently stimulated in grapevine leaves after bacterial application in 2004 (Fig. 1). Maximal chitinase activity was reached two months post-treatment with each single bacterium (late-July, called pré-véraison, results from Magnin-Robert et al. (2007) with permission), while with bacterial mixtures, chitinase activity remained high over a longer period of time (till late-August or early-September, called véraison). Compared to the control, chitinase activity was almost two to three folds higher following treatments with single bacteria. *Acinetobacter lwoffii* Al1, *B. subtilis* (Bs), *P. agglomerans* Pa2, *P. fluorescens* Pf1 and Pf2 were the most effective compared to Al2 and Pa1. With bacterial mixtures, chitinase activity was in several cases up to five fold higher than the control. Mixtures consisting of AL, PA and PF were the most inducers of this activity. Treatments with mixtures containing 3–6 bacteria resulted also in an enhancement of chitinase activity at a level at least comparable to that induced by single bacteria. Interestingly, some treatments with mixtures containing *P. agglomerans* (AL + PA, Bs + PA, AL + Bs + PA, AL + PA + PF, Bs + PA + PF) led to a maintained chitinase activity over time.

In grape berries, chitinase activity was also stimulated transiently in 2004 whatever the bacterial treatment (Fig. 2). Nevertheless, chitinase activity was lower than in leaves and occurred later (starting mainly from late-September). Treatments with most of single bacteria (Al1, Al2, Bs, Pa1, Pf1, Pf2) in 2003 (Table 2s, supplemental data) and in 2004 (Fig. 2) resulted in enhancement of chitinase activity, especially at full ripening [late-September 2004 and also October for Pa1, results from Magnin-Robert et al. (2007) with permission]. Some mixtures containing *Pantoea* spp. and/or *Acinetobacter* spp. and/or *Pseudomonas* spp. (PF, AL + PA, AL + PF, Bs + PA, PA +PF) also induced chitinase activity, while the others were weakly effective in 2003 and led only to a slight induction of chitinase activity in grape berries when compared to the control (Table 2s, supplemental data). In 2004, also few mixtures of bacteria significantly stimulated chitinase activity in grape berries (late-September and also October for AL + PF). Mixtures containing *A. lwoffi* and *B. subtilis* and/or *P. fluorescens* (AL + Bs, AL + PF, Bs + PF, AL + PA + PF, AL + Bs + PA + PF) were the

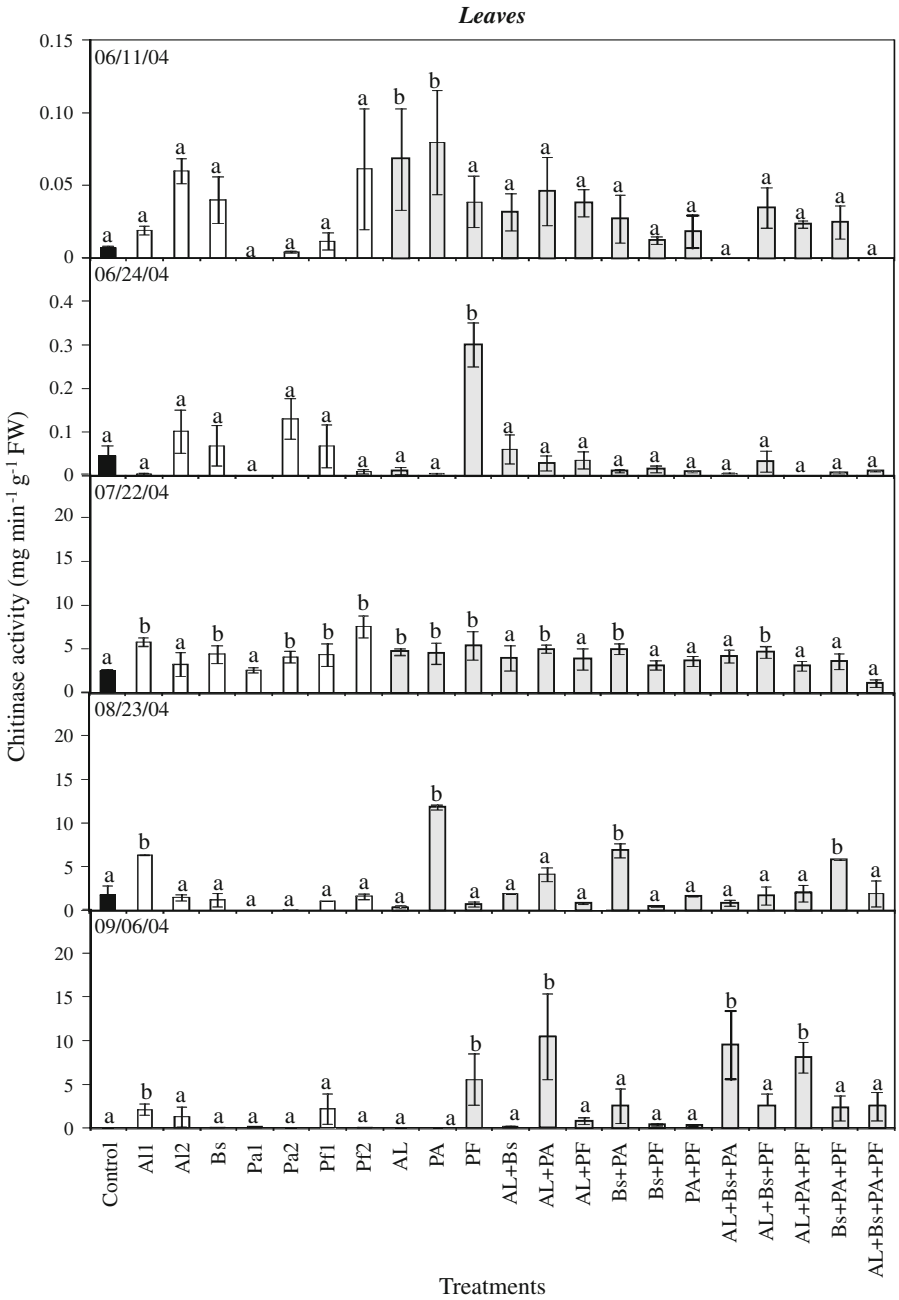

Fig. 1 Chitinase activity in leaves of grapevine plants treated with single (white bars) or mixtures (grey bars) of selected bacteria by soil drenching in vineyard. Each bacterium or bacterial mixture was applied at a final density of 1×10^8 CFU ml^{-1} at the beginning of the 2004 season. Treatments consisted of control (without bacteria, black bar) and different strains of *A. lwoffii* (Al1 and Al2), *B. subtilis* (Bs), *P. agglomerans* (Pa1, Pa2) and *P. fluorescens* (Pf1, Pf2), either in single or in mixtures of two *A. lwoffii* (AL), two *P. agglomerans* (PA), two *P. fluorescens* (PF), or other possible combinations of different genera (AL + Bs, AL + PA, AL + PF, Bs + PA, Bs + PF, PA + PF, AL + Bs + PA, AL + Bs + PF, AL + PA + PF, Bs + PA + PF, AL + Bs + PA + PF). Chitinase activity was determined during the 2004 growing season. Results of single bacteria were from Magnin-Robert et al. (2007) with permission. Data are means of three replicates with three independent measurements. Columns headed by different letter are significantly different ($P \leq 0.05$) according Duncan's multiple range test ($F_{21,176} = 2.97$, 3.19, 2.87, 5.29 and 37.12 and $P < 0.0001$, <0.0001, <0.0001, <0.0001 and <0.0001 the 06/11/04, 06/24/04, 07/22/04, 08/23/04 and 09/06/04, respectively). Bars represent SE

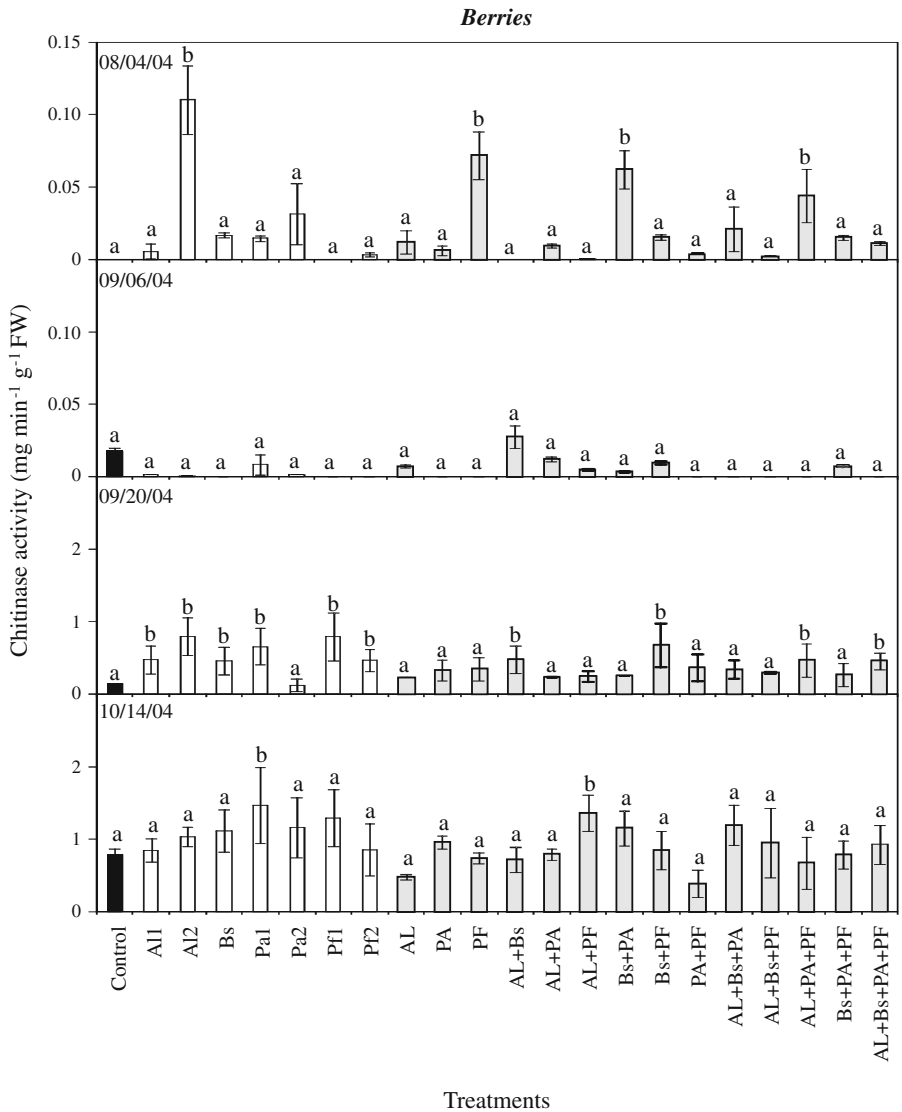

Fig. 2 Chitinase activity in berries of grapevine plants treated with single (white bars) or mixtures (grey bars) of selected bacteria by soil drenching in vineyard. Each bacterium or bacterial mixture was applied at a final density of 1×10^8 CFU ml^{-1} at the beginning of the 2004 season. Legend as in Fig. 1. Results of single bacteria were from Magnin-Robert et al. (2007) with permission. Data are means of three replicates with three independent measurements. Columns headed by different letter are significantly different ($P \leq 0.05$) according Duncan's multiple range test ($F_{21,176} = 3.37$, 1.63, 4.85 and 5.40 and $P < 0.0001$, 0.046, <0.0001 and <0.0001 the 08/04/04, 09/06/04, 09/20/04 and 10/14/04, respectively). Bars represent SE

most effective in inducing chitinase activity in grape berries (Fig. 2). Other bacterial mixtures, especially those containing the *Acinetobacter* spp. and *Pantoea* spp. (AL + Bs + PA, AL + PA + PF, AL + Bs + PA + PF) were also effective in enhancing chitinase activity in berries.

β-1,3-Glucanase activity in grapevine leaves and berries after bacterial treatments

β-1,3-Glucanase activity was measured in July and/or September 2003 and during the whole 2004 growing season following bacterial treatments on May 20th, 2003 and May 20th, 2004. Data showed that β-1,3-glucanase activity was stimulated in grapevine leaves and berries following the application of selected bacteria (Fig. 3; Tables 3s and 4s, supplemental data). In the leaves, β-1,3-glucanase activity appeared biphasic, with a weak level during "pré-véraison" (late-July) and a second peak with a higher magnitude (2.5 times) during pre-harvest stage (early-September) (Fig. 3). The first peak remained low in most treatments with single bacteria and bacterial mixtures. Chronologically, the highest activity of β-1,3-glucanase occurred after the onset of chitinase stimulation in leaves with single bacteria (late-July, results from Magnin-Robert et al. 2007 with permission). With single isolates, the highest activity in the leaves was

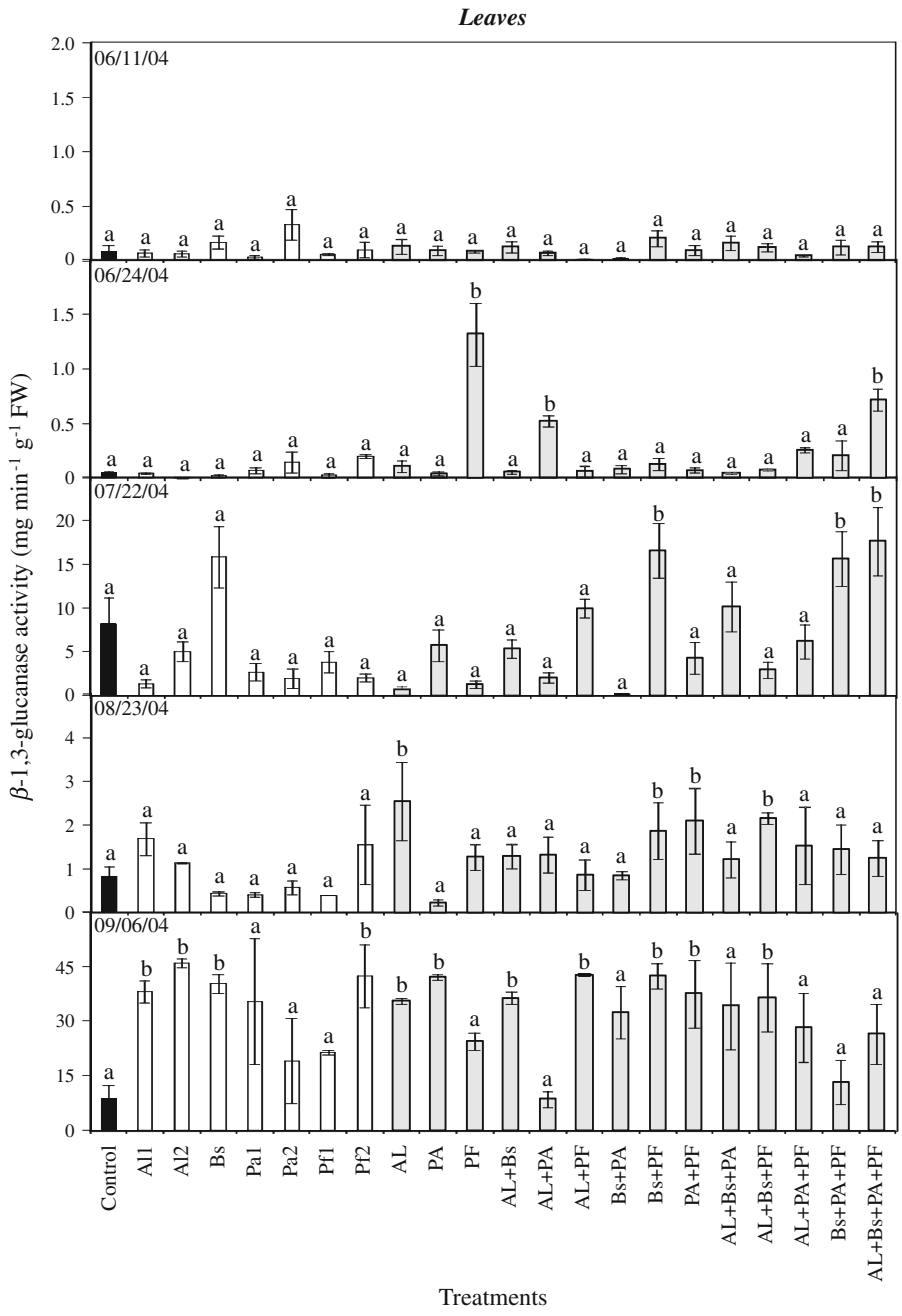

Fig. 3 β-1,3-Glucanase activity in leaves of grapevine plants treated with single (white bars) or mixtures (grey bars) of selected bacteria by soil drenching in vineyard. β-1,3-Glucanase activity was determined during the 2004 growing season. Legend as in Fig. 1. Results of single bacteria were from Magnin-Robert et al. (2007) with permission. Data are means of three replicates with three independent measurements. Columns headed by different letter are significantly different ($P \leq 0.05$) according Duncan's multiple range test ($F_{21,176} = 1.51$, 3.25, 4.20, 6.37 and 4.9 and $P = 0.078$, <0.0001, <0.0001, <0.0001 and <0.0001 the 06/11/04, 06/24/04, 07/22/04, 08/23/04 and 09/06/04, respectively). Bars represent SE

observed in plants treated in 2004 with *A. lwoffii* (Al1, Al2), *B. subtilis* (Bs) or the *P. fluorescens* Pf2 (Fig. 3), and in a lesser extent in 2003 with *P. fluorescens* (Pf1, Pf2) or *P. agglomerans* Pa1 and Pa2 (Table 3s, supplemental data). Compared to the control, most of the mixtures were only effective in September 2004 (Fig. 3). Mixture containing both *B. subtilis* and *Pseudomonas* spp. (Bs + PF) appeared to be the sole active over time (Fig. 3). A significant increase of β-1,3-glucanase activity was also observed following treatments with mixtures of the two *Acinetobacter* spp. or the two *Pantoea* spp. (AL, PA). Treatments combining *Acinetobacter* spp. or *Pantoea* spp. with other genera (mixtures with 3–7 isolates, i.e. AL + Bs, AL + PF, PA + PF, AL + Bs + PF, Bs + PA + PF, AL + Bs + PA + PF) also stimulated

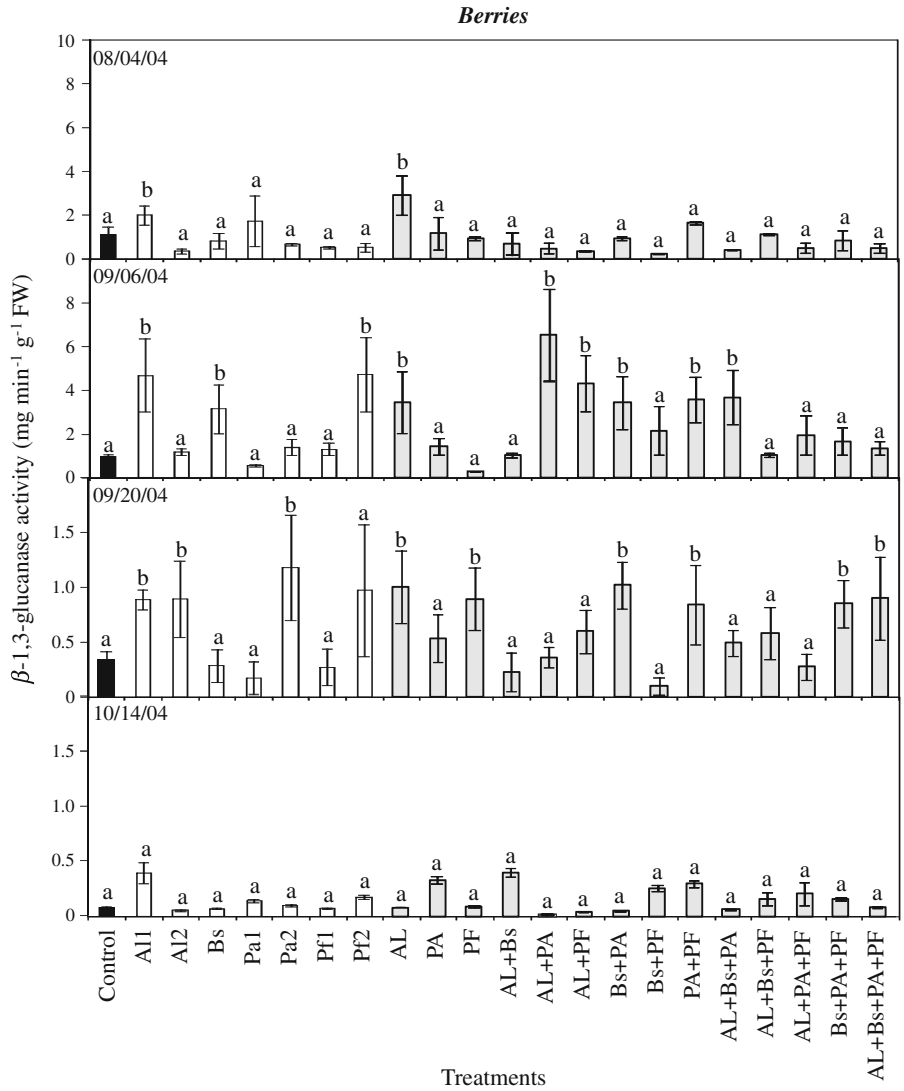

Fig. 4 β-1,3-Glucanase activity in berries of grapevine plants treated with single (white bars) or mixtures (grey bars) of selected bacteria by soil drenching in vineyard. β-1,3-Glucanase activity was determined during the 2004 growing season. Legend as in Fig. 1. Results of single bacteria were from Magnin-Robert et al. (2007) with permission. Data are means of three replicates with three independent measurements. Columns headed by different letter are significantly different ($P \leq 0.05$) according Duncan's multiple range test ($F_{21,176} = 5.84$, 3.12, 2.32 and 2.16 and $P < 0.0001$, <0.0001, 0.0015 and 0.0035 the 08/04/04, 09/06/04, 09/20/04 and 10/14/04, respectively). Bars represent SE

β-1,3-glucanase activity compared to the control, excepted for plants treated with AL + PA or Bs + PA which exhibited a lower β-1,3-glucanase activity. Interestingly, in the presence of other bacteria, the mixture AL + PA resulted in an enhanced level of β-1,3-glucanase activity. This effect was especially observed with the mixture of seven bacteria (AL + Bs + PA + PF) and in a lesser extent with the mixtures of five (AL + Bs + PA) or six isolates (AL + PA + PF).

In grape berries, the β-1,3-glucanase activity was also enhanced after the onset of that in leaves, but to a lesser extent (Fig. 4). It peaked already in end-July/early-August with the single strains Al2, Pa1 and Pf1 in 2003 (Table 4s, supplemental data) or with Al1 in 2004 (Fig. 4). Most of mixtures containing *A. lwoffii*, *P. agglomerans* and *P. fluorescens* (AL + Bs, AL + PA, AL + PF, Bs + PA, AL + Bs + PF, AL + PA + PF) applied in 2003 (Table 4s, supplemental data) or AL in 2004 (Fig. 4) also showed an enhanced β-1,3-glucanase activity in berries in end-July/early-August. It also peaked in September 2004 with most of bacterial treatments and returned to the basal level in October. Chronologically, β-1,3-glucanase activity was stimulated earlier than chitinase activity in grape berries. A strong activity of β-1,3-glucanase was observed in September 2004 in plants treated with Al1, Al2, Bs, Pa2 or Pf2, and also in August with Al1

(results from Magnin-Robert et al. 2007 with permission). In the presence of the two *A. lwoffii* (AL) β-1,3-glucanase activity remained high from early-August to late-September 2004 (ripening), while with the two *P. fluorescens* (PF) β-1,3-glucanase activity was high only at ripening (Fig. 4). As indicated above, the β-1,3-glucanase activity also increased in most of assays with mixtures of *A. lwoffii*, *B. subtilis* or *P. fluorescens* both in end-July 2003 and September 2004. Strongest activity was observed with mixture containing *Pantoea* spp. and *Acinetobacter* spp. (AL + PA) in September 2004 (Fig. 4). Curiously, compared to the control, β-1,3-glucanase activity was weakly or not at all stimulated in the assay combining *A. lwoffii*, *B. subtilis* and *P. fluorescens* (AL + Bs + PF) in 2003 (Table 4s, supplemental data) and 2004 (Fig. 4), respectively.

Reduction of *B. cinerea* development in grapevine leaves and berries

To verify the capacity of bacterial treatments to protect grapevine against *B. cinerea*, assays were monitored both in berries and leaves after drenching the plant soil with single or various mixtures of selected bacteria since 2004, as none significant *Botrytis*-attack occurred during 2003 in vineyards. Leaves were detached from treated plants and afterwards challenged with the phytopathogen *B. cinerea*. Within seven days post-inoculation, leaves from control plants developed large necrotic lesions (diameter >16 mm), whereas bacterized plants showed reduced disease development by 22 % (13.3 mm necrosis) to 75 % (4.2 mm necrosis) compared to control plants in the 2004 season (Fig. 5a). The percentage of leaf protection was defined as the reduction in the lesion size relative to the control and used to estimate the protective effect of the bacterial treatments. In response to single bacteria (results from Magnin-Robert et al. 2007 with permission), the highest protection was observed with *A. lwoffii* Al1 or Al2, *P. agglomerans* Pa2, or *P. fluorescens* Pf1 or Pf2. *B. subtilis* (Bs) or *P. agglomerans* Pa1 alone remained less efficient. Treatments with mixtures of both strains of *P. agglomerans* (PA) induced a better leaf protection (53 %, 8.8 mm necrosis) than did treatments with the two strains of *A. lwoffii* (AL) or the two *P. fluorescens* (PF). Mixtures consisting of 3–7 bacteria indicated that most of treatments combining both *Acinetobacter* spp. and *Pseudomonas* spp. resulted in a poor leaf protection, as for AL + PF (four bacteria), AL + Bs + PF (five bacteria) or AL + Bs + PA + PF (seven bacteria). Interestingly, the mixture AL + PA + PF (six bacteria) or all other combining 3–6 bacteria (AL + Bs, AL + PA, Bs + PA, Bs + PF, PA + PF, AL + Bs + PA, Bs + PA + PF) strongly protected the leaves (45–75 %, 4.2–9.2 mm necrosis).

Clusters were also analysed in the same trials at ripening of the 2004 growing season, when the degree of infection of clusters in control plants had reached about 45 %. The protective effect of bacterial treatments was estimated by the use of the percentage of berry protection, which was defined as the reduction in the *Botrytis* infection relative to the control. As can be seen in the Fig. 5b, treatments with the *P. agglomerans* Pa1 alone or in mixtures (PA) were among the most effective in protecting grape berries against *B. cinerea* (20–24 % protection, 34.8–36 % contamination). In contrast, assays with *P. fluorescens*, *B. subtilis* or *A. lwoffii* as single or in dual mixtures did not induce any significant protective effect. Treatments with mixtures of 3–7 isolates also yielded a protection ranging from 0 to 24 %. The best protection (10–24 %) was observed with the treatments combining *Pantoea* spp. to other bacteria, except in assays combining both *Pantoea* spp., *Acinetobacter* spp. and *Pseudomonas* spp. which did not at all protect grape berries against *B. cinerea*.

Persistence of grapevine protection without renewed treatment

Leaves from control plants in vineyards infected with *B. cinerea* in 2005 season developed large necrotic lesions diameter (>16 mm, Fig. 6a). In plants treated with selected bacteria in previous years, leaves infected with *B. cinerea* showed that disease reduction might reach 67 % (5.4 mm necrosis) at year 3 (2005). With single bacterium (Magnin-Robert et al. 2007), the highest protection (ranging from 52 to 63 %, 6 to 7.8 mm necrosis) was observed in plants which had received *B. subtilis* (Bs), *P. agglomerans* (Pa1) or the two strains of *P. fluorescens* (Pf1, Pf2). Leaves from vines treated with the two *P. agglomerans* (PA) or the two *P. fluorescens* (PF) showed a disease reduction by about 52 % (7.8 mm) and 53 % (7.6 mm), respectively. The level of protection reached only 23.4 %

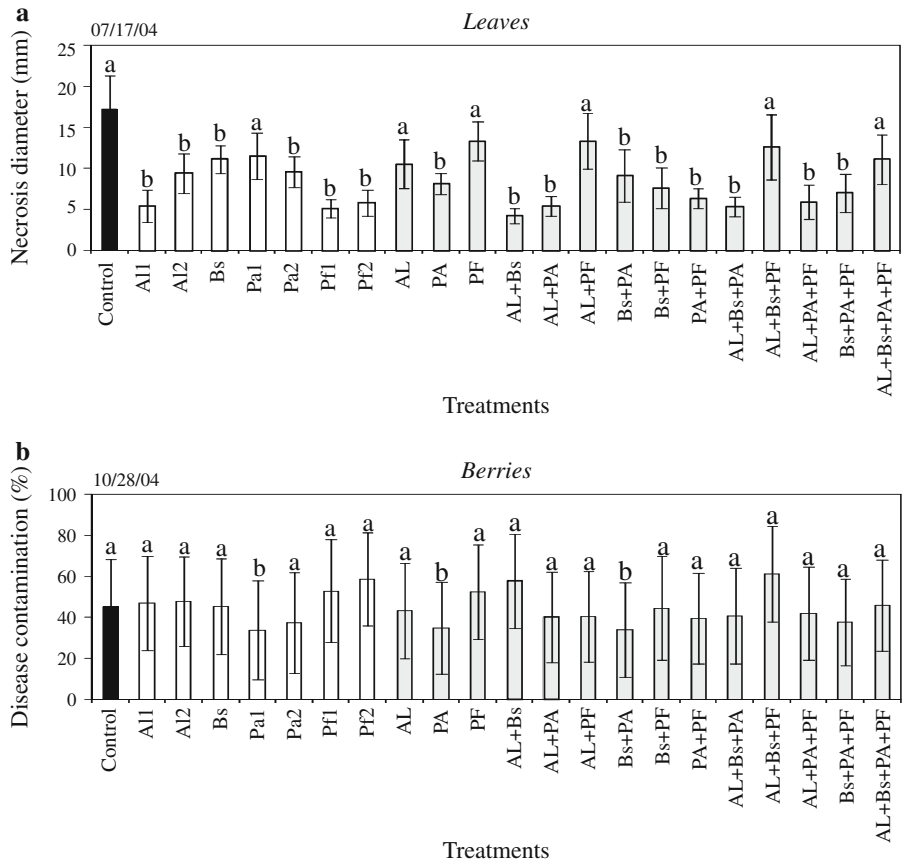

Fig. 5 Development of grey mould disease caused by *B. cinerea* in leaves (**a**) and berries (**b**) of grapevine plants treated with single bacteria (white bars) or with mixtures (grey bars) by soil drenching in vineyard. Disease development was determined during the 2004 growing season. After two months, young leaves were detached and challenge-inoculated with *B. cinerea*, then disease was evaluated at seven days post-challenge by measuring necrosis development diameter. In berries, disease severity was measured as percentage of infected berries per cluster at full ripening. Legend as in Fig. 1. Results of single bacteria were from Magnin-Robert et al. (2007) with permission. Data are means of three independent measurements with 30 leaves and 100 clusters per treatment. Columns headed by different letter are significantly different ($P \leq 0.05$) according Duncan's multiple range test ($F_{21,638} = 2.37$ and $P < 0.001$ in (**a**), $F_{21,2178} = 3.04$ and $P < 0.0001$ in (**b**)). Bars represent SE

(12.4 mm) in plants treated with mixture of the two *A. lwoffii* (AL). Leaves from plants treated with mixtures containing 3–7 bacteria were strongly protected against *B. cinerea*. In most of cases, a great homogeneity of protection was observed (ranging from 53 to 67 %, 5.4 to 7.6 mm necrosis).

Clusters from control plants in 2005 season showed a degree of *B. cinerea* infection of about 14 %. Plants treated in previous years (2003 and 2004 seasons) with *A. lwoffii* Al1 or Al2 or in mixture (AL), or with *P. agglomerans* alone or in mixture (Pa1, Pa2, PA), showed a consistent grey mould reduction in berries at 2005. This protection reached a level of about 44–82 % (2.4–7.5 % contamination). In the same conditions, most of plants treated with single bacteria showed a protection of berries that did not exceed 36 % (Fig. 6b). Disease reduction in grape berries is significant for a half of treatments with the bacterial mixtures. It ranged from 42 to 62 % (5.1–7.9 % contamination), especially with mixtures of three (Bs + PA), four (AL + PA, AL + PF), five (Bs + PA + PF) or six bacteria (AL + PA + PF). Interestingly, this protection was systematically observed in plants previously treated with mixtures containing *Pantoea* spp. or *Acinetobacter* spp., although a non-significant protection appeared in assays where *Pantoea* spp. were combined with *Pseudomonas* spp. (PA + PF). However, the

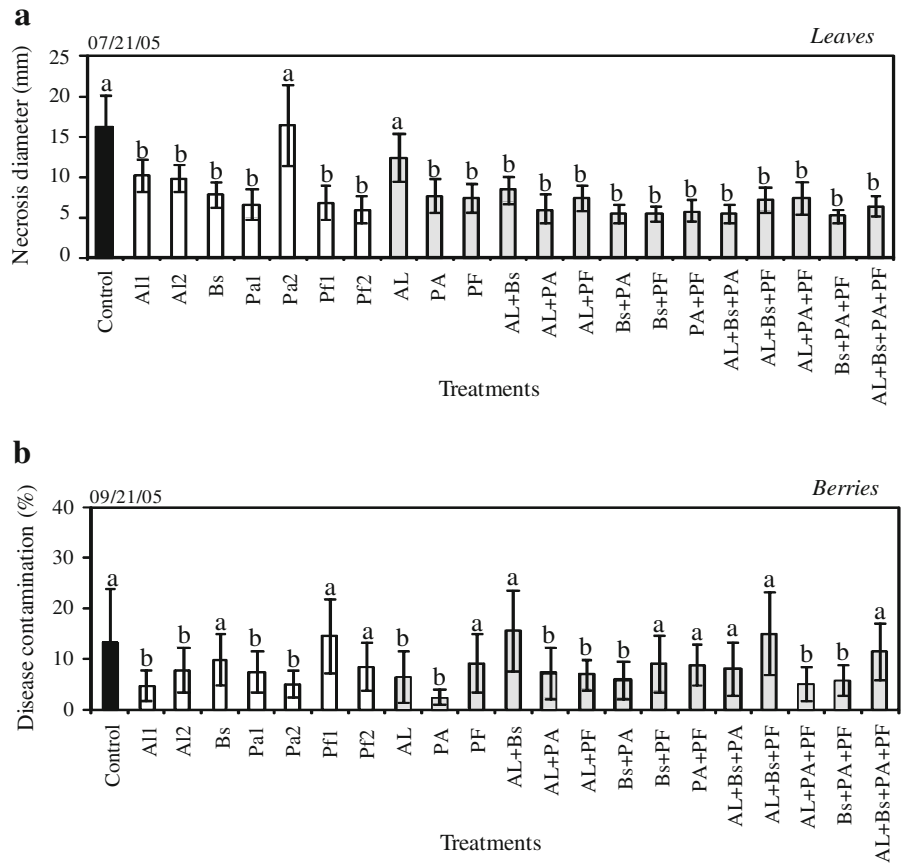

Fig. 6 Development of grey mould disease caused by *B. cinerea* in leaves (**a**) and berries (**b**) of grapevine plants pre-treated in 2003 and 2004 with single bacteria (white bars) or with mixtures (grey bars) by soil drenching in vineyard. Disease development was determined during the 2005 growing season. Bacteria were not applied in 2005, but disease was evaluated using young leaves after seven days of inoculation with *B. cinerea* as described before. In berries, disease severity was measured as percentage of infected berries per cluster at full ripening. Legend as in Fig. 1. Data are means of three independent measurements with 30 leaves and 100 clusters per treatment. Columns headed by different letter are significantly different ($P \leq 0.05$) according Duncan's multiple range test ($F_{21,638} = 2.67$ and $P < 0.0001$ in (**a**), $F_{21,2178} = 5.86$ and $P < 0.0001$ in (**b**)). Bars represent SE

protection was low in plants pre-treated with *Acinetobacter* spp. combined with *Bacillus* spp. (AL + Bs, AL + Bs + PF), or even in the presence of *Pantoea* spp. in the mixture (AL + Bs + PA, AL + Bs + PF + PA).

Discussion

The use of grapevine-associated bacteria to manage grey mould disease in vineyards appears to be promising as alternative to chemical fungicides. The efficacy of selected strains of *P. agglomerans*, *P. fluorescens*, *B. subtilis* and *Acinetobacter lwoffii* spp. to control *B. cinerea* was already shown when they were applied individually (Magnin-Robert et al. 2007; Trotel-Aziz et al. 2008). The biocontrol activity was clearly associated with an enhancement of plant resistance towards the phytopathogen and a systemic induction of defense reactions in grapevine involving jasmonic acid pathway (Magnin-Robert et al. 2007; Trotel-Aziz et al. 2008). In the present study, we further demonstrated differential biocontrol effectiveness of these bacteria applied as mixtures of two to seven isolates by drenching the plant soil in vineyards. We showed that, depending on the growing season,

grapevine leaves and berries displayed variable degree of grey mould contamination which is significantly contrasted in most cases with plants treated with bacteria.

During 2004 season, disease symptoms caused by *B. cinerea* were significantly reduced in both leaves (up to 67 %) and berries (up to 24 %) of plants treated with single bacteria. This protection was at least equivalent or more effective when plants were pre-treated with mixtures of selected bacteria (up to 75 % for leaves and 24 % for berries). Considering the spatial separation of applied bacteria at the root level and the phytopathogen on leaves or berries, the induced protection by selected bacteria as single (Magnin-Robert et al. 2007) or mixtures (this work) could result from an ISR in grapevine plants. In this sense, the greatest levels of systemic resistance were observed in the presence of mixtures containing *P. agglomerans* strains, while most of treatments with mixtures containing together *A. lwoffii* and *P. fluorescens* isolates induced only a slight resistance level, especially in berries. This is consistent with the fact that a concerted action of different microorganisms with different mechanisms such as direct antagonism and induction of ISR could be responsible for a highly efficient disease reduction (De Boer et al. 2003). Combining bacteria inducing SA-dependent and SA-independent ISR is another possibility to increase their effectiveness towards the phytopathogens (van Wees et al. 2000). *P. agglomerans* has been described as an efficient epiphytic and rhizospheric biocontrol agent (Amellal et al. 1998; Han et al. 2000; Nunes et al. 2001, 2002), that induces resistance as effectively as 2,6-dichloroisonicotinic acid in other plants (Han et al. 2000). In our study, both *P. agglomerans* PTA-AF1 and PTA-AF2 were isolated from the grape leaves. Furthermore, PTA-AF1 displayed antagonistic activity against *B. cinerea*, as for *P. fluorescens* PTA-CT2 (Trotel-Aziz et al. 2008).

The low resistance induced by the mixtures containing *A. lwoffii* and *P. fluorescens* could result from possible interactions between bacteria in applied mixtures, and also from interactions with local microflora in the particular vineyard used for this study. Based on tests of co-inoculation in vitro, we did not find any incompatibility between bacteria constituting the mixtures (B. Verhagen, personal communication). This also suggests that the poor protection obtained with certain mixtures of bacteria can be attributed to non-complementary mechanisms of action. Differences in effectiveness of the various bacterial mixtures might also result from the different origins of each isolate and/or from its concentrations in the rhizosphere or in colonized tissues during plant growth (Raaijmakers et al. 1995; Mercado-Blanco and Bakker 2007), even if the concentration applied was similar for all mixtures. Moreover, *P. fluorescens* PTA-268 and *B. subtilis* PTA-271 were rhizospheric, *P. fluorescens* PTA-CT2 was also endophytic, while *Acinetobacter* strains were isolated from the roots (Trotel-Aziz et al. 2008).

A significant systemic protection against grey mould was also observed in the next year (2005) following bacterial treatments (up to 62 % for leaves and 82 % for berries). Although this protection occurred when the intensity of *B. cinerea* contamination was less important than in 2004, this clearly indicates that biocontrol activity of applied bacteria might be maintained in vineyards, at least over year $n + 1$. These results may reflect an adaptation of selected bacteria to their native environment which thus preserve their biocontrol activity. Such process might impact on the density of some bacteria and their performance especially over a moderate *B. cinerea* contamination. Similar results have been reported in wheat plants (Schippers et al. 1987) showing that some bacteria applied at year n could survive in soil or on plant residues until year $n + 1$ leading to a strong reduction of plant diseases. Janisiewicz and Korsten (2002) also suggested that the increase of the genetic diversity of biocontrol agents through the use of mixtures of microorganisms would lead to more persistent protection over time and for a broader range of environmental conditions. Interestingly, we showed that the mixtures containing both *P. agglomerans* strains (PTA-AF1 and PTA-AF2) were also among the most effective in 2005 in reducing systemically the grey mould symptoms in grape plants.

Induced resistance was correlated in most cases to an activation of some defense reactions during treatment with single or mixtures of bacteria, in similar extent to earlier results of Trotel-Aziz et al. (2008) and Verhagen et al. (2011) under controlled conditions, and strongest correlations were mainly observed with the treatments containing *Pantoea* spp. combined with the other bacterial isolates. Indeed, during the 2004 season drenching the plant soil with bacterial mixtures resulted in a significant and successive activation of

chitinase and β-1,3-glucanase in leaves and berries. The activity of these PR proteins started to increase three weeks after the onset of bacterial application. Interestingly, chitinase activity of leaves was up to two fold higher after treatment with bacterial mixtures than with single bacteria. This is especially apparent in berries after treatment with mixtures combining *A. lwoffii* with *B. subtilis*, *A. lwoffii* or *B. subtilis* with *P. fluorescens*, or those containing *Acinetobacter* spp. and *Pantoea* spp. Such a delayed stimulation of chitinase activity in grapevine leaves and berries might contribute to enhance systemic resistance towards *B. cinerea*. Both chitinase and glucanase activities should participate in the plant defense by hydrolyzing fungal cell wall components (van Loon and van Strien 1999). They also should amplify the plant defense by the released chitin and glucan fragments as elicitors (Côté et al. 1998; Trotel-Aziz et al. 2006; Aziz et al. 2007). It has been reported that there is not always a strong correlation between expression of defense-related genes and ISR (Ahn et al. 2007), and that other defense-related mechanisms such as callose deposition, accumulation of active oxygen species, and papillae formation may be activated (Verhagen et al. 2004; Wang et al. 2005). Phytoalexin (e.g. resveratrol and viniferin) accumulation would be another important part of grapevine defense that is often involved in disease resistance (Aziz et al. 2003, 2006). Such a specific accumulation of phytoalexins was recently observed both in leaves and cells of grapevine treated with some of the bacteria used in this study (Verhagen et al. 2011) or with other inducing ISR-*Pseudomonas* spp. (Verhagen et al. 2010). These observations were in concordance with induction of phenylalanine ammonialyase activity in bacterized plants (Magnin-Robert 2007; Trotel-Aziz et al. 2008). Therefore, it appears likely that inhibition of *B. cinerea* development, which is a marked trait of bacterial effect, is certainly a consequence of the induced defense reactions.

To conclude, this is the first report providing evidence that some applications of bacterial mixtures in vineyards might induce systemic resistance in grapevine against *B. cinerea* and stimulate some of the plant defense-reactions simultaneously. Treatments with mixtures containing different bacteria together with *P. agglomerans* appeared as the most protectors and resulted in high and maintained chitinase and glucanase activities. Interestingly, the induced resistance could be maintained with a better homogeneity during the season $n + 1$ without renewal of bacterial treatments. Further investigations are still required to better understand the mechanisms involved in the induced resistance of grapevine against *B. cinerea*, as well as complexity of microbial interactions and impacts of environmental factors on biocontrol efficiency in vineyards.

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