# Phyllosphere microbiome-based biocontrol solution against the filamentous pathogens *Botrytis cinerea* and *Plasmopara viticola* in grapevine

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**Abstract**

Plants harbor different microbial communities in their different organs. This difference is due to the various conditions to which the different parts of the plant are subjected. As a result, the phyllosphere microbiome may be drastically different than the root microbiome. Since each microbiome is supposed to be well adapted to its environment, we hypothesize that bacterial communities from the leaves should be more effective in protecting the plants against the pathogen attacking the aerial part of the plant than the ones from the soil commonly used as biocontrol agents. In a previous study, we demonstrated that many phyllosphere bacteria isolated from the grapevine phyllosphere were indeed very effective against such plant pathogens under *in vitro* conditions, inhibiting their mycelial and spore development. In this project, we investigated the biocontrol ability of these phyllosphere bacteria *in planta* against two filamentous pathogens colonizing the aerial part of the plant: *Botrytis cinerea*, the ascomycete responsible for gray mold and *Plasmopara viticola* the oomycete responsible for downy mildew. Our results show that more than 40 strains were able to inhibit at least one pathogen in their spore physiology or infection progress. Among these strains, 27 strains were able to impact both pathogens and 20 were also capable of stimulating plant defenses and. Finally, when used in consortia, bacteria showed synergistic effects, resulting in a much stronger protection than that obtained by the application of the strains alone. These data suggest that phyllosphere bacteria could be a promising tool to help wine grower to fight against grapevine diseases by providing a strong long-lasting protection on their crops.

Keywords: *Vitis vinifera*, *Botrytis cinerea*, *Plasmopara viticola*, plant protection, biocontrol, plant defense,

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## Introduction

Plant microbiota form a rich and complex network that is organized into different communities. Each part of the plant provides a specific environment with conditions favorable to the development of different species of bacteria (Compant et al., 2019). Thus, within the same plant, bacterial communities found in roots are different from those found in leaves and flowers (Zarraonaindia et al., 2015). Furthermore, because the composition of each community is determined by the sum of all conditions they have to face, the bacteria found at the surface or inside the plant of the same organ are also different (Dastogeer et al., 2020). Many recent studies have shown that the plant microbiota contributes to its host's health by modulating its growth, inhibiting pathogen development and promoting plant defenses (Brader et al., 2017; Rai et al., 2023) and such bacteria are good candidates for developing biocontrol solution.

However, it appears that current biocontrol solutions only rely on organisms isolated from the soil or roots, even when the aim is to control pathogens affecting the aerial part of the plant (Syed Ab Rahman et al., 2018; Pascale et al., 2020, EU Pesticide database)

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53 In this study, we therefore wanted to test the potential of bacteria isolated from the
54 phyllosphere (the leaves and their surrounding environment) to protect plants against leaf-
55 borne pathogens. We selected grapevine as a model plant to test such biocontrol solutions, for
56 several reasons : i) it is a culture with a strong economic interest worldwide; ii) it is a perennial
57 plant, and such plants are expected to harbor more adapted phyllosphere microbiota compared
58 to annual plants, since perennial plants have longer-lasting interactions with their associated
59 bacterial communities (Primieri et al., 2022); iii) most grapevine cultivars are highly susceptible
60 to leaf-borne pathogens which makes this culture one the most fungicide consuming crops in
61 the world, requiring several applications of copper, sulfur and other chemical compounds which
62 can exceed the amount of one hundred kg per hectare each year (W. J. Li et al., 2011). Biocontrol
63 is of particular interest for such cultures, that need alternative and novel effective solutions, to
64 limit the toxicity linked to the excessive use of phytosanitary products.

65 Biocontrol phyllosphere bacteria have a wide range of modes of actions and can act either
66 directly on the pathogens or indirectly, through the plant. Thus, they can have a direct inhibitory
67 effect through the production of metabolites that can disturb the pathogen's growth and
68 physiology, impair their virulence or they can also compete for space and nutrient, e.g. by
69 trapping vital resources such as iron through the secretion of siderophores. They can also have
70 an indirect effect on the pathogen by improving plant health through the production of plant
71 growth promoting compounds or the induction of a local or systemic defense response (Legein
72 et al., 2020). In grapevine, this defense response may involve the production of stilbenic

73 compounds, a group of phytoalexins of major importance for this crop due to their high toxicity
74 for fungal pathogens. This family includes a large number of inducible molecules that are
75 produced in several forms from monomers to tetramers. Their distribution and concentration
76 are cultivar and organ dependent and the resistant cultivars have been shown to synthetize high
77 amounts of the more active forms such as viniferins (Waffo-Teguo et al., 2008; Viret et al., 2018;
78 Guerrero et al., 2020).

79 In view of these multiple modes of action, combining different strains into a mixed microbe-
80 based biocontrol solution could provide a multi-layer protection by combining several
81 mechanisms that may lead to synergies and increased plant protection. Several studies already
82 highlighted that consortia of beneficial microbes can help to improve the effectiveness of the
83 protection or at least decrease its variability (Guetsky et al., 2001; Yu et al., 2022; Wang et al.,
84 2023).

85 In a previous study, we demonstrated that the use of the grapevine microbiota isolated from the
86 phyllosphere contained many powerful strains that could effectively inhibit the development of
87 leaf pathogens when co-cultivated in *in vitro* experiments (Bruisson et al., 2019). Thus, several
88 bacteria were able to strongly inhibit the mycelial growth of the fungus *B. cinerea* and the
89 oomycete *Phytophthora infestans* in *in vitro* conditions. They were also able to disrupt the
90 physiology of these pathogens by affecting their spores. We concluded that the use of such
91 bacteria was a promising strategy to increase the effectiveness of microbe-based biocontrol
92 solutions, but that more advance testing using plants was required.

93 In this work, we show that grapevine phyllosphere bacteria used as single strains or consortia
94 can provide a very effective protection *in planta* against two of the most detrimental grapevine
95 diseases caused by filamentous organisms, downy mildew caused by the oomycete *Plasmopara*
96 *viticola*, and gray mold caused by the ascomycete *Botrytis cinerea*. Our results suggest that this
97 protection is achieved by combining direct effects on the pathogens, affecting their spores and
98 their mycelium development, as well as indirect effects linked to their ability to increase the
99 expression of defense-related genes and the production of specialized metabolites involved in
100 plant defense.

101

102 **Materials and methods**

103 **Biological material and culture media**

104 Bacterial strains isolated from grapevine phyllosphere as described in (Vionnet et al., 2018) and
105 screened for their biocontrol capacity in (Bruisson et al., 2019) were grown on LB (Lysogeny
106 Broth) medium in the dark and at room temperature. LB medium was prepared by mixing 12.5
107 g/L of LB Broth Miller and 10 g/L of LB Broth Lennox (Fisher Bioreagents) in distilled water. For
108 solid medium, 15 g/L of Agar-Agar Kobe I (Roth) were added.

109 *Botrytis cinerea* strain 2905 was provided by the Mycology group at the Agroscope of Changins
110 and was grown in potato dextrose agar (PDA) (Roth). PDA was prepared by dissolving 39 g/L of
111 PDA powder (Sigma-Aldrich) in distilled water.

112 *Plasmopara viticola* sporangia were provided by the Agroscope of Changins. They were
113 harvested from infected leaves in vineyards each year to reflect the genotypes currently
114 infecting the grapevines at the time of the experiments.

115 Cuttings of *Vitis vinifera* cultivar Gamay, Gamaret and the interspecific hybrid Divico (Gamaret x
116 Bronner). After 2 months, plants were transferred into growth chambers with a 16h/8h
117 photoperiod, 21/20 °C day/night temperature and 40 % relative humidity (RH). Two Phillips
118 master TL-D super 80 36W/840 neon bulbs were used to provide light.

119 **Effect of bacterial strains on *B. cinerea* spore germination**

120 Spores of *B. cinerea* grown on PDA plate were harvested by watering the plate with 5 mL of
121 sterile water and filtration through glass wool to remove hyphae. The spore solution was then
122 adjusted to 5 x $10^4$ spores/mL and 12 µL of this solution was mixed in an Eppendorf tube with 36
123 µL of bacterial suspension adjusted at $OD_{600}$=1 in V8 medium, clarified using a 0.2 µm Millex®-FG
124 filter (Merck). The mixture was transferred into a 24-well plate and the plates were incubated at
125 21 °C at light for 8 hours. Three technical replicates were performed for each modality and the
126 control consisted in spore solutions mixed with filtered V8 without bacteria. After 8 h pictures
127 were taken using an imaging plate reader (Cytation5, Biotek). Spores were counted according to
128 their development stage (not germinated, normal germination, delayed germination) and the
129 results were analyzed with a chi-square test using the package fifer on R with * = $p < 0.05$, ** = $p$
130 $< 0.01$ and *** = $p < 0.001$.

131 **Effect of bacterial strains on *P. viticola* zoospore motility**

132 Zoospores of *P. viticola* were released from sporangia harvested by suction from sporulating
133 lesions with a filter-containing pipette tip connected to a vacuum device. The sporangia were
134 resuspended in sterile water and their concentration was adjusted to 5 x $10^4$ sporangia/mL in a
135 falcon tube. The tube was placed in a shaker at 25 rpm at room temperature and in the dark for
136 2 hours for zoospore release. After 1 h, the zoospore solution concentration was adjusted to 5 x
137 $10^4$ zoospores/mL. The effect of bacteria on zoospore motility was assessed by mixing 500 µL of
138 a solution of bacteria diluted at $OD_{600nm}$ = 2 in sterile water with 500 µL of the zoospore solution
139 in a 1.5 mL Eppendorf. The Eppendorf was then incubated at room temperature in a shaker at 25
140 rpm for 30 minutes. After incubation, the solution was transferred into a counting chamber and
141 observed under a microscope. After counting, percentage of motile and non-motile zoospores
142 were computed and compared to the control (zoospore mixed with distilled water only).
143 Differences between bacterial treatments and the control were assessed by using an ANOVA
144 followed by a Dunnett's test with $* = p < 0.05$, $** = p < 0.01$, and $*** = p < 0.001$. Zoospores
145 were then used for leaf disc infection following the procedure described below.

146 **Electron microscope observation of zoospores**

147 Pellets of P. viticola zoospores, bacteria, or bacteria in contact with zoospores released using the
148 method described above were prepared according to Roland and Vian (1991). Briefly, the pellets
149 were centrifuged for 5 minutes at 2000 rpm and resuspended in a solution of 3%
150 glutaraldehyde–2% paraformaldehyde in 0.14 M PIPES buffer (pH 7) for prefixation. Samples
151 were then centrifuged for 20 minutes at 2000 rpm, the supernatant was removed, and the
152 samples were washed twice with 0.07 M PIPES buffer. They were embedded in 2% agarose and

153 postfixed with a solution of 1% OsO4. The samples were then dehydrated in a graded series of
154 ethanol solutions (30–50–70–95–100% v/v) and infiltrated in a solution of ethanol mixed with
155 propylene oxide (1:1) and pure propylene oxide, followed by a series of epoxy resin mixed with
156 propylene oxide (1:3, 1:1) for 2 hours each. Finally, they were infiltrated with epoxy resin for 17
157 hours. After polymerization for 48 hours at 60°C, a staining step was performed by floating on a
158 50°C solution of 1% methylene blue, 1% sodium borate, and 1% azure II for 10 seconds for semi-
159 thin sections. Thin sections were cut and stained with 2% uranyl acetate followed by lead citrate,
160 according to Reynolds (1963). The thin sections were observed with a Tecnai G2 Spirit BioTWIN
161 transmission electron microscope (TEM) equipped with a Gatan UltraScan High-resolution CCD
162 camera.

163 **Leaf discs infection assays**

164 Leaf discs were cut from 2-month-old grapevine leaves, taken from leaf stages 4 to 6. Leaves
165 were taken randomly from at least ten plants and were rinsed with tap water and surface
166 sterilized by immersion in hypochlorite calcium (40 g/L) for 7 minutes followed by rinsing in
167 sterile water prior to cutting. Leaves were then dried with sterile filter paper, used to cut leaf
168 discs using a 1.7 cm diameter punch and placed in sterile water. Ten leaf discs were taken
169 randomly and placed on top of a filter paper moistened with sterile water placed inside a 9 mm
170 Petri dish with the abaxial side facing upward and placed in a growth chamber with a 16h/8h
171 photoperiod, 21/20 °C day/night temperature and 40 % RH. For each modality, three Petri dishes
172 containing ten leaf discs each were used. After 24 h each plate was inoculated with 500 µL of
173 bacterial solution diluted in sterile water and adjusted to $OD_{600nm}$ = 1, using a sterile glass

174 atomizer and placed back in the growth chamber. Two days after the inoculation, the lead discs
175 were infected with *P. viticola* or *B. cinerea*. For *P. viticola*, the infection was performed using 500
176 µL of solution adjusted at 5 x $10^4$ zoospores/mL for each plate. For *B. cinerea*, the infection was
177 performed by depositing a 20 µL droplet of a spore solution adjusted to 1 x $10^5$ spores/mL in
178 $PDB^{1/4}$ on a wound made by a sterile syringe at the center of the lamina. The same procedure
179 was used with sterile water only for the negative control. For positive controls, plants were
180 treated with a 2 g/L solution of copper hydroxide (Kocide® Opti, Bayer) against *P. viticola* and a
181 0.375 % solution of brown algae (Alginure, Andermatt) against *B. cinerea*. After infection, Petri
182 dishes were sealed with Parafilm® and placed back in the growth chamber. For each modality,
183 one plate containing 10 leaf discs was used to collect material for gene expression analysis by
184 qPCR and stilbene production by HPLC. Five leaf discs were collected, dried and snap frozen in
185 liquid nitrogen after 6 h for qPCR analysis and after 3 days for HPLC analysis. Pictures of each
186 remaining plate were taken one week after infection to assess the development of symptoms.

187 **Whole plant infection with *P. viticola***

188 Half of the leaves from foliar stage 4 to 7 of two-month-old grapevine plants (4 plants per
189 treatment) were sprayed with a solution of 4 mL of bacteria adjusted to $OD_{600nm}$ = 1 on their
190 adaxial face, while the other half was protected with plastic bags to prevent unwanted
191 inoculation. Once the inoculation finished, plastic bags were removed and uninoculated leaves
192 were sprayed with sterile water. Two days after the bacterial inoculation, all leaves from foliar
193 stage 4 to 7 were sprayed with 200 µL of a solution of *P. viticola* zoospores adjusted to 5 x $10^4$
194 zoospores/mL. After a week, leaves were collected, the level of symptoms was quantified and

195 compared to the control (plants inoculated with water only) in search of local and systemic
196 protection from the bacteria.

197 **Symptom assessment**

198 For symptom measurement, pictures of leaves were analyzed in an automated and quantitative
199 manner, a macroinstruction was developed in the freeware ImageJ to measure the necrosis
200 development of *B. cinerea* and the formation of sporangiophores of *P. viticola* as described in
201 (Guyer et al., 2015). The level of inhibition was calculated as a percentage of the level of
202 infection observed in control.

203 **Gene expression analysis**

204 The ability of phyllosphere bacteria to promote defense gene expression on grapevine was
205 analyzed by qPCR in 8-week-old plants. Five leaf discs from infection assays described above
206 were sampled 6 h after infection, snap-frozen in liquid nitrogen and stored at −80 °C. RNA was
207 extracted with the NucleoSpin RNA Plant from Macherey-Nagel with a modified procedure
208 (Schellenbaum et al., 2008). Reverse transcription was performed using the SensiFAST cDNA
209 Synthesis Kit from Bioline. Quantitative PCR reactions were performed using the SensiFAST SYBR
210 Hi-ROX Kit from Bioline. Each reaction was carried out with 5 µL of cDNA (5 ng/µL), 7.5 µL of
211 SYBR Hi-ROX mix, 0.5 µL of each primer and 1.5 µL of sterile water. For amplification, an initial
212 denaturation step at 95 °C for 15 min was done, followed by 45 amplification cycles (95 °C for 15
213 s, 60 °C for 15 s, 72 °C for 30 s). Each reaction was run in duplicate and the experiment was
214 repeated twice. All results were analyzed using the double delta Cq method with uninfected

215 samples treated with water as reference and normalization was performed relative to the
216 expression of the two reference genes Actine and 60SRP (60S ribosomal subunit). Primers were
217 used to monitor the expression of the following defense-related genes, PR-1 (pathogenesis-
218 related protein 1), PR-4 (pathogenesis-related protein 4), STS (stilbene synthase), ROMT
219 (resveratrol O-methyltransferase), PAL (phenylalanine ammonia-lyase) and LOX (lipoxygenase)
220 (Supp. Table 1). A two-way ANOVA followed by Tukey-HSD post-hoc test was performed for
221 statistical analysis with $* = p < 0.05$, $** = p < 0.01$, and $*** = p < 0.001$.

222 **Stilbenic compound measurement**

223 Leaf disc fragments were weighed and placed into microcentrifuge tubes. Samples were
224 immediately snap-frozen and immersed in 300 µL of methanol. The tubes were then heated at
225 60°C for 15 minutes with agitation at 800 rpm, followed by rapid cooling in an ice bath for 5
226 minutes. After cooling, the samples were centrifuged to separate the supernatant. The
227 methanolic extracts were analyzed using Ultra-Performance Liquid Chromatography (UPLC) to
228 quantify the presence of various stilbenic compounds, including piceid, resveratrol, α-viniferin,
229 δ-viniferin, ε-viniferin, isohopeaphenol, and pterostilbene, following the method described by
230 Pezet et al. (2003). The analysis was performed using a Vanquish UHPLC system (Thermo
231 Scientific) with a mobile phase consisting of H2O with 0.1% formic acid (Phase A) and
232 acetonitrile with 0.1% formic acid (Phase B). The gradient elution program started with 5% B and
233 increased to 40% over 17 minutes, followed by an increase to 50% B between 17 and 20
234 minutes. From 20 to 25 minutes, the proportion of B increased to 100%, which was maintained
235 until 29 minutes. The flow rate was set at 0.3 mL/min. Separation was carried out using a

236 Hypersil Gold column (1.9 µm, 100 x 2.1 mm, Thermo Scientific) equipped with a 0.2 µm UHPLC
237 filter (Thermo Scientific), maintained at 30°C. The injection volume was 2 µL, and detection was
238 performed using a UV detector at 280 nm and 310 nm. This method ensures accurate and
239 reproducible quantification of stilbenic compounds in leaf tissues.

240

241 **Results**

242 **Motility disruption of *P. viticola* zoospores after contact with phyllosphere bacteria**

243 We tested all the strains from our collection of bacteria regarding their ability to disturb *P.*
244 *viticola* zoospores *in vitro*. Confrontation assays between bacteria and *P. viticola* zoospores
245 showed that the contact with bacteria can strongly affect zoospore motility. A significant
246 reduction was measured with 28 strains out of the 53 tested. Visual assessment revealed that
247 zoospores either completely lost their motility, or kept motile as in the non-treated controls, but
248 we did not observe slower movement or lesser dispersal. However, the proportion of affected
249 zoospores was different depending on the bacterial strain used. Thus, a group of 16 strains
250 caused very strong reduction, affecting 90%-100% of the zoospores and reaching the level of
251 inhibition achieved with the copper used as a positive control, while a group of 12 strains caused
252 a significant but lower reduction (40%-90 %) (Table 1). A large predominance of bacteria from
253 the *Bacillus* genus were found among the active strains, since 19 of the 22 *Bacillus* strains tested
254 belonged to this group. However, the two most effective strains affecting both 100 % of the
255 zoospores (PIP10 and SOP5) were a *Pantoea agglomerans* and a *Microbacterium hominis* strain,

256 respectively. Interestingly the active strains were composed of a balanced proportion of
257 epiphytic (13) and endophytic (16) strains.

258 After being in contact with the bacteria, the zoospores were observed using a transmission
259 electron microscope (TEM). The zoospores that were in contact with non-active bacteria and
260 had not lost their motility displayed a standard oval shape comparable to the control zoospores.
261 However, the zoospores that had been in contact with the active strain PID6 (*Bacillus cereus*)
262 showed an irregular shape, suggesting that the whole cell had lost its integrity and had been
263 strongly affected by the presence of the bacteria (Fig. 1).

264 After addition of the bacteria, all the solutions containing the zoospores were used to infect leaf
265 discs cut from the susceptible cultivar Gamaret to monitor pathogen development. The
266 observations showed that in the case of 18 strains, the infections performed with the treated
267 zoospores resulted in a significant reduction in symptoms (from ca. 30% to 80 % of the control)
268 compared with the infection performed with non-treated zoospores (Table 1). From these 18
269 strains, only 13 were also significantly impairing motility, suggesting that the loss of motility was
270 not always correlated with symptom reduction and that other mechanisms are involved. Indeed,
271 five strains that had not impaired zoospore motility were nevertheless efficiently reducing the
272 symptoms caused by the zoospores with which they had been co-incubated.

273 Interestingly, four of these five strains belong to different genera than those highlighted
274 previously, namely *Rhodopseudomonas*, *Cupriavidus*, *Variovorax* and *Herbaspirillum.*

275 It seemed that when bacteria isolated from the cultivar Pinot noir were co-inoculated with
276 zoospores, this almost systematically resulted in significant symptom reduction, whereas the
277 two other cultivars harbored a lesser proportion of protective strains in this leaf disc
278 experiment.

279

280 **Phyllosphere bacteria inhibit the germination of *B. cinerea* spores**

281 The ability of phyllosphere bacteria to disrupt the germination of *B. cinerea* spores was assessed
282 by confrontation tests. After being in contact with the bacteria for 8 h, the spores displayed four
283 different morphologies: i) normal germination, with expected formation of a long germ tube, ii)
284 partially inhibited germination, with only the tip of the germ tube visible, iii) complete inhibition
285 of germination, with full inability for the spore to form a germ tube, iv) in a few rare cases,
286 spores could also form a swollen germ tube, revealing an abnormal germination in which the
287 germ tip swelled, stopping its elongation (Fig. 2 B-E).

288 Among the 53 bacteria that were tested, 34 strains were able to strongly disturb the
289 germination of more than 50% of *B. cinerea* spores, either by strongly delaying the formation of
290 the germ tube or by fully preventing spore germination. The six most effective strains SOD14,
291 PIP6, PIP4, PID6, PID13 and PID1 were even capable of fully inhibiting the germination, leaving
292 no spore able to germinate properly. These strains belonged mainly to the *Bacillus* or
293 *Paenibacillus* genera, with the exception of PIP4 (*Cupriavidus*) and of the most effective one,
294 PIP6 (*Herbaspirillum*).

295 **The inhibition of *B. cinerea* spore germination is higher with consortia than with bacteria**
296 **alone**

297 Consortia containing two to three active strains impairing spore germination were used to
298 assess the ability of the bacteria to generate synergies with higher protection abilities. The
299 solution containing the combination of bacteria contained the same final cell concentration than
300 the one used in the previous experiment, so any increase in effectiveness could not be simply
301 due to the increased bacterial density in the mixture. This combination resulted in an overall
302 increase of the strains' germination inhibition abilities. All consortia were able to affect all or
303 almost all spores, with the least effective one (CHP14, PIP6 and SOD2) inhibiting 98 % of the
304 spores and 20 out of the 23 tested consortia inhibiting 100 % of the spores (Fig. 3). The
305 difference between the consortia consisted in the proportion of spores that were fully vs.
306 partially inhibited in their germination. Interestingly, the presence of some strains such as PID6
307 (*Bacillus cereus*) and SOP8 (*Cupriavidus metallidurans*) seemed to increase the effect of all
308 consortia containing them and the use of these two strains alone prevented almost all spores
309 from germinating. On the contrary, the strains SOD2 (*Bacillus halotolerans*) and CHP14 (*Bacillus*
310 *subtilis*) slightly reduced the effectiveness of the consortia which contained them.

311 **Phyllosphere bacteria can inhibit symptom development *in planta* against *P. viticola* and *B.***
312 ***cinerea***

313 The protective abilities of the 53 phyllosphere bacteria were assessed *in planta* on grapevine
314 leaf discs. Bacteria were sprayed onto grapevine leaf discs two days prior to infection, to let
315 them time to adapt and develop in their new environment. Against *P. viticola*, 14 strains were

316 able to significantly reduce the symptoms of the pathogen, to a level of from 24 to 59 % on
317 average (Table 2, Fig. 4), while all other strains did not result in any significant reduction in any
318 significant symptom reduction. These 14 strains included 10 strains already identified as active
319 in the first infection assay carried out with exposed zoospores described above (Table 1), while
320 four other strains that were not active in the previous experiment, SOD6 (*Bacillus cereus)*, CHP2
321 (*Cupriavidus metallidurans*), CHP20 (*Bacillus cereus*) and CHP29 (*Microbacterium proteolyticum*),
322 revealed their antioomycete abilities when pre-inoculated on the leaf discs (Table 2).

323 Higher levels of inhibition were achieved against *B. cinerea* with 15 strains allowing a significant
324 reduction of the necrosis going from 38 to 81 % on average (Table 3). In our conditions, seven
325 strains were able to achieve a better reduction in symptoms (more than 46 %) than the Botector
326 used as positive control: CHP29 (*Microbacterium proteolyticum*), SOP47 (*Variovorax paradoxus*),
327 CHP2 (*Cupriavidus metallidurans*), PIP6 (*Herbaspirillum huttiense*) PIP36 (*Bacillus cereus*), SOP8
328 (*Cupriavidus metallidurans*) and PID2 (*Bacillus simplex*) (Fig.5).

329 **Phyllosphere bacteria can stimulate plant defenses against *P. viticola* and *B. cinerea***

330 Infected leaf discs from the previous experiment were also collected in search for effect on plant
331 defenses. We monitored defense genes expression and stilbenic compound production. A set of
332 defense gene markers linked to the SAR (PAL), ISR (LOX), stilbene biosynthesis pathway (STS and
333 ROMT) and two pathogenesis related protein encoding genes (PR-1 and PR-4) were used for the
334 qPCR analysis. For each pathogen, we tested all strains that were able to significantly reduce symptom
335 development for their ability to induce plant defenses. Among the 14 strains that were effective
336 against *P. viticola*, 11 significantly upregulated the expression of at least three defense genes and

337 three strains had no positive impact on the defense gene expression (Fig. 6). These latter strains
338 (CHP20, CHP29 and SOD31), showed the lowest level of inhibition of symptoms on leaf discs and
339 mainly downregulated gene expression or had no significant effect on the monitored genes.
340 Among the strains that significantly upregulated the expression of several defense-gene, we
341 observed a tendency for upregulation of the genes from the SAR and the stilbene synthesis
342 pathways (PR-1, PAL, STS and ROMT). In contrast, the expression of the genes LOX and PR4 were
343 induced by fewer strains. Furthermore, it seems that when these two genes were upregulated,
344 the fold change was globally lower compared to the genes from the SAR or the stilbene pathway.
345 Interestingly, the strains PIP7, PIP17, SOD13, SOD2 and CHP2 were able to stimulate all or almost
346 all genes tested without downregulating any single gene. Finally, regarding the two best
347 performing strains in term of symptom reduction, PIP27 and CHP15, they had an opposite effect
348 on gene expression: PIP27 upregulated ROMT, PR-1 and PR-4 and slightly downregulated LOX,
349 PAL and STS, while CHP15 caused the opposite effect.

350 For *B. cinerea*, we tested the seven most effective strains that triggered an inhibition higher than
351 the one observed in the positive control. In contrast to *P. viticola* infection, the strains causing
352 the highest inhibition against *B. cinerea* mainly triggered a downregulation of the defense-
353 related genes (Fig. 6). Except for the strain PIP36 (*Bacillus cereus*), which was able to promote
354 the expression of almost all genes and SOP8, which only promoted the expression of PR-4 and
355 had moderate to no effect on the other genes, all the other strains had no significant or
356 negatively affected the expression of the tested set of defense-related genes.

357 Regarding the production of stilbenic compounds, differences were only observed in plants
358 infected with *P. viticola* (Fig. 7). Thus, in infected plants inoculated with protective bacteria, the
359 production of piceid and resveratrol (an inactive storage form and a precursor of more active
360 forms respectively) was stimulated by most of the bacteria. On the contrary, the production of
361 the active compounds, isohopeaphenol, α-viniferin and ε-viniferin was negatively impacted by
362 the bacterial inoculation with most strains. However, few strains were able to stimulate the
363 production of the different active forms. Thus, CHP15 (*Micrococcus yunnanensis*), SOD13
364 (*Paenibacillus xylanexedens*), SOD2 (*Bacillus* subtilis), SOD6 and PID6 (two *Bacillus cereus*) were
365 able to promote the synthesis of at least three of the active forms in infected leaves. Except for
366 CHP15 that triggered an inhibition of symptoms of 46 %, these strains were responsible for an
367 inhibition of 36, 39 and 32 % respectively corresponding to an moderate level among our active
368 strains.

369 **Strains combinations are more efficient than single strains for whole plant protection**

370 The ability of the protective strains to inhibit the symptoms of *P. viticola* on whole plants was
371 assessed using combinations containing three of the best performing strains. The strains used in
372 the consortia were selected based on their ability to affect spore physiology, to inhibit symptom
373 development, to stimulate defenses and to survive when exposed to at least the recommended
374 dose of antifungal compounds commonly used in the vineyard for crop protection such as
375 wettable sulfur and potassium hydrogenocarbonate (Supp. Table 2). Thus, random combinations
376 of CHP2 (*Cupriavidus metallidurans*), PID6 (*Bacillus cereus*), PIP6 (*Herbaspirillum huttiense*),
377 CHP14 (*Bacillus subtilis*), PID13 (*Bacillus endophyticus*), SOP8 (*Cupriavidus metallidurans*) and

378 SOD2 (*Bacillus halotolerans*) were assembled and sprayed on half the leaves of grapevine plants
379 while the other half was sprayed with water prior to the whole plant infection with *P. viticola*
380 zoospores. After a week, symptoms were measured on bacterial-inoculated and uninoculated
381 leaves in search of local and systemic protective effects of the bacteria. Result showed that the
382 strains CHP14, CHP2 and PID13 alone provided the lowest reduction of symptoms along with the
383 highest variability (38, 34 and 31 % respectively), the reduction triggered by CHP2 and PID13
384 being not even significant. However, all combinations were able to significantly reduce the level
385 of symptoms (Fig. 8). For all plants and for all treatments, the severity of symptoms was similar
386 on bacteria-inoculated leaves and in leaves that were spread with only water, suggesting that
387 the presence of bacteria can trigger a systemic response. However, the level of symptom
388 reduction was different between the different treatments. The three best combinations
389 (CHP2/PID6/PIP6; CHP14/PID13/PIP6 and CHP2/CHP14/PID13) led to a symptom reduction
390 ranging from 88.1 to 94.2 % on average, reaching an almost complete protection, however still
391 lower than the positive control (Table 4). The last combination (CHP2/CHP14/SOD2) provided a
392 reduction of 57.6 % which, although significant, was not very consistent (standard deviation of
393 39.2) and remained lower than the protection generated by the strains PID6 and SOP8 alone (82
394 and 68 % respectively).

395

396 **Discussion**

397 The results of this study show that phyllosphere bacteria are powerful agent to fight against
398 grapevine diseases that could be able to provide several layers of protection effective against
399 different kinds of pathogens.

400 **Spore physiology disruption against *P. viticola* and *B. cinerea***

401 The first layer of protection consists in affecting the reproduction of the pathogens trying to
402 colonize the plant. In past studies, bacteria such as streptomyces strains have been showed to
403 negatively affect the motility of *P. viticola* zoospores through the production of oligomycin and
404 pamemycin (Dame et al., 2016). In our study, among the 30 strains able to impair the motility of
405 *P. viticola* zoospores, almost 20 strains belonged to the genus *Bacillus*. This genus is well known
406 for containing many biocontrol species and several studies have highlighted the effect of *Bacillus*
407 strains on zoospores. *Bacillus* sp. 109GG020 is able to affect *Phytophthora capsici* zoospore
408 motility by producing gageopeptins, cyclic lipopeptides resulting in a loss of motility and an
409 abnormal helical instead of straight swimming (Tareq et al., 2015). *B. licheniformis* has also been
410 shown to negatively impair *P. capsici* zoospores by affecting their motility in the same way, as
411 well as causing zoospore lysis (Y. Li et al., 2020). Comparable effects have been described using
412 *Bacillus amyloliquefaciens* against *Phytophthora sojae* (D. Liu et al., 2019) and *Bacillus cereus*
413 against the pathogen *Pythium torulosum* (Shang et al., 1999). However, in our study, the most
414 active strains which, after co-incubation with the zoospores, induced a reduction in the
415 subsequent development of symptoms, were *Pantoea agglomerans*, *Microbacterium hominis*
416 and *Rhodopseudomonas boonkerdii*, species which have so far not been reported to harbor
417 strains affecting zoospores of oomycetes. The origin of the bacteria does not seem to explain

418 their level of inhibition, since the proportion of endophytic and epiphytic strains was
419 comparable among active strains. One might have expected to find more active bacteria among
420 epiphytes, because due to their location they are in direct contact with the zoospores before
421 these enter the leaves. However, previous reports have already shown that other endophytic
422 microorganisms such as beneficial fungi like the ascomycete *Curvularia* sp. M12, were also able
423 to affect *P. capsici* zoospores (Mondol et al., 2017). In our work, after contact with bacteria, no
424 abnormal swimming was observed and the number of zoospores was comparable with the
425 control. The mechanism involved in the loss of motility was not investigated, but it seems that
426 the gageopeptins or cell lysis are not at play since abnormal motility was not observed and there
427 was no apparent sign of cell lysis as seen in Shang et al., 1999 and Tareq et al., 2015. However,
428 the TEM observations suggest that zoospores might be strongly affected by some bacteria since
429 they displayed a complete loss of their oval shape after contact with one of our tested bacteria.
430 It seems probable that the zoospores may have been killed although not lysed. Despite the strong
431 effects of several bacteria on zoospore motility, the zoospores were still able to infect grapevine
432 leaf discs, although with a significant reduction in symptoms compared to the control. Motility is
433 an essential function of the zoospore for plant infection in oomycetes (Chepsergon et al., 2020).
434 It is therefore expected that zoospores unable to swim can no longer infect plants. However,
435 several zoospores were still able to cause infection despite this loss of function. It seems that
436 they were still able to reach an infection site. We can hypothesize that the infection method
437 (spraying of a zoospore solution dissolved in water) could have led to the direct deposition of
438 living zoospores on a stomata or at least close enough for the zoospore to be transported inside

439 the plant with water as they entered the plant by absorption. In all cases it is important to note
440 that even with a complete loss of motility, spores were still able to infect the plant and that a
441 high loss in motility was not always correlated with the highest symptom reduction. Thus,
442 zoospores treated with strains like SOD2 and CHP14 despite an almost full inhibition of motility
443 were still able to successfully infect the plants after subsequent infection. In contrast, some
444 zoospores treated with strains such as PIP27 and PIP4 that were not able to inhibit the spore
445 motility showed a limited capacity to infect plants, suggesting that loss of motility alone is not
446 enough to fully hinder the virulence of *P. viticola* zoospores' and that the contact with bacteria
447 not only affects the motility but also other key functions in the zoospores.

448 The phyllosphere bacteria not only affected the spore motility of *P. viticola* but also the spore
449 germination of *B. cinerea*. The most effective strains regarding this effect belonged to the genera
450 *Herbaspirillum*, *Cupriavidus* and *Bacillus.* The biocontrol abilities for these two first genera have not been
451 well studied so far, but the effect of *Bacillus* strains on fungal pathogens including *B. cinerea* is well
452 known. *Bacillus subtilis* has been reported to affect *B. cinerea* hyphal morphology and to inhibit
453 spore germination through the release of antifungal volatiles (Chen et al., 2008). The use of a
454 broth from *B. cereus* strains also caused comparable effects (Li et al., 2012), as did the
455 application of *B. mycoides* (from the *B. cereus* group) as a biocontrol agent against *B. cinerea* in
456 strawberry (Guetsky et al., 2001a). Interestingly, one strain of *B. thuringiensis* was found among
457 the four best performing bacteria. This species is also a member of the *B. cereus* group and a
458 source of powerful biocontrol agents against insects mainly due to its production of Cry and Cyt
459 toxins (Bravo et al., 2011), but also because of its production of chitinase (Honda et al., 2017).
460 Since *B. cinerea* hyphal and spore cell are also composed of chitin (Chardonnet et al., 1999), this

461 chitinase could be responsible for this germination inhibition and it is possible that *B.*
462 *thuringiensis* could be a very effective biocontrol agent against fungal pathogens and not only
463 insects as suggested by a recent study (Martínez-Zavala et al., 2024). However, this strain was
464 not found in the best performing strain consortia because it was not synergizing well with other
465 bacteria unlike the others aforementioned *Bacillus*, *Herbaspirillum* and *Cupriavidus*. The
466 combination of bacteria from these genera increased tremendously the inhibitory effect on
467 sporulation, especially when different genera were mixed in the same consortia. Thus, consortia
468 of two bacteria from different genera were able to significantly increase the effectiveness of the
469 strains, some of which almost reached a complete inhibition of the germination. The fact that a
470 full of inhibition was reached with two combinations containing bacteria from three different
471 genera supports the hypothesis that the best synergies would be achieved when different
472 genera, supposedly using different mechanisms would be combined. Higher effectiveness of
473 biocontrol solutions using mixtures of bacteria instead of single strains has already been shown
474 for pathogen spore germination (Guetsky et al., 2001). In this case, the mechanisms involved are
475 also unknown, but the authors suggested that when consortia are used, the reliability of the
476 biocontrol is increased while the variability is decreased. This is what we observed in this study
477 as well, but in certain conditions, some associations were less effective. Thus, all combinations
478 containing the strain SOD2 (*B. halotolerans*) and CHP14 (*B. subtilis*) showed a lower effect
479 compared to the same combinations in which this strain was absent or replaced by another one.
480 The mechanism underlying this decreased efficacy is not known but we can rule out antagonism
481 between the strains regarding their growth, since compatibility had been tested for all strains

482 prior to designing the consortia. However, such effectiveness reduction when using
483 combinations of microorganisms in a plant biocontrol context has already been described in
484 banana treated with beneficial fungi (Sukhada et al., 2011)

485 **Preventive inoculation of phyllosphere bacteria prior to infection led to significant protection**
486 **against *P. viticola* and *B. cinerea***

487 Fewer bacterial strains caused a significant reduction of symptoms against *P. viticola* compared
488 with the previous test. Despite the two days between the bacterial inoculation and the
489 pathogen infection to give plants and bacteria time to adapt to each other, it seems that a direct
490 contact with the zoospores was more effective than a plant inoculation. However, the conditions of a
491 bacterial preinoculation are closer to natural conditions or conditions that would be used in the field
492 when applying the strains. If we compare the list of effective strains from the first assay, that were at the
493 same time able to affect motility and to reduce symptom development, with the strains effective in the
494 bacterial preinoculation experiment, only three strains, SOP5, PID6 and PIP17 (*Microbacterium hominis*,
495 *Bacillus cereus* and *Staphylococcus arlettae* respectively) proved effective in both experimental set up,
496 suggesting that the weakening of spores does not play such an important role in natural or field
497 conditions and that other mechanisms are at play. Regarding the type of bacteria that showed activity in
498 this set up, we found once again the genera from the previous experiment *i.e.*: *Cupriavidus, Paenibacillus*
499 *Rhodopseudomonas* and a large representation of *Bacillus* species, but we also found three different
500 strains of *Microbacterium*. So far, this genus has not been often mentioned in the literature as effective
501 against *P. viticola,* but some strains have been shown to be effective at stimulating plant defense and at
502 inhibiting the growth of oomycetes and several fungal pathogens (Barnett et al., 2006; Freitas et al.,
503 2019; Ray et al., 2021; Suárez-Estrella et al., 2023).

504 The same observations have been made against *B. cinerea*, less bacteria caused a reduction in symptoms
505 compared to the test performed with spores, suggesting that for both oomycetes and fungi, activities on
506 spores, although helpful, are not essential for achieving effective biocontrol, it is also possible that the
507 ability of bacteria to disturb the pathogens' spores *in planta* is weaker than *in vitro*. Almost all genera
508 found previously were also found to be active against *B. cinerea* in these conditions, with the addition of
509 one *Variovorax* and one *Plantibacter.* These two genera are known for their plant growth promoting
510 abilities (Giannelli et al., 2024; Han et al., 2013; Mayer et al., 2019; Sun et al., 2018), but their
511 biocontrol abilities are poorly studied. *Variovorax* has shown moderate biocontrol activities in
512 tomatoes against *B. cinerea* and in wheat against *Fusarium* (Besset-Manzoni et al., 2019; Köhl et al.,
513 2020), but to our knowledge, no study has yet reported any biocontrol effects of *Plantibacter*
514 strains.

515 **Several phyllosphere bacteria contribute to enhance grapevine defenses**

516 Bacterial strains successfully inhibiting the development of symptoms when they were
517 inoculated *in planta* prior to infection were tested for their ability to stimulate the expression of
518 several defense genes belonging to different defense pathways. Most of the strains effective
519 against *P. viticola* were able to upregulate several defense genes, but several bacteria such as
520 CHP20, CHP29 and SOD31 show few to no significant effect regarding defense gene upregulation
521 and seemed to even slightly downregulate plant defenses. Most of the strains shared the
522 downregulation of the PR-4 gene a gene encoding a protein with chitinase and chitin-binding
523 activities (Enoki & Suzuki, 2016; M. Y. Li et al., 2020; G. T. Liu et al., 2021). The expression of this
524 protein has been shown to be upregulated in grapevine resistant cultivars against *P. viticola* or in

525 susceptible cultivar upon transgenic overexpression, but not in susceptible cultivars (Enoki & Suzuki,
526 2016; M. Y. Li et al., 2020; G. T. Liu et al., 2021). Other studies mentioned that the expression of PR-4 is
527 upregulated in association with the JA-ethylene pathway against necrotrophic fungi like *B. cinerea* while
528 SA pathway is recruited against biotrophic pathogens such as *P. viticola* (Trusov et al., 2009; Van Loon
529 et al., 2006). This could explain that in our case, the expression of this protein-encoding gene
530 was not promoted since we are using a susceptible cultivar and because the SA pathway,
531 antagonistic to the JA pathway (Vlot et al., 2021), seems to be mainly recruited after infection in
532 our conditions. Thus, for most bacteria stimulating plant defenses against *P. viticola*, PAL, STS,
533 ROMT and PR1 were among the genes that were most frequently upregulated. These data
534 suggest that our active bacteria use different pathways to protect the plant against the pathogen
535 and that several of them do not rely on plant defense promotion. Interestingly, it seems that the
536 most effective strains against *P. viticola*, PIP27 and CHP15, were not the best at stimulating plant
537 defenses, so we may assume that they have a direct and strong effect on the pathogen.

538 When testing induction of defense genes upon infection with *B. cinerea*, we observed that
539 except for PIP36 and SOP8, that triggered the upregulation of LOX and PR-4 (consistent with
540 Trusov et al., 2009 and Van Loon et al., 2006), the other strains negatively affected the
541 expression of the monitored defense related genes. If we compare this observation with the one
542 we made in the case of *P. viticola*, we observe that *Cupriavidus metallidurans* CHP2, stimulated
543 the expression of defense-related genes in presence of *P. viticola*, but mostly inhibited the
544 expression of defense-related genes in presence of *B. cinerea*. This suggests that the effect of
545 this strain is specific to the type of pathogen, whether biotrophic or necrotrophic. But the

546 reduction in plant defense does not appear to be detrimental to the plant, since despite the lack
547 of plant defense stimulation, the bacteria were able to successfully protect the plant against the
548 pathogen suggesting that their effect is more likely acting on the pathogen rather than on plant
549 defenses. However, it is still possible that a different set of defense related genes are involved
550 for the protection against *B. cinerea*. Thus, according to a recent study, the genetic of grapevine
551 resistance is still poorly known, but different defense gene markers such as Ethylene Responsive
552 Factors (ERF) could be involved in the defense against this pathogen (Zhu et al., 2019; Rahman et
553 al., 2024).

554 The inoculation of bacteria also affected the production of stilbenes in leaves. These compounds
555 are phytoalexins with different levels of antifungal properties depending on their structure.
556 Against *P. viticola*, several strains were able to stimulate the production of several of these
557 compounds. Most of the strains promoted the production of resveratrol, synthetized by the
558 stilbene synthase (STS), an active form of stilbene and precursor of several more active
559 compounds such as viniferins and pterostilbenes (Coutos-Thévenot et al., 2001). CHP15, SOD13,
560 CHP2, SOD6 and PID6 (*M. yunnanensis*, *B. xylanexedens*, *C. metallidurans* and two *B. cereus*, and
561 *Cupriavidus metallidurans*) were able to strongly increase the production of at least two
562 different forms of viniferins, one the most active stilbenic compounds against *P. viticola* (Alonso-
563 Villaverde et al., 2011; Pezet et al., 2003; Van Leeuwen et al., 2013). The correlation between the
564 increase of the production of resveratrol and viniferin and the higher expression of The STS
565 genes in several active strains suggest that they indeed positively affect this pathway enough to
566 influence the development of the pathogen. However, no significant amounts of pterostilbenes,

567 a compound with higher antifungal effects than viniferins have been found in any sample,
568 despite the increase in the expression of the ROMT gene encoding the enzyme responsible for
569 its synthesis from resveratrol. This compound is produced in small amounts by grapevine even
570 after being infected by downy mildew (Godard et al., 2009) and a previous study showed that
571 even with higher gene expression and upon infection by a pathogen, levels of pterostilbenes
572 may not change significantly and remain low (Kong et al., 2020; Rahman et al., 2024). It is
573 therefore possible that despite the upregulation of the ROMT gene, the level of pterostilbene
574 production was too low for detection in our conditions.

575 On the contrary, no significant increase of any stilbenic compounds were found against *B.*
576 *cinerea*. These compounds have yet been shown to be effective against *B. cinerea* dormant
577 conidia (Pezet & Pont, 1990; Pont & Pezet, 1990), but when not in its quiescent form, this
578 pathogen produces lytic enzymes able to degrade stilbenic compounds (Pezet et al., 1991; Goetz
579 et al., 1999). The production of such enzymes could explain why no significant changes in
580 stilbene production were observed in our samples after infection with *B cinerea*, both in the
581 presence or absence of phyllosphere bacteria.

582 **Combinations of phyllosphere bacteria are effective *in planta* against *P. viticola***

583 The combination of strains we used on whole plants were all able to significantly protect the
584 plant both locally and systematically with a comparable level of symptom inhibition (Table 4).
585 For each combination, the strains were selected based not only on their abilities to inhibit
586 pathogen growth or to stimulate plant defenses, but also on their abilities to grow well together
587 and to survive in the presence of several phytosanitary products used in the field. Thus, each

588 selected strain was able to develop properly in culture media in presence of potassium
589 hydrogenocarbonate and wettable sulfur. Interestingly, as observed in experiments targeting *B.*
590 *cinerea* spore germination, the combination of strains containing three different genera was the
591 most effective against *P. viticola* compared to the other combinations containing at least two
592 *Bacillus* strains. Thus, the combination CHP2/PID6/PIP6 (*Cupriavidus metallidurans*, *Bacillus*
593 *cereus* and *Herbaspirillum huttiense*) was the most effective one and was able to reach an
594 almost full inhibition of symptoms (94 %). This level of protection was only surpassed by the
595 positive control Alginure®, a product certified for field use against *P. viticola* in Switzerland
596 containing a combination of brown algae, potassium phosphonate and amino acids, acting as a
597 direct fungicide and as a plant defense enhancer. Our best combination of bacteria, despite its
598 lack of formulation, was therefore able to match the protection conferred by a mixture of
599 biocontrol and chemical agents. This result suggests that strains greatly differing in genetic
600 makeup and potentially using different mode of actions are more effective than combinations of
601 very effective but genetically close strains that are more likely to use similar strategies of
602 inhibition. Consistent with the results observed on *B. cinerea* spores, the presence of the strain
603 SOD2 (*Bacillus halotolerans*) had a negative impact on the performance of the consortia in
604 which it was used. Thus, this consortium was the least successful at *P. viticola* inhibition and
605 resulted in lesser inhibition than SOP8 and PID6 alone. This combination also showed the
606 highest variability compared to the other consortia, making it particularly unreliable.

607 The use of combinations of bacteria with other bacterial strains, microorganisms from different
608 kingdoms or even chemical compounds has been reported as successful against several plant

609 pathogens in the past. This solution has several advantages, it allows the addition of several
610 effects such as direct pathogen inhibition, accumulation of more diverse antipathogenic
611 compounds, plant defense stimulation and higher competition for space and resources (Niu et
612 al., 2020). In tomatoes, combination of a *Bacillus* strain with several bactericides and a mixture
613 of two streptomyces was found more effective than the use of each strain or compound applied
614 separately against tomato wilt (Wang et al., 2023; Yu et al., 2022). Similarly, strains of *Bacillus*
615 *mycoides* and *Pichia guilermondii* were more effective at inhibiting *B. cinerea* spore germination
616 and reducing necrotic lesion size in strawberry leaves when used in combination compared to
617 their use alone, showing that organisms from different kingdoms can synergize and also provide
618 more consistent protection (Guetsky et al., 2001). On the contrary, a synthetic microbial
619 community composed of *Pseudomonas*, *Bacillus*, *Trichoderma* and *Rhizophagus* was shown to
620 not synergize against the two fungal pathogens *Fusarium oxysporum* and *B. cinerea* in tomato
621 (Minchev et al., 2021). In this study, the level of protection conferred by the consortium was
622 always equivalent to the best performing organism alone, suggesting that strains assembled in a
623 consortium do not always display synergistic effects in terms of protection against diseases. Even
624 if the use of microorganism combinations as biocontrol agents against plant pathogens has
625 mainly been studied against root pathogens, literature data show that some strains can be more
626 effective than others in term of plant protection. Thus, for bacteria, the genera *Bacillus* and
627 *Pseudomonas* are the most commonly found (Niu et al., 2020). In our study, several different
628 *Bacillus* species were proven effective but no *Pseudomonas* were present. Instead, other genera
629 such as *Cupriavidus*, *Herbaspirillum*, *Microbacterium*, and other less commonly studied species

630 showed high potential. We can speculate that to achieve the best protection possible with
631 bacteria-based biocontrol solutions, the use of combination including such genera is key and
632 that strains showing only moderate effects when used alone could be of high importance to
633 stabilize or increase the effectiveness of a consortium.

634 **Conclusion**

635 This study reports that the use of phyllosphere bacteria as biocontrol agents could be an
636 efficient strategy to fight against two of the most detrimental filamentous pathogens for
637 grapevine cultivation, *P. viticola* and *B. cinerea*. The plant natural microbial communities are a
638 good source of potent biocontrol strains and the use of microorganisms already adapted to the
639 environment and the organ to protect. After optimization, their use can probably show higher
640 effectiveness compared to solutions based on the application of exogenous organisms not fit to
641 live in the aerial part of the plant and consequently less likely to reach their full potential against
642 plant diseases. These bacteria were even more effective when they were used in consortia
643 combining diverse bacterial genera. They were shown to disturb the pathogens' spore
644 physiology, the mycelium development and also to stimulate plant defenses. Thus, combinations
645 of different species that show high effectiveness in these three aspects were able to almost fully
646 inhibit *P. viticola* infection in grapevine susceptible cultivars. Whether such level of protection
647 could also be conferred under field conditions is the next question we will need to address. To
648 this end, it will be important to develop a formulation recipe, since in our experiment, bacteria
649 were used only dissolved in sterile water, without any UV or dessication protection. Our results
650 suggest that a solution based on consortia from grapevine phyllosphere isolates could be a

651 powerful tool to control diseases in open vineyards or at least under plant production conditions
652 in nurseries. We believe that such biocontrol solution, providing several layers of defense against
653 pathogen and based on bacteria already adapted to the organ to protect could bring a long-term
654 protection with no harmful side effects on the plant or the environment. This solution could help
655 the farmer to reduce their use of pesticide without compromising the health of their plant and
656 the safety of their products.

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**Table 1**: Inhibition of *P. viticola* zoospore motility after 30 minutes contact with phyllosphere bacteria and level of symptom development 1 week after leaf disc infection.

| | | motility | | | symptoms | | |
|---|---|---|---|---|---|---|---|
| **strain** | **organism / treatment** | **inhibition (%)** | **SD** | **significance** | **inhibition (%)** | **SD** | **significance** |
| Cu | copper hydroxide | 100 | 0 | *** | 92 | 3.8 | *** |
| PIP10 | *Pantoea agglomerans* | 100 | 0 | *** | 64 | 21.9 | *** |
| SOP5 | *Microbacterium hominis* | 100 | 0 | ** | 61.8 | 29.3 | *** |
| PIP7 | *Microbacterium hominis* | 99.4 | 1.1 | *** | 66.2 | 29.9 | *** |
| PID6 | *Bacillus cereus* | 99.0 | 1.7 | *** | 83.3 | 10.8 | *** |
| SOD2 | *Bacillus halotolerans* | 99.0 | 1.7 | *** | 22.7 | 39.0 | - |
| CHP14 | *Bacillus subtilis* | 98.8 | 2.1 | *** | 38.5 | 39.2 | ** |
| PID5 | *Bacillus altitudinis* | 98.7 | 1.3 | *** | 18.5 | 61.3 | - |
| SOP51 | *Bacillus pumilus* | 98.7 | 1.2 | *** | 49.4 | 34.4 | *** |
| CHD1 | *Bacillus cereus* | 98.5 | 2.6 | *** | 30.1 | 58.7 | - |
| PIP36 | *Bacillus cereus* | 98.2 | 3.2 | *** | 46.7 | 42.2 | *** |
| SOD14 | *Bacillus thuringiensis* | 97.5 | 2.1 | *** | 4.4 | 40.5 | - |
| CHD4 | *Bacillus subtilis* | 97.1 | 5.0 | *** | 25.4 | 36.1 | - |
| SOD31 | *Bacillus zhangzhouensis* | 95.7 | 4.6 | *** | 26.0 | 46.7 | - |
| CHP20 | *Bacillus cereus* | 95.3 | 7.3 | *** | 18.1 | 51.1 | - |
| CHD2 | *Bacillus cereus* | 95.2 | 4.4 | *** | 33.5 | 36.9 | - |
| SOD6 | *Bacillus cereus* | 92.2 | 4.7 | *** | 39.4 | 47.6 | - |
| PIP2 | *Sphingomonas zeae* | 84.0 | 27.7 | ** | 50.6 | 36.5 | ** |
| PIP17 | *Staphylococcus arlettae* | 81.4 | 12.3 | ** | 39.1 | 42.8 | ** |
| SOD5 | *Bacillus licheniformis* | 79.9 | 28.3 | ** | 10.3 | 48.6 | - |
| PID2 | *Bacillus simplex* | 77.9 | 13.6 | *** | 74.5 | 28.4 | *** |
| CHP29 | *Microbacterium proteolyticum* | 76.3 | 30.7 | ** | 5.0 | 55.0 | - |
| SOD20 | *Bacillus stratosphericus* | 75.5 | 3.3 | * | 19.3 | 50.2 | - |
| PID1 | *Paenibacillus amylolyticus* | 64.1 | 22.9 | *** | 8.2 | 48.8 | - |
| CHP35 | *Bacillus aryabhattai* | 60.2 | 17.5 | * | 35.0 | 58.6 | - |
| CHP6 | *Rhodopseudomonas boonkerdii* | 56.3 | 13.8 | *** | 13.4 | 55.6 | - |
| PID13 | *Bacillus endophyticus* | 51.9 | 16.4 | ** | 52.3 | 41.1 | *** |
| SOP16 | *Bacillus butanolivorans* | 43.1 | 11.1 | ** | 14.9 | 49.8 | - |
| CHD6 | *Micrococcus aloeverae* | 39.7 | 11.1 | ** | 23.1 | 46.7 | - |
| PIP27 | *Rhodopseudomonas boonkerdii* | 36.3 | 31.2 | - | 63.7 | 24.5 | *** |
| PIP4 | *Cupriavidus metallidurans* | 28.0 | 18.3 | - | 60.3 | 32.3 | *** |
| SOD13 | *Paenibacillus xylanexedens* | 35.6 | 13.9 | - | 47.3 | 31.6 | *** |
| PIP3 | *Variovorax paradoxus* | 7.7 | 28.0 | - | 50.6 | 62.6 | *** |
| PIP6 | *Herbaspirillum huttiense* | 3.8 | 22.6 | - | 31.8 | 36.5 | * |

∗ = $p < 0.05$; ∗∗ = $p < 0.01$; ∗∗∗ = $p < 0.001$; - = not significant

**Table 2**: *P. viticola* symptom inhibition 1 week after infection in grapevine leaf discs inoculated with phyllosphere bacteria relative to uninfected control. Only strains that generated significant inhibition are displayed.

| strain | organism / treatment | inhibition (%) | SD | significance |
|---|---|---|---|---|
| Cu | copper hydroxide | 89.9 | 4.9 | *** |
| PIP27 | *Rhodopseudomonas boonkerdii* | 58.6 | 41.8 | ** |
| CHP15 | *Micrococcus yunnanensis* | 46.1 | 49.1 | * |
| PIP7 | *Microbacterium hominis* | 41.6 | 34.1 | ** |
| CHP14 | *Bacillus subtilis* | 39.7 | 32.8 | ** |
| PIP17 | *Staphylococcus arlettae* | 39.1 | 47.3 | * |
| SOD6 | *Bacillus cereus* | 39 | 41.7 | ** |
| SOD13 | *Paenibacillus xylanexedens* | 37.9 | 52.5 | * |
| SOD2 | *Bacillus halotolerans* | 36.6 | 45.5 | ** |
| CHP2 | *Cupriavidus metallidurans* | 35.5 | 47.2 | * |
| PID6 | *Bacillus cereus* | 32.4 | 42.7 | * |
| SOP5 | *Microbacterium hominis* | 28.9 | 24.3 | ** |
| CHP20 | *Bacillus cereus* | 24.8 | 42.4 | * |
| CHP29 | *Microbacterium proteolyticum* | 24 | 28.3 | * |
| SOD31 | *Bacillus zhangzhouensis* | 23.8 | 31.5 | * |

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**Table 3**: *B. cinerea* symptom inhibition 1 week after infection in grapevine leaf discs inoculated with phyllosphere bacteria relative to uninoculated control. Only strains that generated significant inhibition are displayed.

| strain | organism / treatment | inhibition (%) | SD | significance |
|---|---|---|---|---|
| CHP29 | *Microbacterium proteolyticum* | 81.4 | 10.5 | *** |
| SOP47 | *Variovorax paradoxus* | 75.4 | 9.7 | *** |
| CHP2 | *Cupriavidus metallidurans* | 70.8 | 19.1 | *** |
| PIP6 | *Herbaspirillum huttiense* | 69.3 | 19 | *** |
| PIP36 | *Bacillus cereus* | 64.8 | 16.1 | ** |
| SOP8 | *Cupriavidus metallidurans* | 63.8 | 19.4 | * |
| PID2 | *Bacillus simplex* | 62.4 | 11.6 | * |
| Botector | *Aureobasidium pullulans* | 61.1 | 14.8 | *** |
| PIP26 | *Plantibacter flavus* | 59.2 | 25.2 | * |
| SOP16 | *Bacillus butanolivorans* | 55.8 | 25.4 | ** |
| SOD13 | *Paenibacillus xylanexedens* | 54.2 | 25.5 | ** |
| SOP48 | *Staphylococcus xylosus* | 52.1 | 25.3 | * |
| CHP26 | *Bacillus drentensis* | 50 | 40.6 | ** |
| CHP20 | *Bacillus cereus* | 49.8 | 43.7 | ** |
| CHD1 | *Bacillus cereus* | 39.4 | 22.2 | * |
| SOD7 | *Bacillus psychrosaccharolyticus* | 38.6 | 33.6 | * |

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**Table 4**: Local and systemic *P. viticola* symptom inhibition 1 week after infection in whole plants inoculated with phyllosphere bacteria relative to uninoculated control

| | | local | | | systemic | | |
|---|---|---|---|---|---|---|---|
| **Strain** | **organism / treatment** | **inhibition (%)** | **SD** | **significance** | **inhibition (%)** | **SD** | **significance** |
| **Alginure** | **Alginure (brown algae)** | 98.2 | 0.9 | *** | 98.2 | 0.9 | *** |
| **PID6** | ***Bacillus cereus*** | 82.4 | 21.7 | *** | 68.5 | 38.5 | *** |
| **SOP8** | ***Cupriavidus metallidurans*** | 68.3 | 29.5 | *** | 74 | 19.8 | *** |
| **PIP6** | ***Herbaspirillum huttiense*** | 63.4 | 35.9 | *** | 29.6 | 55.5 | N.S. |
| **SOD2** | ***Bacillus halotolerans*** | 52.6 | 31.9 | *** | 58.3 | 18.1 | *** |
| **CHP14** | ***Bacillus subtilis*** | 38 | 64.4 | * | 31.1 | 73.5 | * |
| **CHP2** | ***Cupriavidus metallidurans*** | 33.8 | 46.6 | N.S. | 25.8 | 44.6 | N.S. |
| **PID13** | ***Bacillus endophyticus*** | 31.2 | 70.8 | N.S. | 32.2 | 53.4 | N.S. |
| **CHP2 / PID6 / PIP6** | ***Cupriavidus metallidurans / Bacillus cereus / Herbaspirillum huttiense*** | 94.2 | 5.5 | *** | 94 | 5.2 | *** |
| **CHP14 / PID13 / PIP6** | ***Bacillus subtilis / Bacillus endophyticus / Herbaspirillum huttiense*** | 88.1 | 8.6 | *** | 90 | 16.2 | *** |
| **CHP2 / CHP14 / PID13** | ***Cupriavidus metallidurans / Bacillus subtilis / Bacillus endophyticus*** | 84.9 | 13.1 | *** | 87.7 | 15.7 | *** |
| **CHP2 / CHP14 / SOD2** | ***Cupriavidus metallidurans / Bacillus subtilis / Bacillus halotolerans*** | 57.6 | 39.2 | ** | 67 | 26.8 | ** |

$* = p < 0.05$; $** = p < 0.01$; $*** = p < 0.001$; - = not significant

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908 **Figure 1:** Transmission electron microscopy image of *P. viticola* zoospores placed for 30 min in

909 contact with water (control), or a solution of the *Bacillus cereus* strain PID6 $OD_{600}$ = 1.

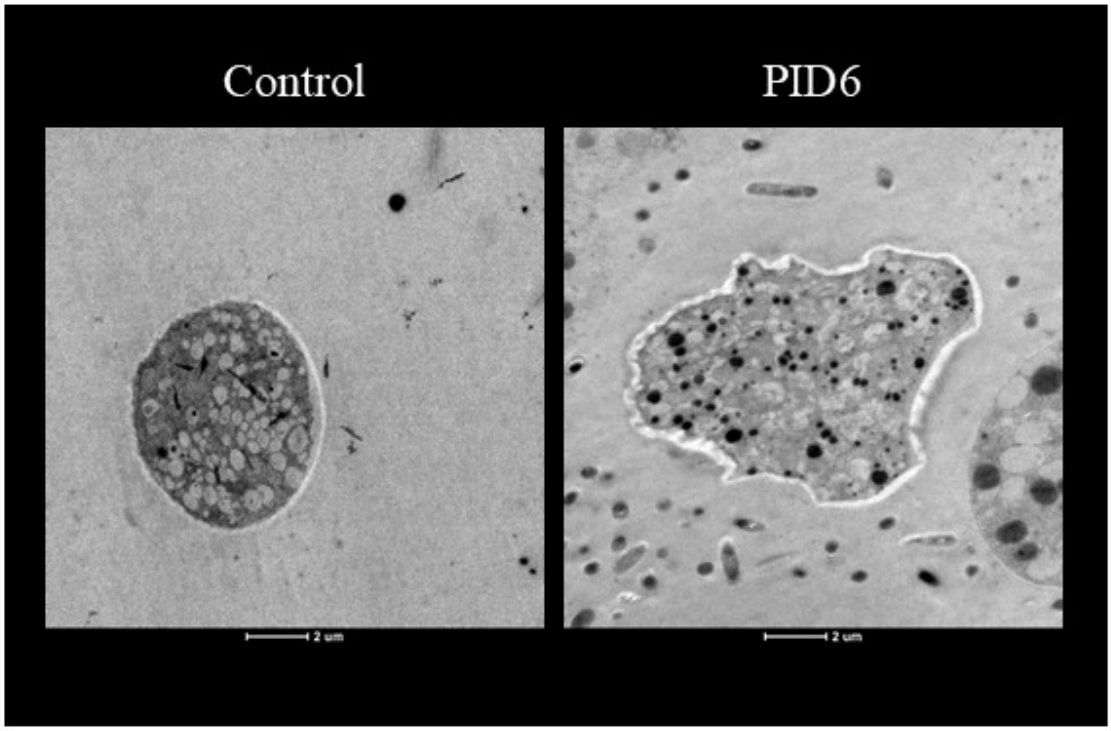


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**Figure 2:** Effect of phyllosphere bacteria on *B. cinerea* spore germination. A: Each colored bar represents the proportion of spores showing the corresponding morphology. Green, normal germination; yellow, partial inhibition (only the tip of germ tube is visible); orange, swelling; and red, no germination. Each treatment with bacteria significantly affected the physiology of the spores, with $p < 0.05$ according to a Chi-square test with Yate's correction. The different types of germination morphologies are shown in (B to E); B: normal germination; C; absence of germination; D: partial germination and E: swelling.

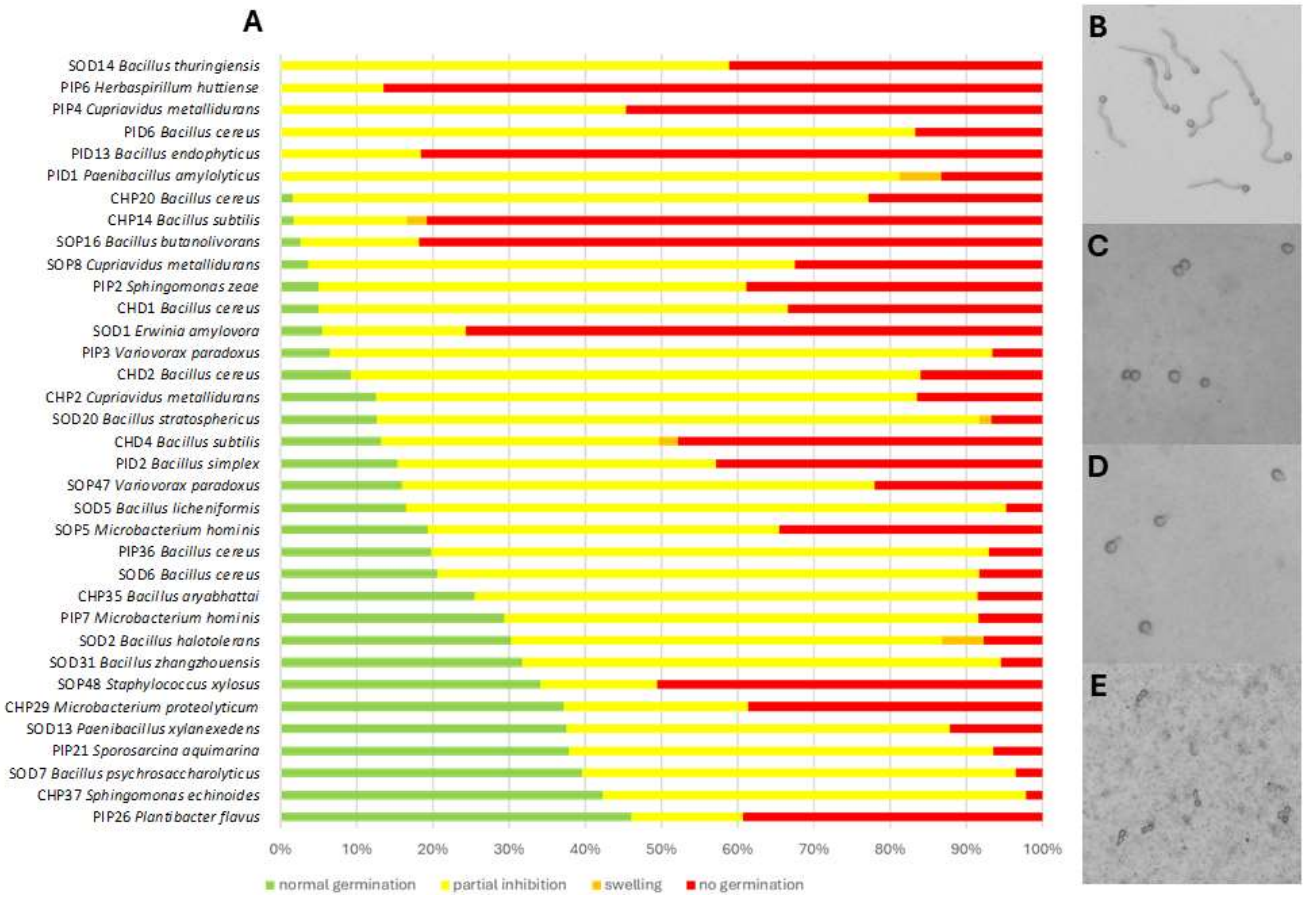
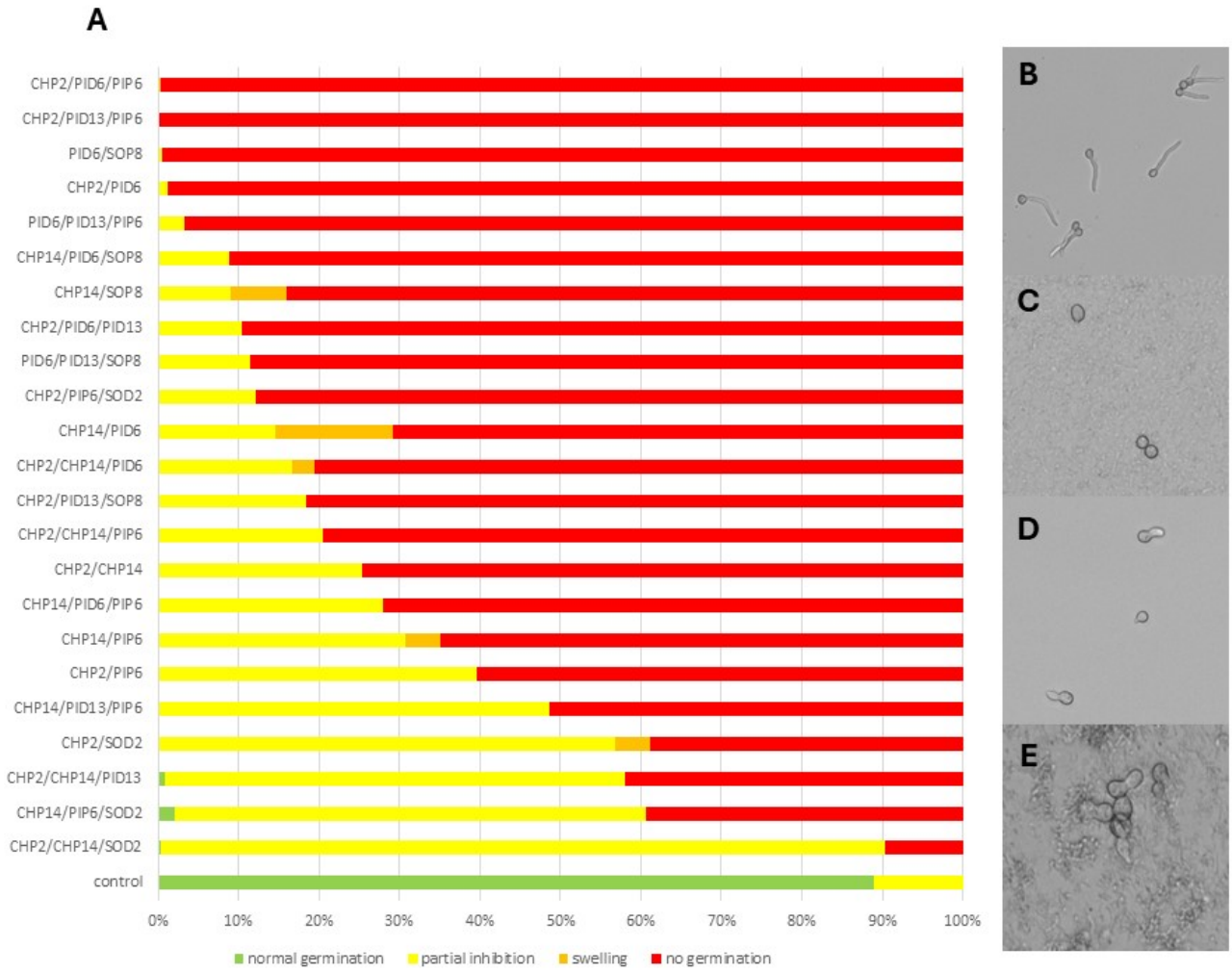


**Figure 3**: Effect of consortia of phyllosphere bacteria on *B. cinerea* spore germination. A: Each colored bar represents the proportion of spores showing the corresponding morphology. green: normal germination; yellow; partial inhibition (only the tip of germ tube is visible); orange: swelling and red: no germination. Each treatment with bacteria significantly affected the physiology of the spores, with $p < 0.05$ according to a Chi-square test with Yate's correction. The different types of germination morphologies are shown in (B to E); B: normal germination; C; absence of germination; D: partial germination and E: swelling.



935 **Figure 4:** Representative pictures of grapevine leaf discs inoculated with phyllosphere bacteria
936 prior to infection with *P. viticola*. Control: leaf disc not inoculated with bacteria and infected with
937 *P. viticola zoospores*; PIP27, SOD13 and SOD31: leaf discs inoculated with *Rhodopseudomonas*
938 *boonkerdii*, *Paenibacillus xylanexedens* and *Bacillus zhangzhouensis* respectively prior to
939 infection. Picture were taken 1 week after infection.

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949 **Figure 5:** Representative pictures of grapevine leaf discs inoculated with phyllosphere bacteria
950 prior to infection with *B. cinerea*. Control: leaf disc not inoculated with bacteria and infected
951 with *B. cinerea* spores; CHP29, PID2 and SOD7: leaf discs inoculated with *Microbacterium*
952 *proteolyticum*, *Bacillus simplex* and *Bacillus psychrosaccharolyticus* respectively prior to
953 infection. Picture were taken 1 week after infection.

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963 **Figure 6:** Level of expression of defense-related gene in grapevine leaf discs inoculated with

964 phyllosphere bacteria that significantly protected the plant against *P. viticola* or *B. cinerea* in the

965 leaf disc assay. Levels of expression were normalized with the two reference genes Actine and

966 60SRP and expressed relative to infected control leaf discs that were not inoculated with

967 bacteria.

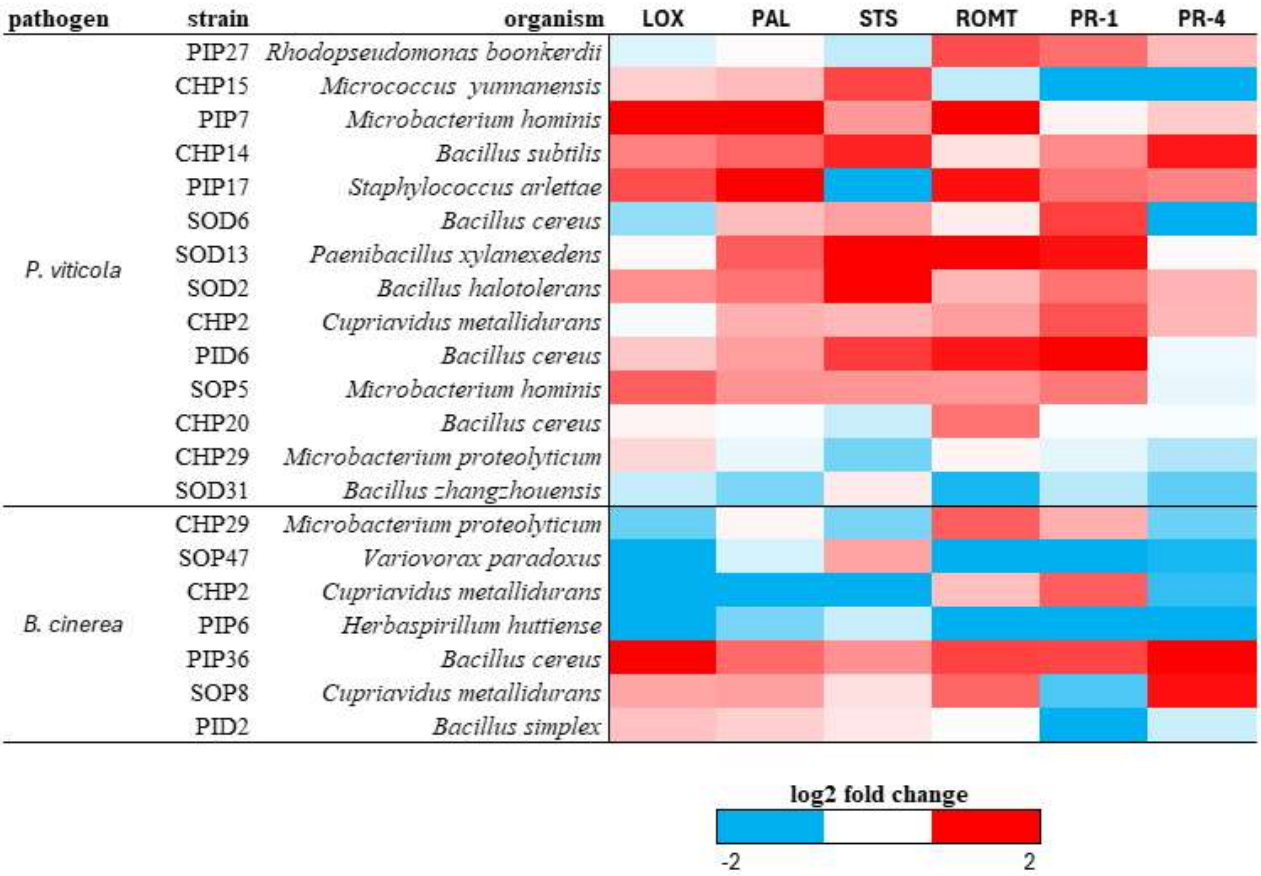


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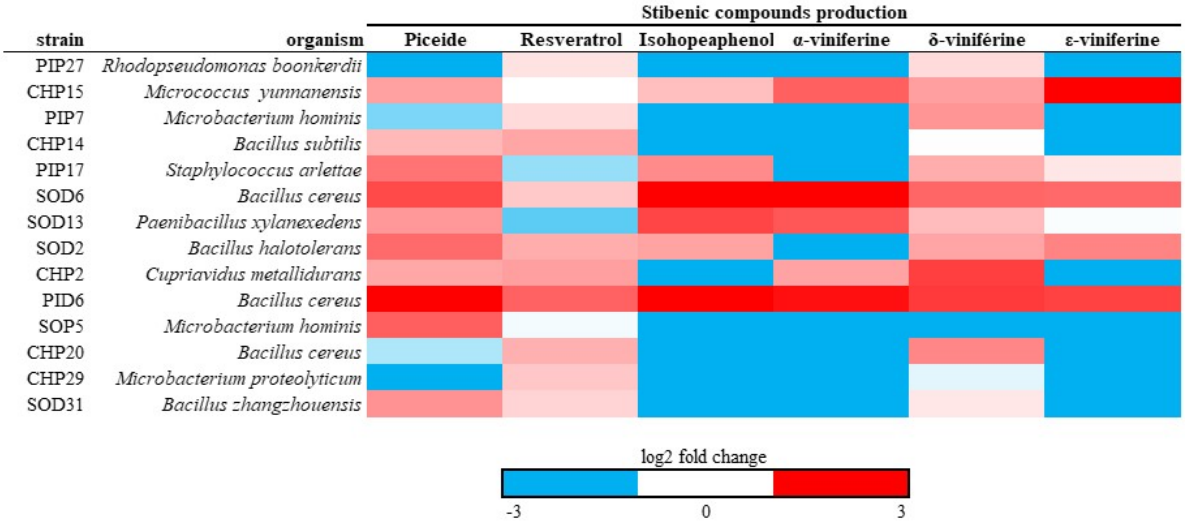
**Figure 7:** Stilbene production levels in grapevine leaf discs inoculated with phyllosphere bacteria 3 days after infection with *P. viticola* zoospores. Levels of production based on dry weight, relative to infected leaves that were not inoculated with bacteria.



984 **Figure 8:** Representative pictures of grapevine leaves from whole grapevine plants inoculated
985 with phyllosphere bacteria prior to infection with *P. viticola*. Leaves were inoculated with
986 different mixtures of the following six strains: *Cupriavidus metallidurans* (CHP2), *Bacillus cereus*
987 (PID6), *Herbaspirillum huttiense* (PIP6), *Bacillus subtilis* (CHP14), *Bacillus endophyticus* (PID13)
988 and *Bacillus halotolerans* (SOD2). Control: non-inoculated leaf infected with *P. viticola zoospores*.
989 Picture were taken 1 week after infection.

CONTROL CHP2/PID6/PIP6 CHP14/PID13/PIP6 CHP2/PID6/PIP6 CHP2/CHP14/SOD2

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