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Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine

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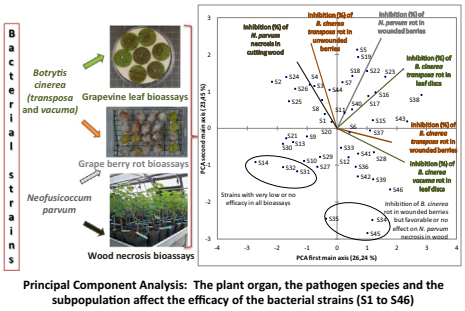
HIGHLIGHTS

- 46 bacterial strains from grapevines screened for antagonism toward two pathogens.
- *Bacillus ginsengihumi* reduced *B. cinerea* rot on leaf discs and wounded berries.
- *Pantoea agglomerans* strains inhibited *N. parvum* necrosis in grapevine cutting stems.
- Synergistic relationships shown between several bacterial strains and the pathogens.

GRAPHICAL ABSTRACT



Principal Component Analysis: The plant organ, the pathogen species and the subpopulation affect the efficacy of the bacterial strains (S1 to S46)

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ABSTRACT

Botrytis cinerea and *Neofusicoccum parvum* are devastating fungal pathogens of grapevines. A total of 46 bacterial strains isolated from grapevines were screened for their antagonistic activity toward *B. cinerea*, which causes gray mold, and *N. parvum*, which causes cankers, trunk diseases and berry rot. *In vivo* screening using detached berries and leaf discs and *in planta* screening using growing grapevine cuttings were compared to select the most efficient biological control agents. On grapevine leaf discs, the most effective strain, *Bacillus ginsengihumi*, exhibited more than 80% antagonistic activity against the two major grapevine subpopulations of *B. cinerea* (*transposa* and *vacuma*). At the berry surface, the efficacy of the bacterial strains strongly depended on the presence or absence of wounds. On stem cuttings, some strains, notably *Pantoea agglomerans*, were able to significantly reduce the length of the necrosis caused by *N. parvum*. Thus, both the plant parts and the pathogen species may significantly affect the efficacy of the bacterial strain. Marked differences between the two fungal pathogen species in terms of strain inhibitory efficacy were observed on wounded berries, as some Bacillus spp. strains efficiently controlled *B. cinerea* but not *N. parvum*. Noticeable differences in bacterial antagonist efficiencies were detected between berries and leaf discs inoculated with *B. cinerea* (*transposa*), e.g., some Paenibacillus spp. strains were efficient on leaves but not on berries. Synergistic relationships were also revealed between several bacterial strains and the pathogens. Some bacterial strains (e.g., *Bacillus licheniformis*) reduced *B. cinerea* rot severity in wounded and unwounded berries, but they increased *N. parvum* necrosis on stem cuttings.

1. Introduction

Botrytis cinerea Pers.:Fr. is a necrotrophic plant pathogen of economic importance that affects more than 200 host plants including vegetables, ornamentals, and fruit crops (Jarvis, 1977; Williamson et al., 2007). In viticulture, this pathogen causes lower yields and significant reductions in wine quality (Elmer and Michailides, 2007; Ky et al., 2012) and is considered a species that is genetically complex and diverse, notably including different transposon genotypes. In vineyards, the two major sympatric transposon genotypes are: (i) *transposa*, which harbors the transposable elements Boty

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(Diolez et al., 1995) and Flipper (Levis et al., 1997) and (ii) *vacuma*, which lacks these transposons. The proportions of these subpopulations in vineyards may change during the season, with the *vacuma* subpopulation detected early in the season, notably at the time of blooming. Meanwhile, the frequency of the *transposa* subpopulation, interestingly characterized by isolates that demonstrate increased virulence on grape berries (Martinez et al., 2005), increases during fruit growth and maturation and becomes largely predominant at harvest (Giraud et al., 1997; Martinez et al., 2008; Walker et al., 2014). Currently, *B. cinerea* is primarily controlled via the application of specific anti-Botrytis synthetic fungicides that favor the development of resistant strains to most chemicals in many viticultural regions in France and in other countries (Elad and Evensen, 1995; Leroux et al., 2002; Walker et al., 2013).

Grapevine trunk diseases (GTDs), including *Eutypa* dieback, Esca and *Botryosphaeria* dieback, are considered today to be major factors limiting grapevine productivity (Bertsch et al., 2013; Bruez et al., 2013). Among several pathogenic fungi associated with GTDs, *Neofusicoccum parvum* (Pennycook & Samuels) Crous et al. (2006), an anamorph of *Botryosphaeria parva* (Pennycook and Samuels, 1985), has been shown in different pathogenicity tests to be one of the most virulent of the fungal species associated with the GTDs (Laveau et al., 2009; Úrbez-Torres and Gubler, 2009). This species has been frequently associated with necrosis and external wood necroses (canker lesions) in grapevine wood (Amponsah et al., 2011; Laveau et al., 2009; Phillips, 2002). In addition, it was reported to cause a bunch rot of grapevine berries, as found with some other species of *Botryosphaeriaceae* (Steel et al., 2013; Wunderlich et al., 2011a,b). To date, since the ban of sodium arsenite in 2001 in Europe due to its toxicity to the environment and humans, no efficient chemical control measure has been developed to control GTDs and/or *Botryosphaeriaceae* infections in vineyards (Decoin, 2001; Larignon et al., 2008). Thus, to prevent the extension of GTDs and to reduce the use of pesticides in the management of grapevine diseases, studies of complementary and/or alternative methods to synthetic chemicals have sparked interest in viticulture. Numerous prior studies showed the antagonistic activities of several species of bacteria, fungi and yeast towards GTD fungal agents (Bertsch et al., 2013; Compant et al., 2013), but no commercial bacterial product has been developed for the control of GTDs.

The majority of methods for selecting BCAs of plant pathogens have been focused on a single biocontrol agent as the antagonist for a single pathogen (Pliego et al., 2011). Using one antagonist or a combination of specific antagonists or a generalist strain of BCA to control more than one plant pathogen remains an unfulfilled goal. Given that screening is the critical first step in the development of BCAs, it is essential to note that there is not always a correlation between *in vitro* inhibition and *in planta* control of infection and/or symptom development (Donmez et al., 2011; Duffy et al., 2003; Köhl, 2009; Köhl et al., 2011; Pliego et al., 2011; Raspor et al., 2010). Thus, it was necessary to develop appropriate screening *in vivo* procedures in grapevine to select potential antagonistic bacteria of interest in a multi-disease approach. Because it has been recommended that antagonistic microorganisms should preferably be derived from their natural environments (Pliego et al., 2011; Raspor et al., 2010), we evaluated 46 bacterial strains that all originated from vineyards (grapevine wood or grape berries) to ensure that the selected BCA candidates would be better adapted to vineyard conditions in the future.

Thus, our major objectives were as follows. First, to evaluate and select novel bacterial strains isolated from grapevine as potential biocontrol agents against two major grapevine fungal pathogen species, *B. cinerea* and *N. parvum*, including two strains from two major vineyard subpopulations of the latter (transposon genotypes: *transposa* and *vacuma*). Second, to compare the efficacy of the bacteria in controlling the two pathogens on susceptible host organs of interest, i.e., comparing berries and leaves for *B. cinerea* and berries and stems of growing cuttings for *N. parvum*. Third, we analyzed all our results to detect possible synergistic relationships between the bacteria and pathogens that could interfere with later disease control.

2. Materials and methods

2.1. Pathogenic fungal strains and culture conditions

2.1.1. B. cinerea

Two pathogenic *B. cinerea* strains were selected from the INRA-UMR 1065 SAVE collection, Bordeaux, as representatives of each major transposon genotype: 213T for *transposa* and 357V for *vacuma*. These were selected on the basis of their aggressiveness, ranging from high virulence (213T) to intermediate-high virulence (357V), compared to other *B. cinerea* strains from the same collection for which virulence had been compared in pathogenicity tests on grape berries (Martinez et al., 2005).

Stock cultures were maintained on malt agar (MA) (15 g L^{-1} of malt from Biokar Diagnostics, and 20 g L^{-1} of Setaxam® agar) at 5 °C before being used in experiments.

To prepare for the inoculation of leaves and berries, strains were cultured in Petri dishes containing MA medium at 22 °C (12 h light/12 h dark). Mycelium plugs with growing mycelia were then obtained after 7–10 days.

2.1.2. N. parvum

The *N. parvum* isolate (“Cou 02”) was selected from the INRA-UMR 1065 SAVE collection, Bordeaux. This strain was originally obtained in 2008 from a Cabernet Sauvignon cultivar in an experimental INRA vineyard near Bordeaux and was characterized as highly aggressive in previous preliminary studies at INRA. The GenBank accession number for rDNA (ITS, 5.8S and ITS2) of *N. parvum* strain is KT306957. The strain was subcultured on MA and incubated at 22 °C (12 h light/12 h dark) for 1 week before being used for artificial inoculation in bioassays with berries and cuttings.

2.2. Bacterial strains

A total of 46 bacterial strains, all isolated from grapevine, including 35 strains from wood tissue and 11 from the grape berry surface, were tested (Table 1). The GenBank accession number for 16S rDNA of all the strains are provided in Table 1. The 11 strains isolated from the grape berry surface originated from “Biological Resources Center for Enology” (University of Bordeaux and Bordeaux Polytechnic Institute). Strains were maintained on Viabank cryogenic storage beads at −20 °C. Before being inoculated inside the plant host, and depending on the bioassay, bacteria were grown in glass tubes (40 mL) or in Corning cell culture flasks for 24 h at 28 °C in the dark in tryptic soy broth (TSB, Difco) or Luria–Bertani (LB) liquid medium (Sigma) depending on the assay. Bacterial growth was determined by comparing the turbidity with a McFarland standard solution (bioMérieux® SA), which is equivalent to 10^8–10^9 CFU/mL (4 on the McFarland scale).

2.3. Plant material

2.3.1. Grapevine leaf discs

Healthy leaves used in the leaf bioassay were detached from *Vitis vinifera* (cv. Cabernet Sauvignon) stem cuttings that were harvested in an INRA greenhouse at 25 °C. The third and fourth

Table 1
List and identification of the bacterial strains, isolated either from grape berries (Martins, 2012) or from grapevine wood tissues, of asymptomatic and esca-foliar symptomatic plants (Bruez, 2013).

Test code	Bacterial species	Bacterial origin	Symptomatic/asymptomatic plant	Wood tissues	GenBank accession number
S1	*Pantoea agglomerans*	Grape berries	NA	Surface	HE648133.1
S2	*Pantoea agglomerans*	Grape berries	NA	Surface	HE648432.1
S3	*Pantoea agglomerans*	Grape berries	NA	Surface	HE648134.1
S4	*Pantoea agglomerans*	Grape berries	NA	Surface	HE588003.1
S5	*Pantoea agglomerans*	Grapevine wood	Asymptomatic	Necrotic cordon	KT363714
S6	*Pantoea agglomerans*	Grapevine wood	Asymptomatic	Non-necrotic cordon	KT363715
S7	*Bacillus pumilus*	Grapevine wood	Asymptomatic	Necrotic cordon	KT363710
S8	*Pantoea agglomerans*	Grapevine wood	Asymptomatic	Necrotic cordon	KT363691
S9	*Paenibacillus turicensis*	Grape berries	NA	Surface	HE587983.1
S10	*Paenibacillus turicensis*	Grapevine wood	Symptomatic	Non-necrotic cordon	KT363708
S11	*Paenibacillus turicensis*	Grapevine wood	Symptomatic	Non-necrotic Cordon	KT363709
S12	*Paenibacillus illinoisensis*	Grape berries	NA	Surface	HE587985.1
S13	*Paenibacillus illinoisensis*	Grape berries	NA	Surface	HE587984.1
S14	*Paenibacillus barengoltzii*	Grapevine wood	Symptomatic	Inner trunk	KT363707
S15	*Paenibacillus polymyxa*	Grapevine wood	Asymptomatic	Necrotic cordon	KT363711
S16	*Paenibacillus* sp.	Grapevine wood	Asymptomatic	Non-necrotic cordon	KT363724
S17	*Paenibacillus* sp.	Grapevine wood	Asymptomatic	Inner trunk	KT363716
S18	*Paenibacillus* sp.	Grapevine wood	Asymptomatic	Non-necrotic cordon	KT363713
S19	*Paenibacillus* sp.	Grapevine wood	Asymptomatic	Inner trunk	KT363722
S20	*Paenibacillus* sp.	Grapevine wood	Asymptomatic	Inner trunk	KT363712
S21	*Enterobacter cowanii*	Grape berries	NA	Surface	HE588001.1
S22	*Enterobacter cowanii*	Grape berries	NA	Surface	HE587998.1
S23	*Enterobacter* sp.	Grapevine wood	Asymptomatic	Inner trunk	KT363690
S24	*Enterobacter* sp.	Grapevine wood	Asymptomatic	Inner trunk	KT363719
S25	*Acinetobacter radioresistens*	Grape berries	NA	Surface	HE588005.1
S26	*Acinetobacter radioresistens*	Grape berries	NA	Surface	HE588004.1
S27	*Brevibacillus reuszeri*	Grapevine wood	Symptomatic	Necrotic cordon	KT363698
S28	*Brevibacillus reuszeri*	Grapevine wood	Symptomatic	Necrotic cordon	KT363697
S29	*Bacillus licheniformis*	Grapevine wood	Asymptomatic	Necrotic cordon	KT363721
S30	*Brevibacillus reuszeri*	Grapevine wood	Symptomatic	Necrotic cordon	KT363699
S31	*Brevibacillus reuszeri*	Grapevine wood	Symptomatic	White rot	KT363700
S32	*Bacillus pumilus*	Grapevine wood	Asymptomatic	Necrotic cordon	KT363718
S33	*Bacillus licheniformis*	Grapevine wood	Asymptomatic	Inner trunk	KT363723
S34	*Bacillus* sp.	Grapevine wood	Asymptomatic	Inner trunk	KT363701
S35	*Bacillus pumilus*	Grapevine wood	Symptomatic	White rot	KT363702
S36	*Bacillus ginsengihumi*	Grapevine wood	Symptomatic	White rot	KT363693
S37	*Brevibacillus reuszeri*	Grapevine wood	Asymptomatic	Inner trunk	KT363720
S38	*Bacillus ginsengihumi*	Grapevine wood	Asymptomatic	Necrotic cordon	KT363694
S39	*Bacillus ginsengihumi*	Grapevine wood	Symptomatic	White rot	KT363695
S40	*Brevibacillus reuszeri*	Grapevine wood	Symptomatic	Necrotic cordon	KT363703
S41	*Bacillus firmus*	Grapevine wood	Symptomatic	Necrotic cordon	KT363704
S42	*Curtobacterium* sp.	Grapevine wood	Asymptomatic	Outer trunk	KT363692
S43	*Bacillus* sp.	Grapevine wood	Asymptomatic	Necrotic cordon	KT363705
S44	*Bacillus licheniformis*	Grapevine wood	Symptomatic	White rot	KT363706
S45	*Xanthomonas* sp.	Grapevine wood	Asymptomatic	Necrotic cordon	KT363696
S46	*Bacillus* sp.	Grapevine wood	Symptomatic	White rot	KT363717

leaves counting from the apex were detached and rinsed with distilled water. Leaves were surface disinfected for 10 min in 50 g L^{-1} sodium hypochlorite solution at pH 7.2, rinsed one time with sterile water, and then dried on sterile filter papers. Eight discs (2.5 cm in diameter) were excised from each leaf with a cork borer. Seven leaf discs were distributed in one Petri dish with the abaxial surface facing upward. Beforehand, the bottom of each dish had been covered with filter paper dampened with 10 mL of sterile water.

2.3.2. Grape berries

For all tests, table grapes (cultivar: Thompson Seedless) originating from the supermarket were washed for 15 min under continuous tap-water flow. Then, they were surface disinfected via immersion in calcium hypochlorite solution (50 g L^{-1}; pH 7.2) for 10 min, rinsed three times with distilled water and left to dry at room temperature. Undamaged grape berries were selected visually and carefully cut off from the grape bunches, using scissors, with the pedicel attached.

2.3.3. Grapevine wood cuttings

Rooted cuttings (cultivar: Cabernet Sauvignon), originating from INRA experimental vineyards near Bordeaux, were used in the *N. parvum* wood inoculation bioassay. They were processed and prepared as described by Laveau et al. (2009). During the period of incubation for the two bioassays, between April and October 2013, the plants were grown in an open greenhouse in which air temperature (*T*) and air relative humidity (RH) were monitored using Vaisala HMP 35C probes (Campbell Sci., Logan, UT). The average *T* was 21 °C and 66% for RH.

2.4. Bioassays

2.4.1. Leaf bioassays

The antagonistic activity of the 46 bacterial strains was tested on grapevine leaf discs against two pathogenic *B. cinerea* strains corresponding to the two main transposon genotypes of epidemiological importance in vineyards: 213T for the *transposa* subpopulation and 357V for the *vacuma* subpopulation (Martinez et al., 2005, 2008).

Two bioassays were performed with the two transposon genotypes of *B. cinerea*. Each bioassay involved two tests: the first test was carried out with 26 bacterial strains (S1–S26) and the second with 20 strains (S27–S46). For each test, 7 leaf discs were distributed per Petri dish, and four dishes were replicated for each

treatment corresponding to the combination of one bacterial strain and one pathogenic strain (n = 28 leaf discs per combination).

The control treatments were as follows: (i) UUC (untreated uninoculated control) untreated with the bacteria and uninoculated with the pathogen (n = 14 discs); (ii) UC (untreated control) inoculated with pathogen mycelium plugs only (n = 30 discs); (iii) BC (bacteria control) treated with bacterial suspension only (n = 28 discs per strain); (iv) MC (medium control) uninoculated, treated only with the sterile bacterial culture medium TSB (n = 14 discs); (v) fungicide control FC (n = 28 discs) with a fungicide solution of Fluazinam (Sekoya, Syngenta France SAS, 50% a.i., 250 g a.i. 100 L).

Application of the bacterial suspensions and the fungicide solution was carried out by spraying to runoff the leaf discs using a sprayer EcoSpray (A 520, Labo Chimie France).

Petri dishes were then placed at 23 °C for 24 h in controlled growth chambers (Conviron CMP-5090; Winnipeg, Manitoba, Canada). Then, depending on the bioassay, one mycelial plug (4 mm in diameter) of 213T or 357V was placed at the center of each leaf disc using a sterile scalpel (mycelia facing the leaf surface). The Petri dishes were then incubated at 23 °C with a 12-h light photoperiod. Disease development on leaf discs was measured as the average of two perpendicular diameters for every typical rotted lesion 5 days post inoculation with the pathogen. The lesion diameter reduction rate was ultimately expressed as a percentage based on the UC.

2.4.2. Grape berry rot bioassays with B. cinerea and N. parvum

All bacterial strains were evaluated for their biocontrol potential against *B. cinerea* (213T) and *N. parvum* (Cou02) (at 23 °C and 28 °C, respectively). The strain of *B. cinerea* used was chosen based on its high virulence on grape berries (Martinez et al., 2005).

Two bioassays were performed corresponding to the two pathogens. For methodological reasons, for each bioassay there were two tests: the first with 26 strains (S1–S26) and the second with 20 strains (S27–S46). In both tests, the efficacy of bacterial strains against both *B. cinerea* and *N. parvum* was tested on wounded and unwounded berries. In the first test, the experimental design consisted of 20 berries per treatment (strain × pathogen × wounded–unwounded), whereas in the second test, the sample number was reduced to 15 berries per treatment.

For wounded berries, three artificial wounds (1–1.5 mm in diameter) were made in a triangle on the equatorial zone of the berry using a sterile pipette tip. Then, 10 µl of each bacterial strain suspension was introduced into each wound site.

In the tests, unwounded berries were dipped in the bacterial suspension with TSB medium contained in a Corning cell culture flask.

The control treatments with wounded and unwounded berries consisted of: (i) UUC (untreated uninoculated control) untreated with the bacteria and uninoculated with the pathogen, (ii) UC (untreated control) inoculated with mycelium plugs of the pathogen only, (iii) MC (medium control) untreated and uninoculated controls sprayed only with sterile bacterial culture medium TSB, (iv) FC (fungicide control) berries sprayed to runoff, using an EcoSpray sprayer (A 520) with a fungicide solution of Fluazinam (Sekoya, Syngenta France SAS, 50% a.i., 250 g a.i. 100 L).

Regarding incubation, the berries were placed on a metallic grid in high humidity chambers (plastic boxes, 19 × 13 × 4 cm) at a rate of 20 or 15 berries per box depending on the test. In each chamber, 100 mL of distilled water was added and the chambers were placed into a controlled growth incubator (Conviron CMP-5090; Winnipeg, Manitoba, Canada) in the dark at 28 °C for 24 h to allow bacteria to better colonize the berries before pathogen inoculation.

After 24 h incubation, the wounded and unwounded fruits were inoculated with mycelial plugs (4 mm in diameter) of either *B. cinerea* or N. parvum. One plug was deposited on the equator of each berry with the mycelial side facing the berry surface. As for wounded berries, the plug was placed at the center of the three wounds. The humidified chamber boxes for B. cinerea and N. parvum were then placed in the growth chambers at 23 °C and 28 °C, respectively. For each berry, the percentage of rotten berry surface area was visually scored, and the average rot severity of each treatment was calculated as described by Calvo-Garrido et al. (2014). Development of *B. cinerea* rot severity was assessed at 7 dpi on wounded berries and at 11 dpi on unwounded berries. With *N. parvum*, rot severity was assessed at 11 dpi on wounded berries and at 12 dpi on unwounded berries.

2.4.3. Stem disease bioassay

The ability of the bacterial strains tested to suppress typical stem disease symptoms (canker and necrosis) following *N. parvum* artificial inoculation was determined by performing a wood cuttings bioassay under open greenhouse conditions. Two tests were performed: the first with 26 bacterial strains (S1–S26) and the second with 20 strains (S27–S46). In the first test, the trial layout was a completely randomized block design with two blocks, each containing 232 plants that corresponded to plants of the cultivar Cabernet Sauvignon. Twenty-nine treatments (with 8 plants per treatment in each block) were tested corresponding to the twenty-six bacterial strains co-inoculated with the pathogenic fungus and three control treatments including: (i) uninoculated untreated control (UUC), in which samples were not inoculated with the fungus and not treated with bacteria, (ii) untreated control (UC), with samples inoculated with the fungus *N. parvum* only, and (iii) fungicide control (FC), treated with a fungicide solution of Fluazinam (Sekoya, Syngenta France SAS, 50% a.i., 250 g a.i. 100 L). The UUC cuttings were treated with sterile bacterial culture medium (TSB for the first test and LB for the second test) and with sterile MA plugs. In the second test, 268 plants were used. Eleven plants were tested for every bacterial strain co-inoculated with the pathogenic fungus. The same controls as in the first test were used with 16 plants for each control treatment.

In both bioassays, the stem of each cutting was surface-sterilized by rubbing with paper towel, with 95% ethanol. Then, in the central part of each stem cuttings below the upper bud, an artificial wound was made by drilling a hole in the bark (4 mm in diameter) with a drill. The fresh wound was immediately drop-inoculated with 40 µl of bacterial suspension. Once the liquid was dried 20–40 min at air temperature, the hole was filled with a *N. parvum* mycelium plug cut off from the margin of a fresh mycelial MA culture, with the mycelium facing the inner part of the stem. The treated wounds were then, individually, covered with Parafilm® (Scellofrais film) for protection during the incubation period. After an incubation period of 104 ± 7 days, the presence (incidence) and the length of an external canker were assessed visually on every cutting. Then, the stem of each plant was cut longitudinally in the middle, and the length of the internal vascular lesions (or necroses) in the cutting wood, just under the bark, was recorded visually by measuring the necrotic lesions upwards and downwards from the wound-inoculation hole. To better detect and expose necrotic zones, wood tissue around the top-margin of the necrotic zone was sometimes removed with a scalpel.

2.5. Statistical analysis

Statistical tests were performed to identify and quantify the major relationships among the main seven variables characterizing the inhibition rates from the different bioassays. Standard correlation tests (Pearson correlation coefficient) were used and an associated principal component analysis (PCA) was also performed. Six variables were submitted to the PCA as active major variables

corresponding to the inhibition rates of (i) *B. cinerea vacuma* in leaf-disc tests, (ii) *B. cinerea transposa* in leaf-disc tests, (iii) *B. cinerea transposa* in unwounded-berry tests, (iv) *B. cinerea transposa* in wounded-berry tests, (v) *N. parvum* in wounded-berry tests, and (vi) *N. parvum* in stem cuttings. The unique variable corresponding to the inhibition rate of *N. parvum* in the unwounded-berry tests was considered a supplementary variable because of the relatively low levels of symptoms expressed in the inoculated control by the pathogen in this type of experiment. For analysis of variance (ANOVAs) as well as for PCA analysis, two statistical software packages were used: StatBox (Version 6.6, Grimmer© Logiciels, Paris) and XLSTAT (Version 2014.4.04, Addinsoft©, www.xlstat.com).

3. Results

3.1. Leaf bioassays

The results of the severity reduction are shown in Fig. 1. After 5 days of incubation, the mean *Botrytis* rot severity at the leaf surface in the untreated controls inoculated only with *B. cinerea* reached (i) 90.3% and 88.2% in the two tests for the strain representative of the *transposa* subpopulation and (ii) 50.7% and 97.7% in the two tests for the strain representative of the *vacuma* subpopulation. The leaf discs exhibited no symptoms in two supplementary control treatments, either with no pathogen inoculation or following spraying with a specific fungicide (Fluazinam) before *B. cinerea* inoculation (data not shown).

Regarding the effects of bacterial strains, the results showed that most of the strains tested were able to inhibit one or both strains of *B. cinerea* (Fig. 1). When tested with *B. cinerea vacuma*, 15 strains significantly reduced ($P = 0.05$) the *B. cinerea vacuma* leaf symptoms when compared with the corresponding control, with inhibition levels ranging from 45.2% to 94.18%. In this bioassay, seven bacterial strains (in decreasing order of efficacy: S38, S42, S27, S43, S46, S39, S41) exhibited more than 80% inhibition of gray mold. Three strains (S34, S37, S45) demonstrated inhibition rates between 60% and 80%, and nine bacteria (S6, S22, S28, S29, S1, S17, S16, S3, S19) displayed inhibition levels ranging from 40% to 60%. Following inoculation with *B. cinerea transposa*, 12 strains significantly reduced ($P = 0.05$) lesion diameters attributable to the pathogen with inhibition levels ranging from 41.5% to 90.78%. In this bioassay, only one strain, S38, achieved an inhibition level greater than 80%. Three strains (S18, S5, S17) exhibited inhibition levels between 60% and 80%, and eight strains (S15, S23, S43, S19, S16, S22, S29, S28) achieved inhibition levels between 40% and 60%.

One bacterial strain, S38 (*Bacillus ginsengihumi*), isolated from grapevine wood, demonstrated the highest inhibition rate for lesion development caused by each *B. cinerea* strain (94.1% and 90.7% with *vacuma* and *transposa*, respectively). The percentage of inhibition achieved by this strain was significantly different from that of the untreated control (UC) and all the other strains in the *B. cinerea transposa* tests. In the *B. cinerea vacuma* tests, it was significantly greater than the control and 35 other bacterial strains.

Some strains exhibited an inhibitory capacity against only one strain that was representative of a transposon genotype of *B. cinerea*. For example, three strains (S37, S42, S45) did not exert any significant antagonistic effects toward *B. cinerea transposa*, but they notably controlled the *vacuma* strain with a minimal efficacy of 60%. Conversely, one strain (S5) did not significantly inhibit *B. cinerea vacuma*, whereas it was significantly effective against *B. cinerea transposa*.

3.2. Grape berry rot bioassays with B. cinerea and N. parvum

Severity reduction results are shown in Figs. 2 and 3. The untreated control UC (berries inoculated with the pathogen only) in both bioassays showed notable necrotic disease symptoms. In the *B. cinerea* bioassay, overall rot severity reached 76.4% and 91.8% with the untreated control (UC) treatments in the tests using

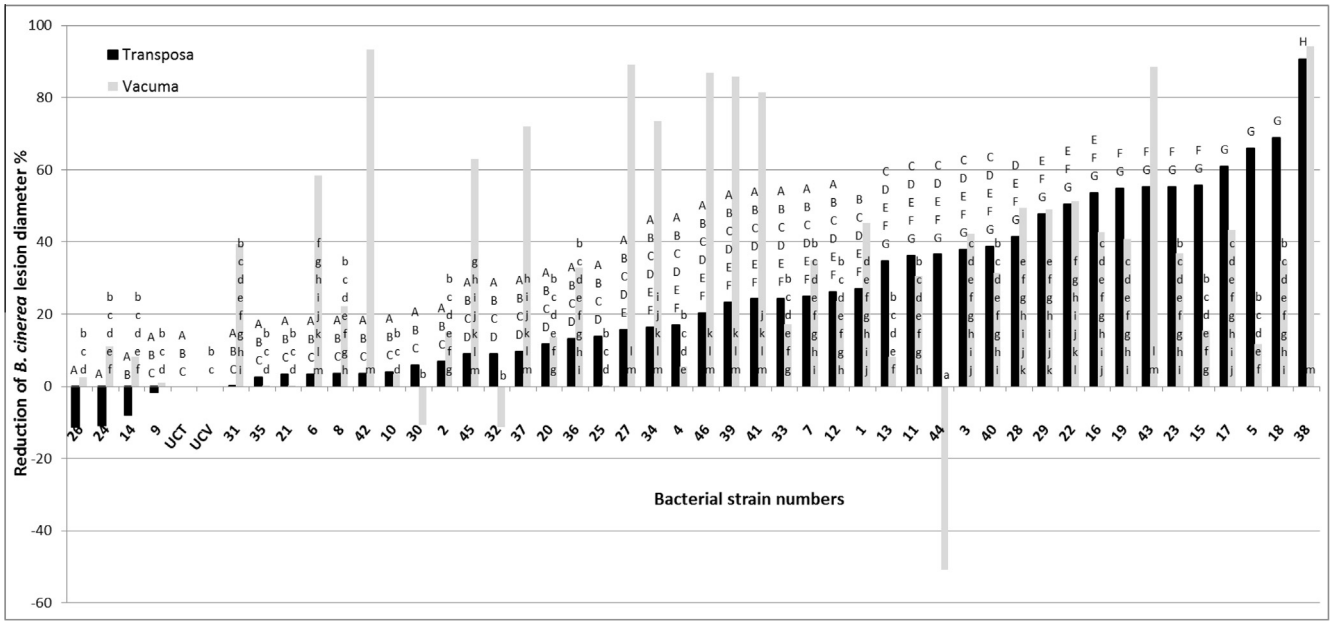

Fig. 1. Percentage of symptom inhibition exhibited by 46 bacterial strains in terms of lesion diameter caused by *B. cinerea* infection. Two subpopulations of *B. cinerea* were inoculated onto grapevine leaf discs: *transposa* (black bars) and *vacuma* (gray bars). Leaf discs (cv. Cabernet Sauvignon) were sprayed with bacterial suspensions and incubated at 23 °C for 24 h and then inoculated with one mycelium plug of one pathogen subpopulation. The percentage of inhibition was scored after a 5-day incubation period at 23 °C. The untreated controls (one for each *B. cinerea* strain; UCV and UCT) corresponded to inoculation with the pathogen only. Each value represents the mean of 28 leaf discs for each strain and 42 discs for each control. For each *B. cinerea* subpopulation, overall means linked by the same letter are not significantly different according to Newman and Keuls' test after ANOVA ($P = 0.05$).

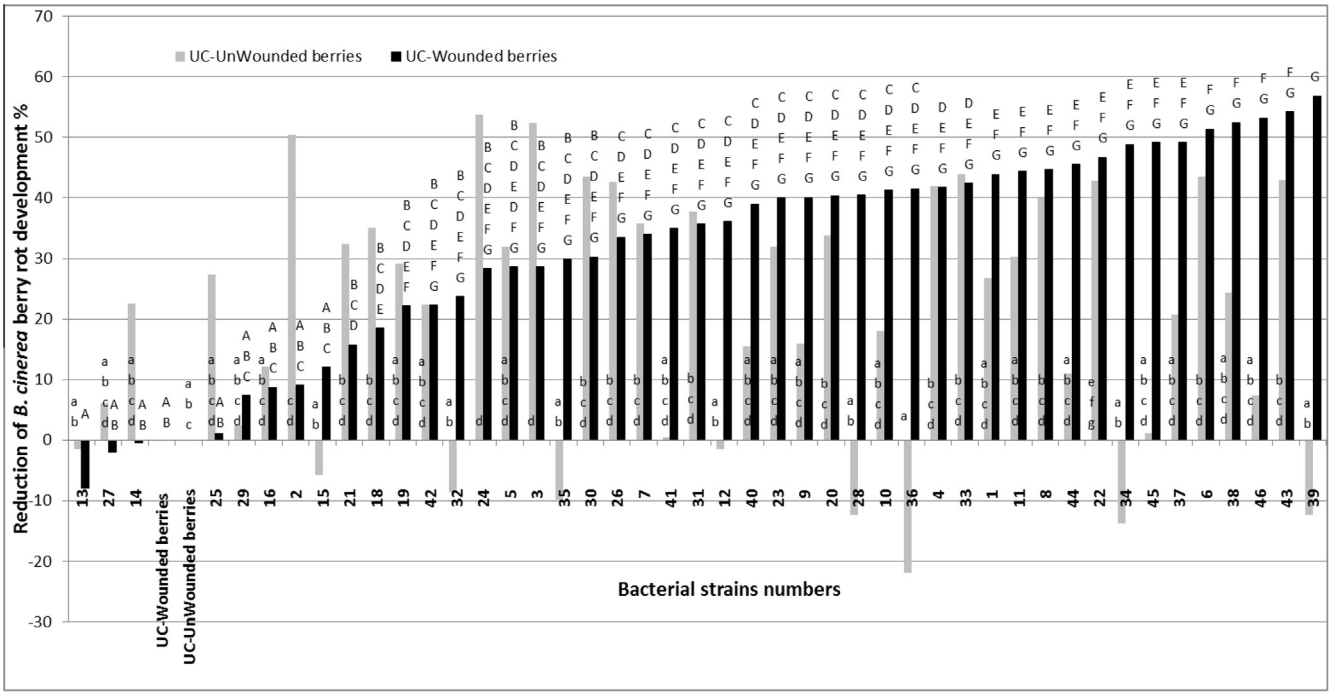

Fig. 2. Percentage of symptom inhibition exhibited by 45 bacterial strains on rot development due to *B. cinerea.* Wounded (black bars) and unwounded (gray bars) grape berries (cv. Thompson Seedless) were inoculated with bacteria before incubation for 24 h at 28 °C and then inoculated with one mycelium plug of *B. cinerea* (*transposa*). The untreated controls (one for wounded berries, UC-wounded berries, and one for unwounded berries, UC-unwounded berries) corresponded to inoculation with pathogen only. Each value represents the mean of 15 or 20 berries according to the test (see Section 2.4.3). Bars with the same letter are not significantly different at $P = 0.05$ according to Newman and Keuls' test after ANOVA (one ANOVA per type of berries, wounded or unwounded). The bacterial strain S17 was not tested in this bioassay.

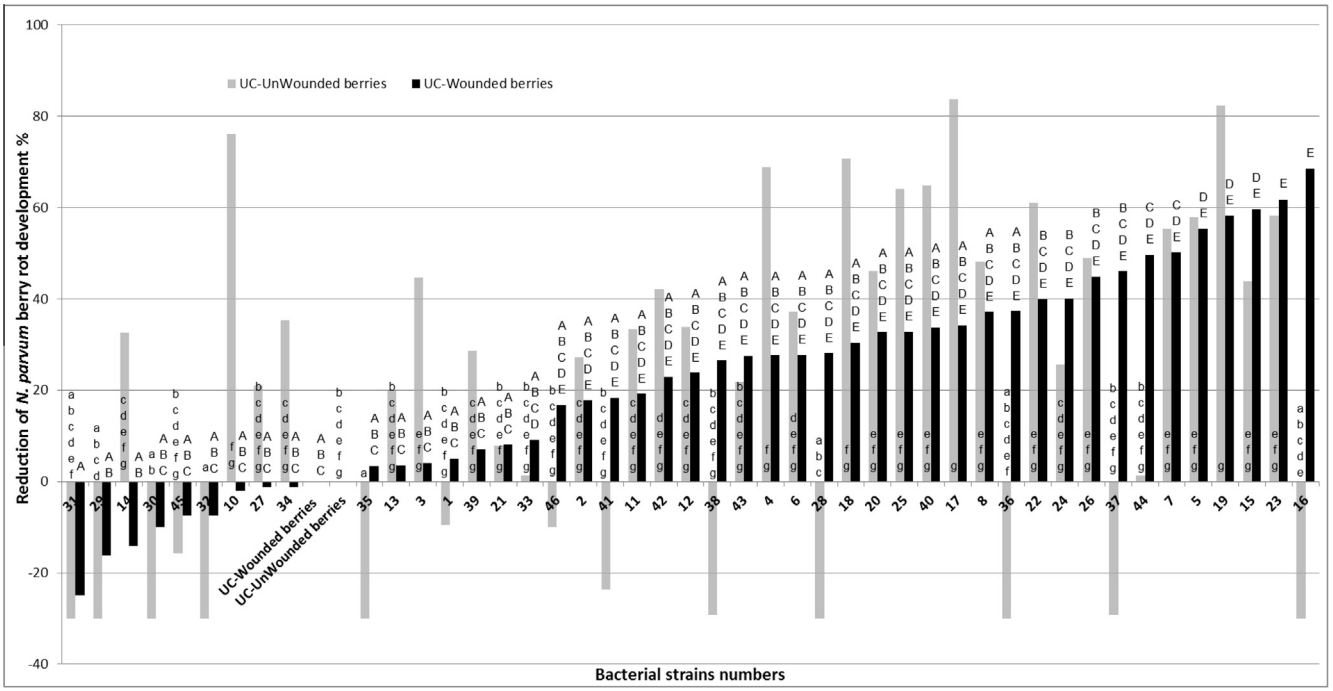

Fig. 3. Percentage of symptom inhibition displayed by 45 bacterial strains on rot development due to *N. parvum.* Wounded (black bars) and unwounded (gray bars) grape berries (cv. Thompson Seedless) were inoculated with bacteria before incubation for 24 h at 28 °C and then inoculated with one mycelium plug of *B. cinerea* (*transposa*). The untreated controls (one for wounded berries, UCW, and one for unwounded berries, UCUW) corresponded to inoculation with pathogen only. Each value represents the mean of 15 or 20 berries according to the test (see Section 2.4.3). Bars with the same letter are not significantly different at $P = 0.05$ according to Newman and Keuls' test after ANOVA (one ANOVA per type of berries, wounded or unwounded). The bacterial strain S9 was not tested in this bioassay.

wounded berries. With unwounded fruit, these control mean percentages were 60.1% and 82% (data not shown). Similarly, in the *N. parvum* bioassay, the overall rot severity values reached with the untreated controls (UC) were 51.7% and 64.9% in the two tests with wounded berries and 20.3% and 43.0% in the two unwounded-fruit tests (data not shown). In both bioassays (one per pathogen),

grape berries for uninoculated UUC and MC controls were always asymptomatic during the entire incubation period. The control fruit "FC" treated with fungicide (Fluazinam) applied at the registered dosage significantly reduced *B. cinerea* symptoms, leading to an overall rot severity of 2.3% and 7.5% in the two tests using the most susceptible wounded berries, respectively. Similarly, in wounded berries inoculated with *N. parvum*, overall rot severity reached 0.0% and 11.1% in the two tests, respectively. The decrease in berry rot severity due to the fungicide was similar in the less susceptible unwounded berries in these bioassays (data not shown).

3.2.1. Reduction of B. cinerea severity by bacterial strains

The inhibition rates of 45 bacterial strains for controlling rot development due to *B. cinerea transposa* at the surface of detached grape berries are shown in Fig. 2. On wounded berries, 27 strains significantly protected grape berries from Botrytis bunch rot compared with the untreated control (UC) inoculated with pathogen only. Their inhibition levels ranged from 33.5% to 56.8%. Twenty-one out of the 27 bacterial strains tested exhibited inhibition levels between 40% and 60%. In unwounded berries, only two strains, S3 (*Pantoea agglomerans*) and S24 (*Enterobacter* sp.), were significantly different from the UC control ($P = 0.05$). The inhibition levels were 52.4% for S3 and 53.3% for S24. However, 11 bacterial strains were characterized by inhibition rates in the 40–60% range.

Importantly, these results showed that the inhibitory efficacies of bacterial strains against *B. cinerea* on grape berries depend on the presence or absence of wounds at the fruit surface (Fig. 2). For instance, five strains (S12, S28, S36, S34 and S39) exhibited significant efficacy levels in wounded berries against *B. cinerea*, but these strains failed to control the pathogen in unwounded berries. Furthermore, pretreatment with eleven strains (S13, S27, S14, S15, S32, S35, S12, S28, S36, S34 and S39) tended to increase the *B. cinerea* symptoms in wounded or/and unwounded fruit (although this was not significant at $P = 0.05$ compared with the corresponding untreated control). However, these bacterial strains exhibited significant differences compared to other strains, notably S36 (*B. ginsengihumi*) leading to a rot severity level in unwounded berries that was significantly higher than 16 other bacterial strains demonstrating inhibition rates exceeding 32.3%.

3.2.2. Reduction of N. parvum severity by bacterial strains

The results for the reduction of *N. parvum* rot lesions are shown in Fig. 3. On wounded berries inoculated with *N. parvum*, 5 out of the 45 bacterial strains tested (S5, S19, S15, S23, S16) significantly reduced ($P = 0.05$) *N. parvum* rot lesions compared with untreated controls (inoculated with pathogen only). Their inhibition levels ranged from 55.3% to 68.4%. In these tests, 9 tested strains demonstrated inhibition rates ranging from 40% to 60%. Two strains isolated from the surface of grape berries (S23 *Enterobacter* sp., S16 *Paenibacillus* sp.) exceeded 60% inhibition of the pathogen.

On unwounded berries, none of the bacterial strains were significantly different from the untreated control. However, nine bacterial strains exhibited inhibition levels between 40% and 60%. Eight other strains reached more than 60% inhibition; including two strains of *Paenibacillus* sp., S17 and S19, that exceeded 80%; these were isolated from the surface of grape berries. Conversely, on unwounded berries, two strains of *Bacillus pumilus* (S32 and S35) isolated from grapevine wood led to significantly greater *N. parvum* rot lesions than the untreated control.

3.3. Stem disease bioassay

3.3.1. Internal necrotic lesion lengths affected by the bacterial strains tested

All the stem cutting (cv. Cabernet Sauvignon) co-inoculated with a tested bacterial strain and *N. parvum* exhibited internal necrotic lesions. The lesions developed both upward and downward from the inoculation point. In the control cuttings inoculated with the pathogen only (untreated controls, UC), the mean lengths of the necrotic lesions reached 86.2 mm and 85.9 mm at the end of the incubation period of 104 ± 7 dpi for the two tests in this stem cutting bioassay (data not shown).

Evaluation of the antagonist potential of the bacterial strains against *N. parvum* indicated that some strains exhibited a significant difference ($P = 0.05$) compared with the control (Fig. 4). The inhibition rates of five strains (S1, S2, S3, S4, S24) were significantly different ($P = 0.05$) from the untreated control (UC), demonstrating marked antagonistic abilities. Their efficacies in decreasing internal necrotic lesion length varied between 32.3% and 43.5%. Moreover, the first four strains (S1–S4) were identified as *P. agglomerans* and were all isolated from the surface of grape berries (Table 1). The other efficient strain, S24 (*Enterobacter* sp.), was isolated from grapevine wood. Furthermore, although not significant compared with the control, 8 strains tended to favor *N. parvum* necrosis, and notably the two strains S35 and S45 demonstrated negative inhibition rates of approximately −20%. These two strains were *B. pumilus* (S35) and *Xanthomonas* sp. (45).

3.3.2. Relationship between the development of internal necrotic lesions and external canker expression

In the bioassays using growing grapevine cuttings in the greenhouse, the lengths of external cankers resulting from *N. parvum* inoculation and developing from the point of inoculation were measured at the end of the incubation period (104 ± 7 dpi). A highly significant Pearson's correlation at $P = 0.01$ (Pearson's $R = 0.79$, dF = 45) was established between the length of the necrosis and the length of cankers following *N. parvum* artificial inoculation of the growing grapevine cuttings (Fig. 5).

4. Relationships between inhibition rate variables in different bioassays

Interrelations between the different variables corresponding to the inhibition rates attributable to the 46 bacterial strains in the different bioassays were investigated by PCA (Fig. 6). The first two major factorial axes account for 49.7% of the total variance. The first composite axis was mainly representative of the inhibition rates obtained in the leaf-disc bioassays inoculated with *B. cinerea*. The two variables, corresponding to the inhibition rates of *B. cinerea vacuma* and of *B. cinerea transposa* in the leaf-disc bioassays, contributed to this axis at the rates of 28.3% and 27.2%, respectively. The inhibition rate of *B. cinerea transposa* in wounded-berry bioassays also contributed to this main first axis, but to a lesser extent (18.4%).

The second major axis (Fig. 6) largely represented inhibition rates resulting from grape berry bioassays. Two variables corresponding to the inhibition rate of *N. parvum* in wounded berries and that of *B. cinerea transposa* in unwounded berries contributed to this axis at the rates of 33.0% and 29.3%, respectively. The inhibition rate of *N. parvum* in stem cuttings contributed also to this second main axis, but to a lesser extent (17.9%). Interestingly, the PCA analysis identified a group of interesting strains, notably including the strains 2, 3, 4 and 24, which markedly inhibited the development both of *N. parvum* necrosis in stem cuttings and *B. cinerea transposa* in unwounded berries. However, this group of strains was located in diametric opposition to the variable corresponding to the inhibition rate of *B. cinerea vacuma* on leaf-discs, clearly indicating a low or moderate effect by the strains under these conditions (inhibition rates of 15.2%, 42.3%, 5.4% and 11.1%, respectively). Some strains (S14, S31, S32) exhibited moderate or no efficacy in all bioassays (Fig. 6). Another group of strains

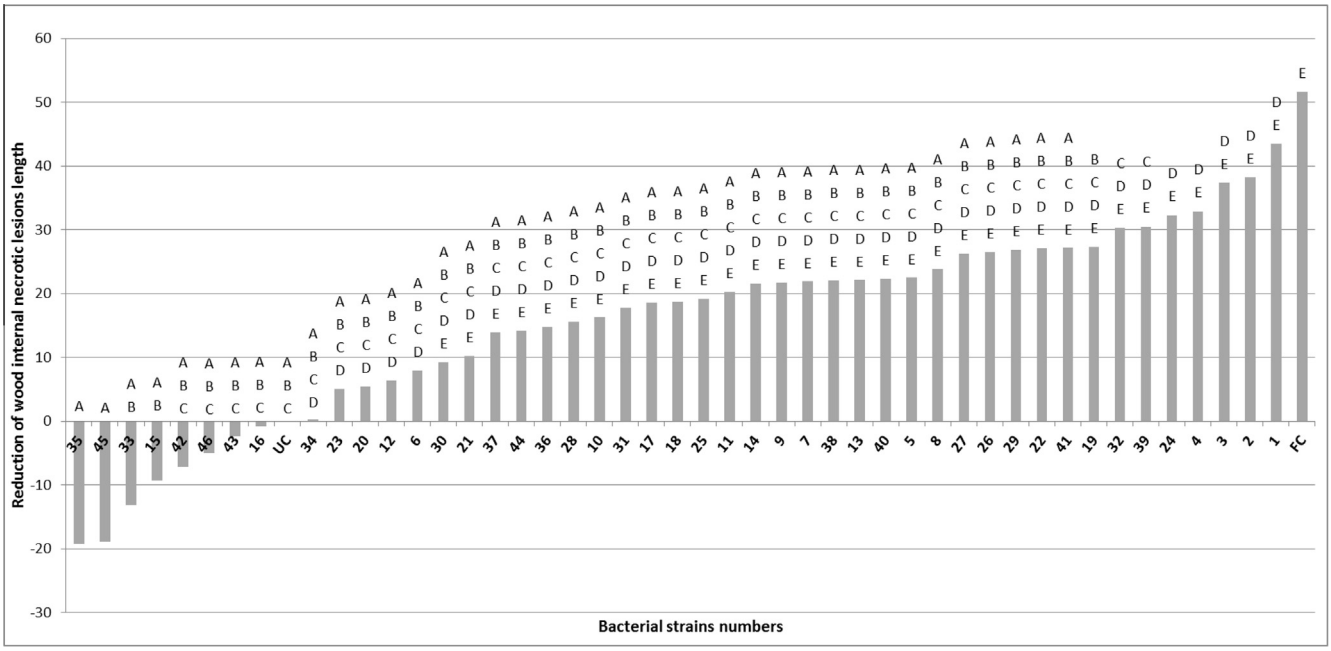

Fig. 4. Percentage of symptom inhibition displayed by 46 bacterial strains on the development of wood inner necrosis due to *N. parvum*. Cutting stems (cv. Cabernet Sauvignon) were co-inoculated with bacteria and one plug of mycelium before incubation in an open greenhouse for 104 ± 7 days. The untreated controls (UC) were inoculated with the pathogen only. Each value represents the mean of 16 or 11 cuttings depending on the bioassay (see Section 2.4.1). Bars with the same letter are not significantly different at $P = 0.05$ according to Newman and Keuls' test after ANOVA.

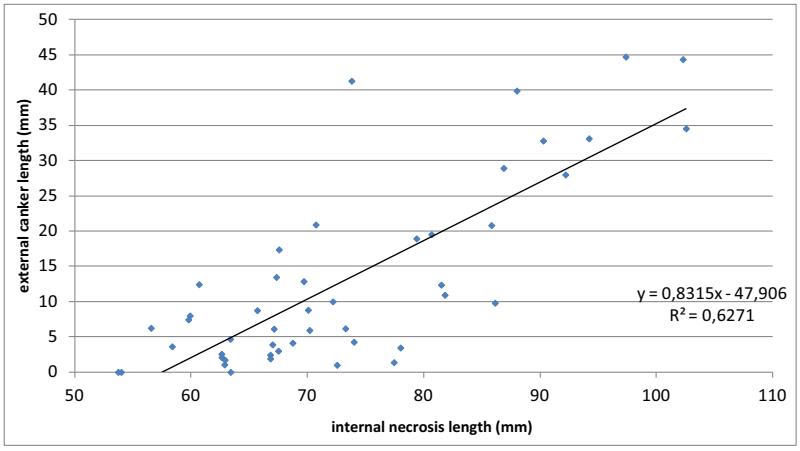

Fig. 5. Correlation between the length of necrosis and the length of cankers in grapevine cutting stems co-inoculated with *N. parvum* and the bacterial strains. Every dot represents the data obtained with one bacterial strain and one dot corresponds to the main inoculated control (untreated with any bacterial strain).

inhibited *B. cinerea* on wounded berries but increased (S35, S45) or did not affect (S34) *N. parvum* necrosis.

5. Discussion

These comparative *in vivo* screening assays showed that there are clear differences in the efficacy ranking of the bacterial strains against the two fungal grapevine pathogens *B. cinerea* and *N. parvum*, on the basis of three different grapevine tissues (berries, leaves and the cutting stems). Control of gray mold using antagonist bacteria has been studied extensively *in vitro* as well as *in vivo*, notably in strawberry (Essghaier et al., 2009) and grapevine leaves (Loqman et al., 2009; Magnin-Robert et al., 2007). The bioassays showed that the inhibitory effects of the bacterial strains differed notably according to the grapevine plant part but also to the *B. cinerea* transposon genotype under consideration.

The results from the bioassays on grapevine leaf discs allowed us to detect *B. ginsengihumi* (S38) as the most efficient strain, which significantly controlled both *B. cinerea* transposon genotypes, reducing the levels of rot expression by over 80%. This species has not been previously reported as a *B. cinerea* antagonist. These bioassays also revealed other bacterial strains that were efficient at controlling both subpopulations with high levels of protection greater than 41%, notably including S17 *Paenibacillus* sp., S19 *Paenibacillus* sp., S16 *Paenibacillus* sp., S22 *Enterobacter cowanii*, S29 *Bacillus licheniformis* and S28 *Brevibacillus reuszeri*. *B. licheniformis* has been shown to control several pathogens, such as B. cinerea and Phytophthora capsici, on many plant hosts by producing various antifungal substances including glucanase, chitinase, peptides and lipopeptides (Cui et al., 2012; Essghaier et al., 2009; Wang et al., 2014). Although no previous study reported inhibitory effects of *B. reuszeri* on *B. cinerea*, different

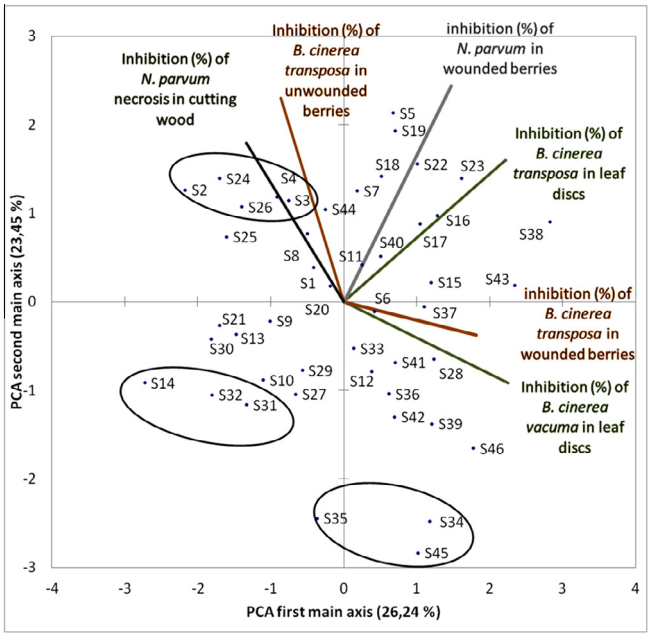

Fig. 6. Principal component analysis (PCA) of interrelations between the six different variables corresponding to the inhibition rates due to the 46 bacterial strains.

species of *Brevibacillus*, such as *Brevibacillus brevis* and Brevibacillus laterosporus, are known to be effective antagonists against different phytopathogenic fungi, including P. capsici and Fusarium oxysporum. These *Brevibacillus* species may produce antibiotics and/or extracellular antagonistic substances such as enzymes (Bapat and Shah, 2000; Zhao et al., 2012). In particular, *B. brevis* reduces *B. cinerea* germination both in vitro and in vivo, on tomato and lettuce, mainly by producing the antifungal antibiotic gramicidin S (Edwards and Seddon, 2001; Seddon et al., 2000; Wafaa et al., 2013). Furthermore, this is the first time to our knowledge that marked differences have been shown in the screening results for grapevine leaf discs based on the *B. cinerea* subpopulation tested (*vacuma* or *transposa*). Three bacterial strains (S37, S42, S45) did not inhibit *B. cinerea transposa*, but they markedly controlled the *vacuma* transposon genotype. Conversely, two strains (S5 and S44) were effective against the *transposa* strain but were not gainst the *vacuma* one. From an epidemiological point of view, it may be more important to control the *B. cinerea transposa* genotype because it is known as (i) the predominant subpopulation in vineyards, particularly on grape berries at the end of the season, and (ii) is more virulent than *vacuma* (Martinez et al., 2008, 2005). Thus, it seems advisable that further screening studies of potential BCAs against *B. cinerea* should take into consideration the transposon genotype within the pathogen.

Differences in nutrient availability between wounded and unwounded grape berries might be the main factor accounting for the differences in the inhibitory effects of *B. cinerea* on grape berries. In the grape berry bioassay, the inhibitory efficacy of the bacterial strains against *B. cinerea* markedly depended on the presence or absence of wounds at the fruit surface. On one hand, 5 strains provided important levels of protection on wounded berries, specifically *B. ginsengihumi* (S38 and S39), *Bacillus* sp. (S43 and S46), and *P. agglomerans* (S6). On the other hand, on unwounded berries, three different strains exhibited similar high levels of protection, specifically *Enterobacter* sp. (S24) and *P. agglomerans* (S2 and S3).

Although *N. parvum* is a secondary grape berry pathogen, biotests using wounded grape berries that were artificially infected with this pathogen were successful. However, in unwounded berries, the overall mean rot severity was lower (approximately 20% in the two tests) than that observed with *B. cinerea*. The difference in the degree of infection between wounded and unwounded berries in the *N. parvum* bioassay can be explained by the fact that the fruit wounds may provide nutrients that are likely required by *N. parvum* for initiating the pathogenic process (Amponsah et al., 2011; Ni et al., 2012). We showed that, in wounded berries inoculated with *N. parvum*, 5 bacterial strains tested significantly reduced rot lesion severity, including two strains (S23 *Enterobacter* sp. and S16 *Paenibacillus* sp.). When considering *B. cinerea* inhibition by these last two strains, S16 was not efficient in the grape berry bioassays, and S23 markedly inhibited *B. cinerea* in wounded berries. Importantly, such results confirmed that the antagonist effects of the bacterial strains may strongly depend upon the pathogen and/or the pathosystem considered.

In the present study, we have demonstrated, for the first time, the effectiveness of *P. agglomerans* and *Enterobacter* sp. as potential biological control agents against *N. parvum* in our main screening bioassays in grapevine cuttings cultivated in pots under greenhouse conditions. This finding is of prime importance because *N. parvum* is a pathogen for which no efficient treatment exists today. Moreover, the evaluation of efficacy under greenhouse conditions has been recommended as the most convincing strategy to select a potential biocontrol agent for disease management (Duffy et al., 2003; Pliego et al., 2011). However, there have been no studies assessing the potential of beneficial bacteria to control *Botryosphaeriaceae* infection or other fungi associated with GTDs using grapevine growing cuttings under greenhouse conditions. Among the 46 bacterial strains we tested, notably four strains of *P. agglomerans* (S1, S2, S3, S4) and one *Enterobacter* sp. (S24) strain exhibited strong antagonistic activities against *N. parvum*. Their efficacy in decreasing internal *N. parvum* necrotic lesion length reached 43.5% for S1.

Conversely, we found that some strains tended to increase internal necrotic lesion length compared with the control, i.e., *B. pumilus* (S35) and *Xanthomonas* sp. (S45). *B. pumilus* has been reported as a plant pathogenic bacterium (Bathily et al., 2010; Galal et al., 2006; Saleh et al., 1997). Similarly, on grapevine, *Xanthomonas* is the causative agent of diverse diseases such as bacterial canker of grapevine caused by *Xanthomonas campestris* pv. *Viticola* and leaf spot caused by *Xanthomonas* sp. (Araujo and Robbs, 2000; Burr and Hurwitz, 1980).

All of these different *in vivo* screening assays showed that the organ host as well as the pathogen considered may considerably affect the bacterial strain ranking based on their antagonist efficiency. For example, by comparing the results from the two bioassays on leaves and berries inoculated with *B. cinerea transposa*, there was an important difference in bacterial strain ranking based on their inhibition efficiency. For example, some strains such as *P. agglomerans* (S2, S3) and *Enterobacter* sp. (S24) markedly inhibited *B. cinerea* on unwounded berries, but their antagonist efficiency was very low on leaves (<40%). This clearly showed the prime importance and effects of the plant organ on the output of a biological control screening procedure. Many factors can affect the effectiveness of BCAs in different bioassays, notably nutrient availability, which may be increased at the surface of mature grape berries compared to leaf discs. As another example, S43 (*Bacillus* sp.) was among the most efficient strains against *B. cinerea* but it improved the symptoms caused by *N. parvum* in cuttings. This last finding clearly shows the importance of carrying out comparative screening bioassays on a multi-pathogen basis to select potential BCAs in a particular crop. The multi-organ and multi-pathogen screenings may also allow the development of control strategies based on the application of mixed BCAs and/or differential application timing adapted to various epidemiological scenarios.

In conclusion, our results demonstrate that *B. ginsengihumi*, a recently described species that has not been well-investigated, has great potential as a biocontrol agent against *B. cinerea* diseases on grapevine. Thus, further studies on such strains should be performed. We strongly recommend multi-organ screening for the detection of potential bacterial control agents in grapevine and possibly in other fruit crops. Multi-organ screening may minimize the risk of missing the detection of BCA candidates. Furthermore, some bacteria were shown to be efficient against one tested pathogen, but they improved the development of the other. Such strains should not be used as field BCAs in the future because of their capacity to stimulate other diseases than that being targeted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2015.09.003.

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