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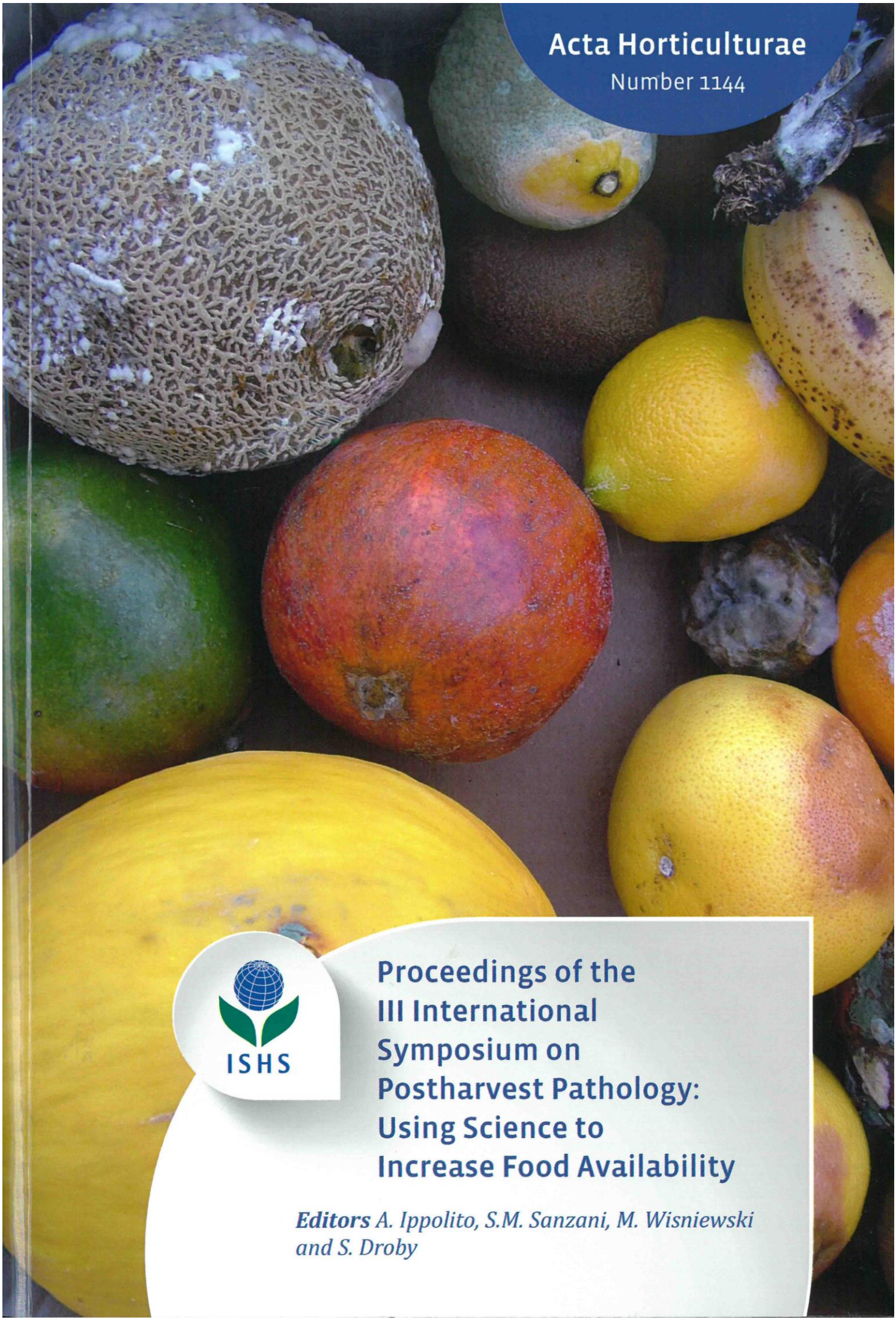

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In vitro and in vivo screening of antagonistic bacterial strains from vineyards to control *Botrytis cinerea* in grapevine tissues

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Abstract

Botrytis bunch rot, caused by *Botrytis cinerea,* is an important fungal disease of grapevine with high economic importance in wine grape production and postharvest storage of table grapes. Biological control by antagonistic bacteria is a promising strategy for reducing the common use of synthetic fungicides to control this pathogen. A total of 45 bacterial strains, isolated from grapevine tissues in 'Bordeaux' vineyards were screened in vitro for their potential antifungal activity against two major vineyard subpopulations of *B. cinerea,* i.e., *transposa* and *vacuma*. These two transposon genotypes differ significantly in virulence on grape berries. The inhibitory effects of the bacterial strains on the mycelial growth were tested in vitro for detecting the potential production of diffusible metabolites and volatile organic compounds. Furthermore, ten strains among the most effective ones were selected and evaluated in vivo on detached grapevine host organs: leaf discs and grape berries. We showed that some of the bacterial strains strongly inhibited *B. cinerea* mycelial growth under in vitro conditions, and also significantly reduced rot severity in vivo. The results suggest the potential to control postharvest gray mold by some of the bacterial strains tested.

Keywords: biological control, volatiles, antibiosis, Botrytis bunch rot, bacteria, *Vitis vinifera*

INTRODUCTION

Botrytis cinerea, the causal agent of gray mold, is responsible for considerable losses in production and storage of economically important crops worldwide, including wine and table grapes (Ky et al., 2012). This pathogen is considered as a species, genetically complex and diverse, including notably different transposon genotypes. In vineyards, the two major sympatric transposon genotypes, corresponding also to subpopulations, are: i) *transposa*, which harbors the transposable elements Boty (Diolez et al., 1995) and Flipper (Levis et al., 1997); ii) *vacuma*, which lacks these transposons (Martinez et al., 2005). This pathogen is mainly controlled by pre- and postharvest specific fungicide treatments which have favored the development of resistant strains to most of these chemicals in many viticultural countries worldwide (Walker et al., 2013). Therefore, developing non-chemical control methods is needed. Biological control based on antagonistic bacteria to prevent this disease, has been increasingly considered as a promising and an attractive alternative. In the literature, numerous studies reported the ability of bacteria to produce, in vitro and/or in vivo, various antifungal substances on different hosts including grapevine (i.e., antibiotics, volatiles) against *B. cinerea* (Zhang et al., 2013; Compant et al., 2013). The ability of bacteria to produce inhibitory volatile substances is considered as an important feature for controlling postharvest pathogens, notably *B. cinerea* (Jamalizadeh et al., 2011; Li et al., 2012). However, many studies reported that there is not always a significant correlation between in vitro and in vivo inhibition of *B. cinerea* infection and/or following symptom development (Sadfi-Zouaoui et al., 2008; Raspor et al., 2010; Sanzani et al., 2012). Lastly,

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there is only one bacterial strain (*Bacillus subtilis*) commercialized as a biocontrol fungicide against *B. cinerea* registered in French vineyards.

The major objectives of this study were to select, in vitro, bacterial strains by assessing and comparing their antifungal activities by diffusible and/or volatile metabolites against two subpopulations of *B. cinerea* (*transposa* and *vacuma*). Based on these in vitro data, ten bacterial candidate strains were selected among the most effective ones. Their potential was evaluated, in vivo, against *B. cinerea transposa* infection on two different grapevine organs: leaf discs and grape berries. Lastly, the potential correlations were investigated between the results of antagonist efficacy in vivo and in vitro.

MATERIALS AND METHODS

Fungal and bacterial strains

1. *Botrytis cinerea.*

Two pathogenic *B. cinerea* strains were selected from the INRA collection (UMR 1065 SAVE), Bordeaux, as representative of each main transposon genotype (Martinez et al., 2005): 213T strain for *transposa* and 357V strain for *vacuma* as described by Haidar et al. (2016).

2. Bacterial strains.

A total of 45 bacterial strains (Haidar et al., 2016) were tested, all isolated from grapevine, including 34 strains from wood tissue (Bruez, 2013) and 11 from the grape berry surface (Martins, 2012). The strains from the grape berry surface originated from "Biological Resources Center Enology" (University of Bordeaux and Bordeaux Polytechnic Institute). For the in vitro essays, strains were grown on Trypto-Casein Soy Agar medium (TSA, Biokar diagnostics) for 24 h at 28°C.

Antifungal in vitro assays

1. Antagonism in dual culture.

Dual cultures were used to test the effect of the 45 bacterial strains on fungal growth of the two *B. cinerea* subpopulations (213T, 357V). A loop of bacterial cells of 1-day-old cultures grown on TSA agar dishes were streaked on a 9-cm petri dish containing potato dextrose agar medium (PDA). Sterile inoculation loops were used to transfer the tested strain by placing it, as a line, at approximately 2.5 cm from the dish center on one side only of the petri dish. Then, 24 h later, one mycelial plug of the *B. cinerea* strain (4 mm in diameter) was inoculated at the centre of each petri dish. As controls, a set of dishes were inoculated similarly but with the pathogen only. Three replicate dishes per bacterial strain-pathogen combination were set up. The dishes were then incubated at 23°C. The radial mycelial growth of the pathogen (measured in millimeter) was assessed after 4 days and the inhibition percentage was calculated using the following growth inhibition equation (GI %) = 100*(R2-R1)/R2.

"R1" represents the minimal distance between the mycelial plug center and the fungal colony margin in the direction of the antagonist bacteria. The control value for fungal colony radius was "R2" assessed, in the same petri dish, as the distance between the mycelial plug center and the fungal colony margin on the opposite side of the bacteria. Bacterial strains were considered as ineffective in suppressing the pathogen when R1>2 cm, i.e., without any noticeable inhibition zone.

2. Effect of antifungal volatile compounds.

An in vitro assay was carried out to evaluate the effects of volatile metabolites produced by the bacterial strains on the radial mycelial growth of the two *B. cinerea* subpopulations. Bacteria of 1-day-old cultures were streaked onto TSA medium and incubated at 28°C for 24 h. One mycelium plug (4 mm) was placed on the center of another

dish containing malt agar medium (MA). Both dishes were then placed face to face (with the TSA bacterial culture at the bottom and pathogen MA culture at the top) preventing any physical contact between the two microorganisms. The dishes were then sealed using several rounds of parafilm and incubated at 23°C. MA dishes containing the pathogen only were used as control. There were three double-dish sets, comprising three replicates, for each bacterial strain and for the control. For *B. cinerea vacuma* and *transposa*, after 14 and 19 days of incubation, two perpendicular colony diameters were assessed to calculate the mycelial inhibition percentage, respectively.

Leaf and grape berry rot bioassays

The antagonistic activity of the 10 bacterial strains selected from the in vitro assays was tested on detached grapevine leaf discs and on detached wounded berries at 23°C against *B. cinerea transposa* (213T) as described by Haidar et al. (2016).

Statistical analysis

Experimental data were compared by an analysis of variance (ANOVA) followed by a Newman-Keuls test (P=0.05). Standard deviations were calculated for all mean values. Two statistical softwares were used: StatBox (Version 6.6, Grimmer© Logiciels, Paris) and XLSTAT (Version 2014.4.04, Addinsoft©, www.xlstat.com).

RESULTS

Antifungal in vitro assays

1. Antagonism in dual culture.

When evaluated in dual cultures against *B. cinerea vacuma*, many of the bacterial strains tested were considered as ineffective in suppressing the pathogen (R1>2 cm). Thus, they were not taken into account in the analysis of variance. As shown in Figure 1, significant differences were demonstrated among the 20 strains of interest, which exhibited from 49.1 to 84% of inhibition after 4 days of incubation. Among these strains, the most effective ones were: S30, S13, S19, S11 and S9. Interestingly, four of these last strains belong to *Paenibacillus* sp. Similarly, the in vitro experiment using *B. cinerea transposa* showed that 20 bacterial strains reduced mycelium growth in the range of 30.9-92.5% (Figure 2). Six of these strains, S43, S30, S32, S13, S23 and S37, reduced the mycelial growth of *B. cinerea* with a percentage of reduction ranging from 75.5 to 92.5%. Among the effective strains, 16 strains exhibited strong antifungal activity towards both *B. cinerea* subpopulations. Out of these 16 strains, five highly efficient strains (S23, S30, S32, S34 and S37) were selected to be tested in further in vivo bioassays against *B. cinerea transposa* on detached wounded berries and leaf discs.

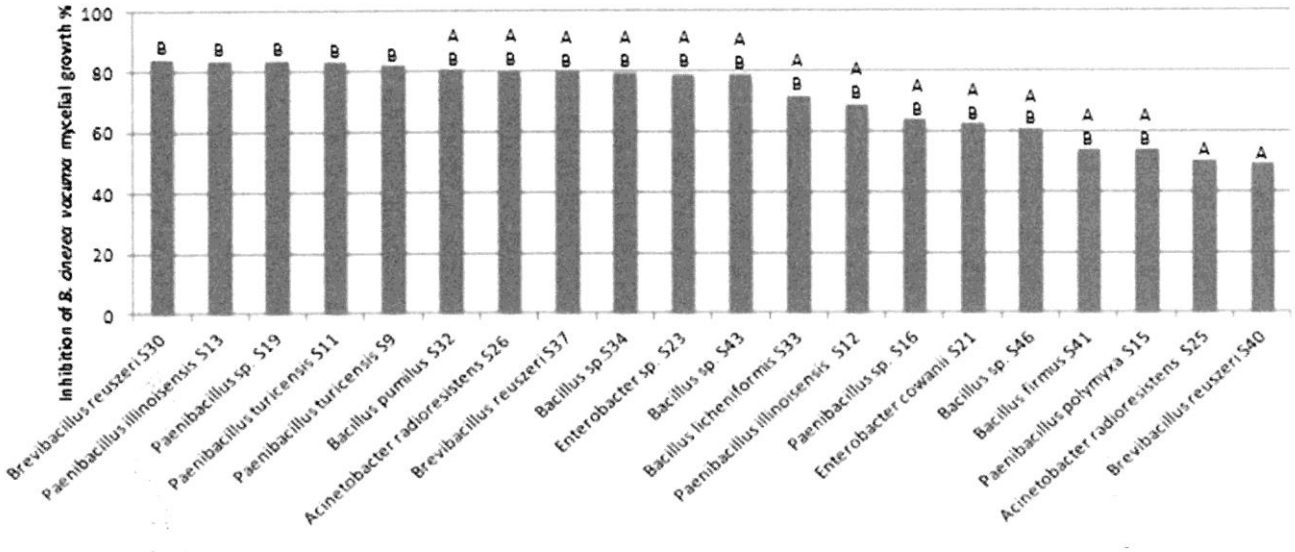

Figure 1. Inhibition (%) by bacterial strains of *B. cinerea vacuma* growth in dual culture on PDA after 4 days of incubation at 23°C. Different letters indicate mean values (three replicates) significantly different at *P*=0.05 according to Newman and Keuls' test.

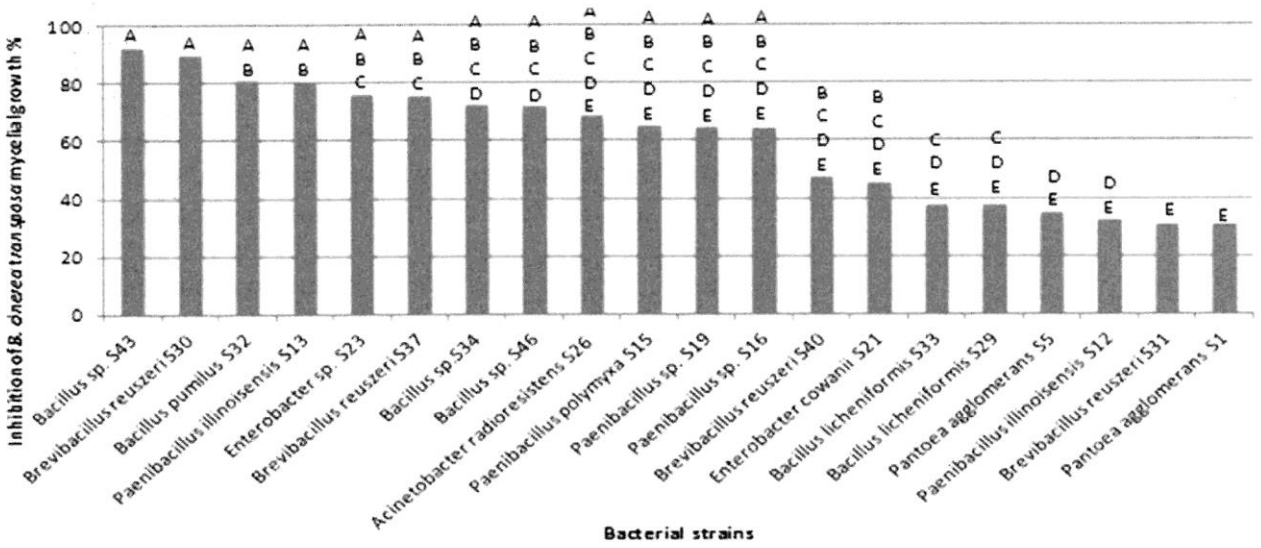

Figure 2. Inhibition (%) by bacterial strains of *B. cinerea transposa* growth in dual culture on PDA after four days of incubation at 23°C. Different letters indicate mean values (three replicates) significantly different at *P*=0.05 according to Newman and Keuls' test.

2. Effect of antifungal volatile compounds.

Figure 3 presents the results of the effect of VOCs produced by 43 bacterial strains, as compared to control, on the growth of *B. cinerea transposa* (S4 and S13 were not tested). Out of these 43 strains, 27 strains exhibited more than 50% inhibition and were classified by the statistical test at *P*=0.05 as "AB" or "B" (control and remaining non-effective strains: "A"). After 19 days of incubation, the mycelial growth of *B. cinerea transposa* was completely inhibited (100% inhibition) by VOCs produced by 3 strains S28, S27 and S20. In addition, 6 other bacterial strains showed a high inhibitory (inhibition rate exceeding 80.0%) efficacy, i.e., S33, S8, S26, S14, S23 and S3 (Figure 3). On the other hand, out of 44 bacterial strains tested against *B. cinerea vacuma*, 34 strains were characterized by the production of volatile antifungal compounds causing fungal inhibition (Figure 3). After 14 days of incubation, 22 strains provided more than 50% inhibition. Five strains showed a very high inhibitory efficacy (over 80%) on this *B. cinerea* subpopulation: S20 (*Paenibacillus* sp.) showed the

highest inhibition of 96.9%, followed by the strains S33, S28, S12 and S8 which demonstrated inhibition rates ranging from 80.7 to 91.6% (Figure 3). In conclusion, five strains exhibited a marked and significant antifungal activity towards both subpopulations of *B. cinerea*: S8, S20, S27, S28 and S33. These were selected for further in vivo bioassays against *B. cinerea transposa* on detached wounded berries and leaf discs.

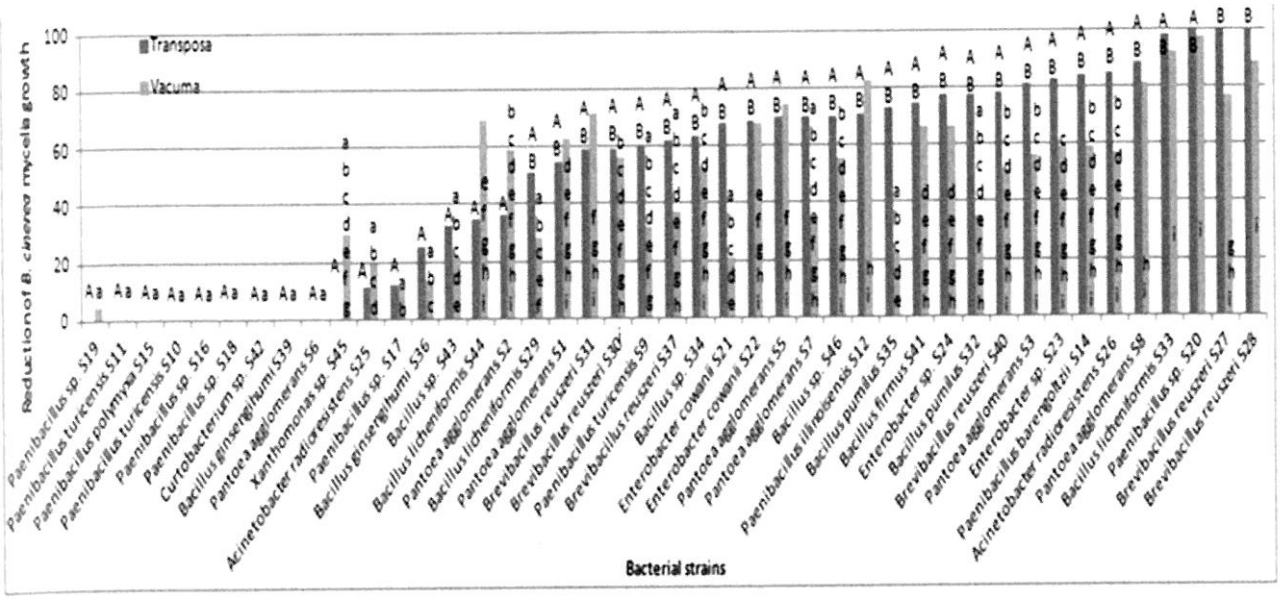

Figure 3. Inhibition (%) by bacterial volatile antifungal compounds of the mycelial growth of *B. cinerea* subpopulations: *transposa* (blue bars) and *vacuma* (orange bars), after 19 and 14 days of incubation, respectively. For each *B. cinerea* subpopulation, overall means (three replicates) with the same letter are not significantly different according to Newman and Keuls' test after ANOVA (P=0.05). (Bacterial strain S13 not tested.)

Reduction of *B. cinerea* rot severity on grapevine fruit and leaf discs by selected bacterial strains

Ten bacterial strains were selected from the previous in vitro tests: S8 (*Pantoea agglomerans*), S20 (*Paenibacillus* sp.), S23 (*Enterobacter* sp.), S27 (*Brevibacillus reuszeri*), S28 (*Brevibacillus reuszeri*), S30 (*Brevibacillus reuszeri*), S32 (*Bacillus pumilus*), S33 (*Bacillus licheniformis*), S34 (*Bacillus* sp.) and S37 (*Brevibacillus reuszeri*) (Haidar et al., 2016). Their efficiency in reducing grey mold rot severity on detached grapevine fruit and leaf discs was assessed. These strains showed different levels of antagonism against *B. cinerea transposa* in the two separate bioassays (Figure 4). In the wounded grape berry test, nine strains reduced significantly at P<0.05 Botrytis bunch rot compared with the control (inoculated with the pathogen only). The significant inhibition levels ranged from 23.7 (S32) to 49.2% (S37). In the leaf discs bioassay, only two strains, S23 and S28, reduced significantly at P<0.05 leaf rot symptoms by 55.2 and 41.5% compared with the control, respectively (Figure 4).

The different screening methods used in this study showed that there were no direct correlation between the inhibition percentage determined in vitro and the in vivo effect of bacterial strains on *B. cinerea transposa* (on berries or leaf discs) (Figure 5).

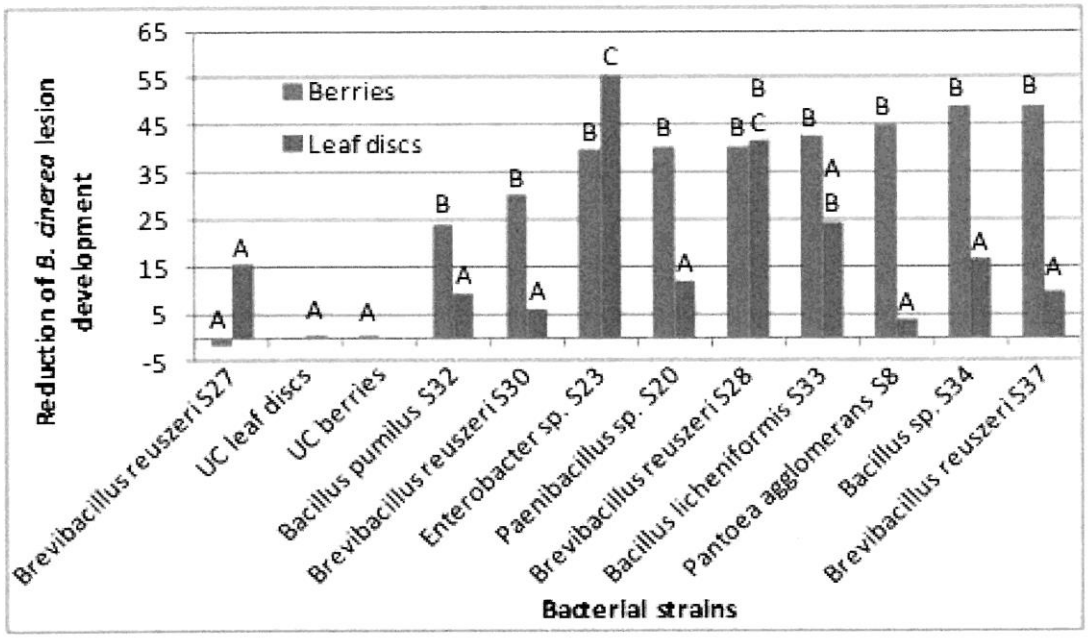

Figure 4. Percentage of symptom inhibition exhibited by 10 bacterial strains in terms of lesion diameter caused by *B. cinerea transposa* infection on berries (blue bars) and on leaf discs (red bars) after 5 and 7 days of incubation at 23°C respectively. The untreated controls (one for each bioassay; UC berries and UC leaf discs) corresponded to inoculation with the pathogen only.

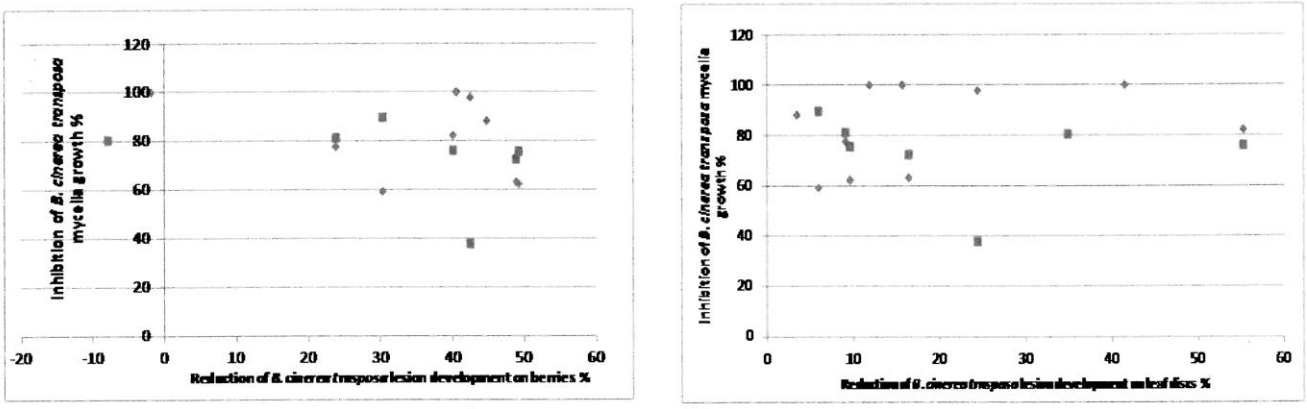

Figure 5. Correlation between the percentage of inhibition displayed by ten bacterial strains on mycelia growth of *B. cinerea transposa* in vitro and in vivo: (A) on berries and (B) on leaf discs. Blue diamond: double Petri dish experiment. Red square: dual cultures.

DISCUSSION

The two in vitro assays (diffusible and volatile compounds) showed that both the screening method and the *B. cinerea* transposon genotype (*vacuma* or *transposa*) can significantly affect the efficacy and the associated ranking of the bacterial strain. In our study, 16 of the tested bacteria that showed antagonism towards *B. cinerea transposa* in dual cultures were also effective against *B. cinerea vacuma* under the same conditions. The observed antibiosis suggests a possible production of diffusible antibiotic compounds produced by these bacteria. In the literature, there are numerous studies on antibiotic-producing bacteria tested against *B. cinerea* (Zhang et al., 2013; Silva et al., 2014).

In the double petri dish assay, many of our bacterial strains exhibited a high inhibitory efficacy on both pathogen subpopulations, reaching more than 80%. Since there was no direct contact between the pathogen mycelium and the bacterium, the volatiles antifungal substances produced by the bacteria must have inhibited the fungal growth. Interestingly, mycelial growth of *B. cinerea transposa* was fully inhibited when exposed the following

strains: S28, S27 and S20. These three strains belong to the *Bacillaceae*, which is a taxonomic family known for comprising several strains characterized by an important antibiosis capacity. The production of volatile compounds by bacteria is considered as a valuable feature especially for effective protection against postharvest pathogens. Volatile compounds were reported effective for suppression of *B. cinerea* growth (Li et al., 2012; Wan et al., 2008). Furthermore, significant differences in the bacterial strain efficacy were observed by comparing the results from the two bioassays on leaves and berries inoculated with *B. cinerea transposa*. Strain S37 (*Brevibacillus reuszeri*) was found as the most effective strain on berries against the pathogen, whereas S23 (*Enterobacter* sp.) was the most efficient strain on leaf discs. Therefore, an important effect of the grapevine organ was noticeable following these biological control screening procedures. This is also an important conclusion reported by Haidar et al. (2016). We also demonstrated and confirmed possible inconsistencies between in vitro and in vivo assays. These results are in agreement with conclusions of other authors (Raspor et al., 2010; Sadfi-Zouaoui et al., 2008). However, S37 (*Brevibacillus reuszeri*) and S23 (*Enterobacter* sp.) that showed strong antifungal effects on grape berries and on leaf discs, respectively, were proved effective also in reducing the *B. cinerea* mycelium growth in dual culture assay. This suggests that, for these two strains, one of their major mechanisms of biological control may be antibiosis. Moreover, for these two strains and particularly S23, their efficacy in in vivo tests may be due to production of inhibitory Volatile Organic Compounds (VOCs), notably against the *Botrytis transposa* strain.

It is widely accepted that species in the *Bacillaceae* family have the ability of producing various antifungal substances, notably antibiotics (Zhang et al., 2013; Ongena and Jacques, 2008). Many *Bacillus* species, as well as *Brevibacillus brevis*, were reported to reduce *B. cinerea* by producing antifungal antibiotics in vitro and/or in vivo (Touré et al., 2004; Compant et al., 2013). Similarly, several studies have investigated *Enterobacteriaceae* (e.g., *Pantoea agglomerans*) as potential source of biocontrol agents against *B. cinerea.* This has been investigated in vitro but also in various plants such as grapes, lentils and apples (Magnin-Robert et al., 2013; Nunes et al., 2002). In conclusion, our results demonstrate the potential of bacterial antagonist strains issued from vineyards. *B. reuszeri* (strain S37) and *Enterobacter* sp. (strain S23) can be considered as potential candidates for the biocontrol of *B. cinerea* on grapevine. Further studies are needed for a better understanding the underlying mechanisms accounting for the biocontrol efficacy and for testing the potential of these strains under vineyards conditions.

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Literature cited

Bruez, E. (2013). Etude comparative des communautés fongiques et bactériennes colonisant le bois de ceps de vigne ayant exprimé ou non des symptômes d'esca. Thesis (Bordeaux 2).

Compant, S., Brader, G., Muzammil, S., Sessitsch, A., Lebrihi, A., and Mathieu, F. (2013). Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. BioControl *58* (*4*), 435–455 http://dx.doi.org/10.1007/s10526-012-9479-6.

Diolez, A., Marches, F., Fortini, D., and Brygoo, Y. (1995). Boty, a long-terminal-repeat retroelement in the phytopathogenic fungus *Botrytis cinerea.* Appl. Environ. Microbiol. *61* (*1*), 103–108 PubMed.

Haidar, R., Deschamps, A., Roudet, J., Calvo-Garrido, C., Bruez, E., Rey, P., and Fermaud, M. (2016). Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine. Biol. Control *92*, 55–65 http://dx.doi.org/10.1016/j.biocontrol.2015.09.003.

Jamalizadeh, M., Etebarian, H.R., Aminian, H., and Alizadeh, A. (2011). A review of mechanisms of action of biological control organisms against postharvest fruit spoilage. Bull. OEPP *41* (*1*), 65–71 http://dx.doi.org/10.1111/j.1365-2338.2011.02438.x.

Ky, I., Lorrain, B., Jourdes, M., Pasquier, G., Fermaud, M., Gény, L., Rey, P., Doneche, B., and Teissedre, P.L. (2012). Assessment of grey mould (*Botrytis cinerea*) impact on phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages. Aust. J. Grape Wine Res. *18* (*2*), 215–226 http://dx.doi.org/10.1111/j.1755-0238.2012.00191.x.

Levis, C., Fortini, D., and Brygoo, Y. (1997). Flipper, a mobile Fot1-like transposable element in *Botrytis cinerea.* Mol. Gen. Genet. *254* (*6*), 674–680. PubMed http://dx.doi.org/10.1007/s004380050465

Li, Q.L., Ning, P., Zheng, L., Huang, J.B., Li, G.Q., and Hsiang, T. (2012). Effects of volatile substances of *Streptomyces globisporus* JK-1 on control of *Botrytis cinerea* on tomato fruit. Biol. Control *61* (*2*), 113–120 http://dx.doi.org/10.1016/j.biocontrol.2011.10.014.

Magnin-Robert, M., Quantinet, D., Couderchet, M., Aziz, A., and Trotel-Aziz, P. (2013). Differential induction of grapevine resistance and defense reactions against *Botrytis cinerea* by bacterial mixtures in vineyards. BioControl *58* (*1*), 117–131 http://dx.doi.org/10.1007/s10526-012-9474-y.

Martinez, F., Dubos, B., and Fermaud, M. (2005). The role of saprotrophy and virulence in the population dynamics of *Botrytis cinerea* in vineyards. Phytopathology *95* (*6*), 692–700. PubMed http://dx.doi.org/10.1094/PHYTO-95-0692

Martins, G. (2012). Communautés microbiennes de la baie de raisin: Incidence des facteurs biotiques et abiotiques. Thesis (Bordeaux 2).

Nunes, C., Usall, J., Teixidó, N., Fons, E., and Viñas, I. (2002). Post-harvest biological control by *Pantoea agglomerans* (CPA-2) on Golden Delicious apples. J. Appl. Microbiol. *92* (*2*), 247–255. PubMed http://dx.doi.org/10.1046/j.1365-2672.2002.01524.x

Ongena, M., and Jacques, P. (2008). *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol. *16* (*3*), 115–125. PubMed http://dx.doi.org/10.1016/j.tim.2007.12.009

Raspor, P., Miklic-Milek, D., Avbelj, M., and Cadez, N. (2010). Biocontrol of grey mould disease on grape caused by *Botrytis cinerea* with autochthonous wine yeasts. Food Technol. Biotechnol. *48*, 336–343.

Sadfi-Zouaoui, N., Essghaier, B., Hajlaoui, M.R., Fardeau, M.L., Cayaol, J.L., Ollivier, B., and Boudabous, A. (2008). Ability of moderately halophilic bacteria to control grey mould disease on tomato fruits. J. Phytopathol. *156*, 42–52.

Sanzani, S.M., Schena, L., De Cicco, V., and Ippolito, A. (2012). Early detection of *Botrytis cinerea* latent infections as a tool to improve postharvest quality of table grapes. Postharvest Biol. Technol. *68*, 64–71 http://dx.doi.org/10.1016/j.postharvbio.2012.02.003.

Silva, L.J., Crevelin, E.J., Souza, W.R., Moraes, L.A.B., Melo, I.S., and Zucchi, T.D. (2014). *Streptomyces araujoniae* produces a multiantibiotic complex with ionophoric properties to control *Botrytis cinerea.* Phytopathology *104* (*12*), 1298–1305. PubMed http://dx.doi.org/10.1094/PHYTO-11-13-0327-R

Touré, Y., Ongena, M., Jacques, P., Guiro, A., and Thonart, P. (2004). Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. J. Appl. Microbiol. *96* (*5*), 1151–1160. PubMed http://dx.doi.org/10.1111/j.1365-2672.2004.02252.x

Walker, A.-S., Micoud, A., Rémuson, F., Grosman, J., Gredt, M., and Leroux, P. (2013). French vineyards provide information that opens ways for effective resistance management of *Botrytis cinerea* (grey mould). Pest Manag. Sci. *69* (*6*), 667–678. PubMed http://dx.doi.org/10.1002/ps.3506

Wan, M., Li, G., Zhang, J., Jiang, D., and Huang, H.C. (2008). Effect of volatile substances of *Streptomyces platensis* F-1 on control of plant fungal diseases. Biol. Control *46* (*3*), 552–559 http://dx.doi.org/10.1016/j.biocontrol.2008.05.015.

Zhang, X., Li, B., Wang, Y., Guo, Q., Lu, X., Li, S., and Ma, P. (2013). Lipopeptides, a novel protein, and volatile compounds contribute to the antifungal activity of the biocontrol agent *Bacillus atrophaeus* CAB-1. Appl. Microbiol. Biotechnol. *97* (*21*), 9525–9534. PubMed http://dx.doi.org/10.1007/s00253-013-5198-x