# Comparison of efficacy and modes of action of two high-potential biocontrol Bacillus strains and commercial biocontrol products against Botrytis cinerea in table grapes

Héctor Calvo, Jean Roudet, Ana Pilar Gracia, María Eugenia Venturini, Vittorino Novello, Marc Fermaud

Received: 16 April 2021 | Accepted: 17 August 2021 | Published: 3 September 2021
DOI:10.20870/oeno-one.2021.55.3.4688

OENO One
VINE AND WINE OPEN ACCESS JOURNAL

# Comparison of efficacy and modes of action of two high-potential biocontrol *Bacillus* strains and commercial biocontrol products against *Botrytis cinerea* in table grapes

Héctor Calvo[1], Jean Roudet[2], Ana Pilar Gracia[1], María Eugenia Venturini[1] and Marc Fermaud[2]

[1] Plant Food Research Group. Instituto Agroalimentario de Aragón– IA2 - (Universidad de Zaragoza-CITA), Miguel Servet, 177. 50013, Spain.
[2] INRAE, UMR SAVE, Bordeaux Science Agro, ISVV, F-33882, Villenave d'Ornon, France

**corresponding author*: marc.fermaud@inrae.fr

*Associate editor*: Vittorino Novello

**ABSTRACT**

In table grapes (cv. Thomson Seedless), the antifungal activity against *Botrytis cinerea* was further analysed and compared between two high potential bacterial biological control agent (BCA) strains, *Bacillus velezensis* BUZ-14 and *B. ginsengihumi* S38. Two commercial biocontrol products (BPs), served as standards of comparison, Amylo-X® and Serenade Max®, also based on *Bacillus* BCA strains. The main mode of action quantified for all the strains was antibiosis due to hydrosoluble and volatile metabolites and their combinations. The BUZ-14 strain was the most active BCA strain, demonstrating significant disease reduction exceeding 60 % when used in the culture form grown in 863 medium, including living cells (LCs) and cell-free supernatant (CFS). Both BPs exhibited significantly reduced efficacy of their CFS fraction (< 10 %) compared with that of the two BCA strains, confirming their high antibiosis potential. The novel methodology allowed us to demonstrate the significant effect of the BCA culture medium on volatilome (VOC) antagonist efficacy. The S38 strain achieved the highest disease reduction (90 %) owing to the greatest production of VOCs in the richest MOLP (Medium Optimum Lipopetide Production) culture medium, whereas grape juice was the least favourable medium for VOC efficacy for both bacterial strains (BUZ14 and S38). The overall poor activity of living *Bacillus* cells in all the BCA and BPs tested is discussed based on the low capacity of the BCA strains to grow in the berry. Then, the presence of living cells is also discussed with the possibility that these cells are not required in field applications of such BCA strains in this genus. Moreover, different environmental suboptimal conditions, including temperature (22 and 27 °C) and relative humidity (RH) (100 and 85–95 %), were tested, and BUZ-14 exhibited the highest *Botrytis* reduction at both temperatures and RH values. However, no significant differences were observed between temperatures or RH values for the same BCA. Further studies in vineyard conditions and applications, such as biofumigation or active packaging, will be performed to confirm the new findings reported in this investigation.

**KEYWORDS**

grapevine, CFS, grey rot, *B. velezensis* BUZ-14, *B. ginsengihumi* S38, MoA, volatilome

## INTRODUCTION

*Botrytis cinerea* is the causal agent of grey mould, which is one of the most important and damaging diseases affecting wine grapes and table grapes worldwide. This agent is a major necrotrophic fungal pathogen of numerous host plants (Elad *et al.,* 2016a; Elad *et al.,* 2016b) and leads to considerable yield and quality losses in the field as well as in wine production and/or storage (Ky *et al.,* 2012; Sharma *et al.,* 2009). Although several anti-*Botrytis* synthetic fungicides are available, their use in pre- or postharvest conditions is not considered sustainable due to the possible negative consequences, including (i) the appearance of resistant strains (Hahn, 2014; Leroux, 2004) and (ii) the adverse effects of the fungicides on the environment and/or human health (Coleman *et al.,* 2012). An emerging social rejection of the use of such chemicals has been raised given the risk posed to human and animal health and the origin of residues in the fruit and/or wine products. Most of the specific anti-*Botrytis* synthetic fungicides are responsible for relatively frequent contamination of commercial wines according to Esteve-Turrillas *et al.* (2016). The use of these anti-*Botrytis* fungicides represents an issue of worldwide relevance with potentially negative consequences for consumer health and international trade.

Biological control is an alternative to pesticides that are increasingly used and distributed worldwide. Research in this field has been very intense during the last two decades (Chowdhury *et al.,* 2015; Köhl *et al.,* 2019; Rahman *et al.,* 2018). However, the incorporation of biocontrol products or bioproducts (BPs) in the market has been difficult in recent years given the significant costs as well as the long period of time needed to obtain registration for the BPs (Droby *et al.,* 2016). Therefore, only a few large companies can develop and market such BPs. For example, in Spain and France, there were only a few commercial BPs based on antagonist microorganisms registered against grey mould in vineyards in 2018. These BPs include Botector® - BIO-FERM GMBH (*Aureobasidium pullulans* DSM 14940 and DSM 14941); Amylo-X® - CERTIS AG (*B. amyloliquefaciens* subsp. *plantarum* D747) and Serenade MAX - BAYER AG (*B. subtilis* QST 713). In Spain, Serifel® - BASF (*Bacillus amyloliquefaciens* MBI 600) was also included as commercial BP but it has not been approved in France to date (Index Acta Biocontrôle, 2018; MAPAMA, 2018). The genus *Bacillus* has been intensively studied and has become one of the most important genera to control fungal phytopathogens in numerous crops (Altindag *et al.,* 2006; Calvo-Garrido *et al.,* 2018; Calvo-Garrido *et al.,* 2019; Calvo *et al.,* 2017; Farace *et al.,* 2015; Hang *et al.,* 2005; Romero *et al.,* 2007). The production of antifungal metabolites (antibiosis), competition for nutrients and induction of resistance in the plant host are the main mechanisms used by the antagonist strains within different *Bacillus* species, notably against *B. cinerea* (Calvo-Garrido *et al.,* 2018; Calvo-Garrido *et al.,* 2019; Calvo *et al.,* 2017; Haidar *et al.,* 2016a).

The investigations conducted in biocontrol have primarily focused on the mode(s) of action and importantly on the interaction among the pathogen, the host plant and the BCA (biocontrol agent). However, few published studies have addressed the final application, including the development of bioformulates (Köhl *et al.,* 2019; Spadaro and Droby, 2016). Due to the lack of marketed biocontrol products that are constantly and highly efficient in the vineyard (Calvo-Garrido *et al.,* 2019), as also noted in other crops (Spadaro and Droby, 2016; Usall *et al.,* 2016), it was necessary to increase the knowledge of such interactions and improve the selection by comparing new BCA antagonists with high potential anti-pathogenic activity.

In this context, antibiosis is the main mode of action used by BCA microorganisms to prevent plant diseases (Di Francesco *et al.,* 2016; Haidar *et al.,* 2016a; Rahman *et al.,* 2018). In the case of the bacterial genus *Bacillus*, the following metabolites should be distinguished: i) hydrosoluble metabolites remaining in the supernatant and involving mostly lipopeptides, polyketides, siderophores and/or lytic enzymes (Chen *et al.,* 2006; Ongena and Jacques, 2008; Stein, 2005) and ii) volatile organic compounds (VOCs) or volatilomes (Calvo *et al.,* 2020; Gotor-Vila *et al.,* 2017a; Wu *et al.,* 2019). The former substances have been extensively studied and mainly include lipopeptides (Ongena and Jacques, 2008), but volatile metabolites are still under investigation. Interestingly, publications investigating the antifungal VOCs produced by antagonist bacteria using *in vivo* bioassays are scarce, notably concerning bioassays in fruit and vegetables (Almenar *et al.,* 2009; Arrebola *et al.,* 2010; Calvo *et al.,* 2020; Gotor-Vila *et al.,* 2017a). However, many more *in vitro* studies on various BCAs, such as *Bacillus*, *Pseudomonas* or *Trichoderma,* are available (Chen *et al.,* 2008; Haidar *et al.,* 2016b;

Haidar *et al.,* 2016c; Paulitz *et al.,* 2000; Yuan *et al.,* 2012; Zhang *et al.,* 2019). In contrast, *in vivo* methodologies are often not validated; therefore, there is a crucial need for developing new methods to evaluate the antifungal capacity of such bacterial antifungal VOCs. The effect of the nutrients available and used by the BCA strain to produce the antifungal volatiles might significantly influence the production of VOCs both qualitatively and quantitatively (Calvo *et al.,* 2020; Gotor-Vila *et al.,* 2017a; Raza *et al.,* 2016). Additionally, environmental external factors, such as temperature and relative humidity (RH), can affect antifungal activity, and these parameters need to be further investigated (the control of the disease might also change due to different optimal development temperatures or RHs of each interacting microorganism) (Calvo-Garrido *et al.,* 2014a; Landa *et al.,* 2004; Mukherjee and Raghu, 1997).

In this study, the mode of action was further investigated in two recently identified bacterial BCA candidate strains, both of great interest, i.e., *Bacillus ginsengihumi* S38 and *Bacillus velezensis* BUZ-14 (formerly named *B. amyloliquefaciens* BUZ-14). BUZ-14 exhibits significant antifungal activity against several phytopathogens, such as *B. cinerea* in table grapes, *Monilinia* spp. in stone fruits or *Penicillium* spp. in citrus and apples (Calvo *et al.,* 2017). Its major mode of action results from the production of lipopeptides (Calvo *et al.,* 2019) and VOCs (Calvo *et al.,* 2020). A bioformulation has also been developed, and its application in the field is currently being tested in stone fruits and vineyards to validate the previous results. On the other hand, the *B. ginsengihumi* strain S38, which was originally isolated from grapevine wood, has been thoroughly investigated under conditions in vineyards in southwestern France, leading to consistent results throughout the four studied seasons (Calvo-Garrido *et al.,* 2018; Calvo-Garrido *et al.,* 2019). The BCA strain significantly controlled the *Botrytis* bunch rot (BBR), yielding reductions in the average severity ranging from 35 % to 60 %, corresponding to similar (or higher) control rates as products registered for commercial use (Calvo-Garrido *et al.,* 2019). For major and key BCA features, the S38 strain exhibited the following: i) a great survival ability in the vineyard ecosystem, which was also confirmed under two contrasting simulated climatic conditions (Calvo-Garrido *et al.,* 2018; Calvo-Garrido *et al.,* 2019); ii) a high overall strain efficacy based on *in vivo* grapevine biotests (Haidar *et al.,* 2016b), and iii) a potentially highly efficient mode(s) of action (MoA), including antifungal metabolite production and nutrient competition (Calvo-Garrido *et al.,* 2018).

This study aimed to compare the antifungal activity and modes of action of these two high potential bacterial BCA strains against *Botrytis cinerea,* by using adapted *in vivo* methodologies based on mature commercial table grapes under controlled conditions. We aimed to demonstrate the influence of temperature and relative humidity (RH) on the antifungal activity against *B. cinerea* of these two candidate strains. Furthermore, we assessed and compared the antifungal role of BCA living cells and the metabolites generated by the two strains (hydrosoluble and volatile metabolites). Moreover, the efficacy was also compared in relation to two commercial biocontrol products (BPs), i.e., Amylo-x® and Serenade Max®, given that the activity of these agents against *B. cinerea* has already been tested in vineyards (Calvo-Garrido *et al.,* 2019).

## MATERIALS AND METHODS

### 1. Fruit sample origin and preparation

For all tests, table grapes (cv. Thomson Seedless) at commercial maturity originating from the supermarket were washed for 15 min under continuous tap-water flow to remove various debris (flower debris, etc.) in the berry and reduce possible fungicide residues. Then, grapes were surface-disinfected by immersion in calcium hypochlorite solution (50 g $L^{-1}$; pH adjusted: 7.2) for 10 min and subsequently treated with thiophanate methyl at 1.6 g $L^{-1}$ for 5 min. This last treatment allowed us to suppress possible interfering, uninoculated, natural fungal contamination and infection due to saprophytic genera, including *Penicillium* spp., *Rhizopus* spp. and/or *Mucor* spp. (for the choice of the fungicide, see section 2.2 below). Later, grape berries were rinsed thrice with distilled water and left to dry at room temperature. Finally, visually undamaged grape berries were selected and carefully cut off from the grape bunches with the pedicel attached using sterile scissors.

Before performing the inoculation, the berries were wounded to favour the entrance of the pathogen into the fruit to assure infection that potentially mimics naturally occurring infection during the preharvest due to insect and/or climatological damage (heavy rain, hail, etc.). Therefore, a small incision was generated in the centre of the fruit (1 × 1 mm) with a sterile tip pipette, and the fruit was left to dry at room temperature for 30 min before inoculation.

## 2. Pathogenic fungal strain and culture conditions

The *B. cinerea* pathogenic strain 213, which was isolated in 1998 in a Bordeaux vineyard from the cv. Semillon blanc (Martinez *et al.,* 2003), was selected from the INRAE-UMR 1065 SAVE collection in Bordeaux. The pathogen strain belongs to the *transposa* genotype and has been characterised as highly virulent on grapevine berries at different stages (Deytieux-Belleau *et al.,* 2009). Stock cultures were maintained on malt agar (MA) (15 g $L^{-1}$, Oxoid Ltd; Basingstoke, Hampshire, UK) at 5 °C before being used in experiments. To prepare the artificial inoculations of berries, the strain was subcultured in Petri dishes containing MA medium at 22 °C (12 h light/12 h dark). After 5–7 days of incubation under these conditions, mycelium plugs (5 x 5 mm) with growing mycelia were then used for mycelial inoculation. On the other hand, an aqueous conidial suspension prepared from 7-day-old cultures of *B. cinerea* grown in malt agar was used. Conidia were scraped from sporulating Petri plates, filtered through four layers of sterile cheesecloth and transferred to a test tube with 9 mL of sterile distilled water with 0.01 % Tween 80. The suspensions were adjusted to $10^4$ conidia $mL^{-1}$. The concentrations of the conidial suspensions were determined using a hemocytometer and a Leica microscope (Leica Microsystems, Germany).

Based on preliminary *in vitro* tests in our INRAE-SAVE laboratory (data not shown), this strain is highly resistant to thiophanate methyl, a broad spectrum (benzimidazole) fungicide, at a dosage of 1.6 kg/Ha, which is used in vineyards against powdery mildew and *Botrytis*. Thiophanate methyl is also effective against various saprophytic and/or pathogenic fungi. Thus, its use beforehand allowed us to reduce the incidence of other possible undesired fungal contaminations, such as *Penicillium* spp., *Rhizopus* spp. and/or *Mucor* spp. In other preliminary *in vitro* tests (data not shown), the compatibility was also tested between the fungicide and the biocontrol agent strains (*B. velezensis* BUZ-14, *B. ginsengihumi* S38, and the BPs Amylo-X® and Serenade MAX®: *B. amyloliquefaciens* D747 and *B. subtilis* QST 713, respectively).

## 3. Biocontrol strains and culture conditions

Two BCA bacterial strains were selected and compared as these strains demonstrated high potential antifungal activity against the pathogen as previously published (Calvo-Garrido *et al.,* 2018; Calvo-Garrido *et al.,* 2019; Calvo *et al.,* 2019; Calvo *et al.,* 2017). First, *Bacillus ginsengihumi* S38 was originally isolated from grapevine tissues and maintained in the collection of INRAE Bordeaux-Aquitaine (SAVE) (Haidar *et al.,* 2016b). The other major BCA strain was isolated and characterised in *Bacillus velezensis* by the University of Zaragoza, Spain. Originally collected from different orchards, the *B. velezensis* strain was isolated from peaches, and its antifungal activity was demonstrated against several postharvest moulds, including *B. cinerea* (Calvo *et al.,* 2017). For subcultures, the bacterial strains were grown in 250-mL flasks containing 100 mL of TSB (Tryptone Soy Broth, Oxoid) medium for 24 h at 30 °C. Then, two media were used for BCA growth as described in previous antifungal tests at 30 °C and 150 rpm for 96 h: i) 863 medium (10 g $L^{-1}$ peptone, 10 g $L^{-1}$ yeast extract and 20 g $L^{-1}$ glucose, according to Yánez-Mendizábal *et al.* (2011)) and ii) MOLP medium (casein peptone, 30 g $L^{-1}$; saccharose, 20 g $L^{-1}$; yeast extract, 7 g $L^{-1}$; $K_2HPO_4$, 1.9 g $L^{-1}$; $MgSO_4$, 0.45 g $L^{-1}$; citric acid, 0.01 g $L^{-1}$; $CuSO_4$, 0.001 mg $L^{-1}$; $FeCl_3$, 0.005 mg $L^{-1}$; $NaMoO_4$, 0.004 mg $L^{-1}$; KCl, 0.002 mg $L^{-1}$; $MnSO_4$, 3.6 mg $L^{-1}$; $ZnSO_4$, 0.14 mg $L^{-1}$; $H_3BO_3$, 0.05 mg $L^{-1}$; agar-agar, 15 g $L^{-1}$, according to Ahimou *et al.* (2000)). In addition, grape juice was also prepared for bacterial growth by manually squeezing mature grapes (cv. Thomson Seedless) by filtering the juice through a sieve to remove all large particles. According to the Pasteurization method, the juice was then heated to 80 °C in a water bath. Finally, the pH was adjusted to 5.5 with NaOH.

For the external control treatment, two commercial biocontrol product BPs were used as the standard of comparison, i.e., BPs Serenade Max®, which contains the antagonist *B. subtilis* QST 713 strain, and Amylo-X®, which was developed with cells and metabolites of *B. amyloliquefaciens* D747. These products were included to establish and demonstrate possible differences between the two previous biocontrol agent candidate strains and such biocontrol products that have already been formulated and marketed. This last scope was also partly shared with previously published vineyard experiments exclusively involving the candidate BCA *Bacillus ginsengihumi* strain (Calvo-Garrido *et al.,* 2019). Since the commercial form of these BPs is spray-dried, the addition of water was achieved following the manufacturer's instructions before use.

## 4. Comparison of partial efficacy of the BCA *Bacillus* strains based on the ranking of major modes of action (MoAs)

To characterise and rank by order of importance some of the major modes of action of the BCA strains S38 and BUZ-14 and the commercial bioproducts Amylo-X® and Serenade MAX® against *B. cinerea*, we separately tested the culture (living cells + supernatant), living cells, cell-free supernatant and volatiles obtained in 863 medium. The preparation of each sample fraction is described below:

### 4.1 Bacterial inoculum preparation

- Culture (CUL): the combination between cells and supernatant, i.e., including the metabolites produced by the BCA during subculturing ($10^9$ CFU $mL^{-1}$)
- Living cells (LC): Cells were separated from the supernatant by centrifugation at 5000 *x g* for 10 min and subsequently decanted. The resulting pellet was rediluted with 0.1 % peptone water without antifungal metabolites generated from the previous subculturing process ($10^9$ CFU $mL^{-1}$).
- Cell-free supernatant: The supernatant was separated from living cells by centrifugation at 5000 *x g* for 10 min and subsequently decanted. This fraction exclusively contained the metabolites produced by the BCA.
- Volatile Organic Compounds: volatiles generated by BCAs as antifungal compounds.

### 4.2 BCA treatment and pathogen inoculation on wounded berries

With the exception of the volatiles treatment (see methodology in the next paragraph), the berries were wounded at the equator with a sterile tip pipette (1 × 1 mm) and then treated by immersion for 3 min in the culture fraction as described above (see section 2.4.1). Then, the berries were air-dried for 30 min and placed on an aluminium grid in a closed plastic box at a rate of 15 berries per box (19 cm × 13 cm × 4 cm). The box was used as an incubation humid chamber, which was filled in with a slight layer of water at the bottom to maintain a maximum relative humidity (RH), i.e., 100 % (Figure 1). The inoculation of the pathogen was conducted with a 5-d-old culture mycelium plug in malt agar and placed directly onto the wound (mycelium facing the wound).

To study the VOC mode of action, a new experimental adapted device was developed specifically as follows: plastic boxes (19 cm × 13 cm × 4 cm) included two Petri dishes, each containing 15 mL of 863 medium and placed floating on the water immediately under the aluminium grid. Then, the 863 medium was inoculated with 10 µL of a BCA suspension containing $10^7$ CFU $mL^{-1}$ (10 µL per Petri dish). Finally, the berries were placed at the rate of 15 berries per box on the grid, avoiding any direct contact with the Petri dish and therefore, very importantly, with the agent itself. The berries were then inoculated with the *B. cinerea* plug as described above, allowing us to strictly assess the interaction between the pathogen inoculated in the fruit and the volatiles resulting from the bacteria growth during the bioassay duration (Figure 1).

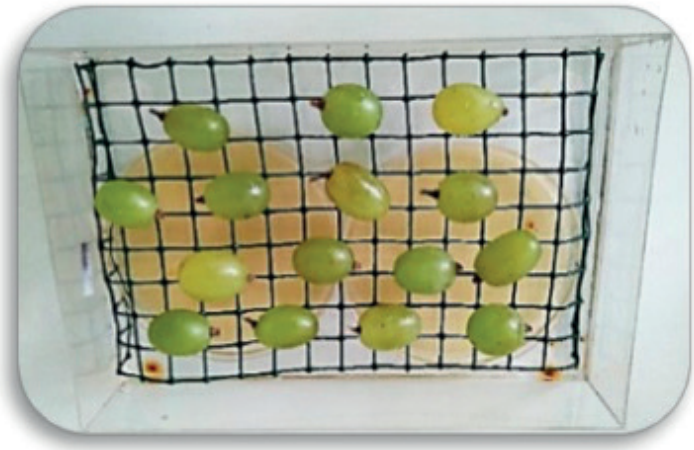

**FIGURE 1.** New experimental bioassay designed specifically for quantifying the anti-fungal effect of the volatile compounds produced by *B. velezensis* BUZ-14 in 863 medium against *B. cinerea* 213 in table grapes (cv. Thomson Seedless).

**TABLE 1.** Overview of the different experimental designs and key experimental features used in this study

| Section[a] | | Temp | RH | Incubation time | BCA | Fraction tested[b] | Replicates |
|---|---|---|---|---|---|---|---|
| Result 1 | Ranking of MoA | 22 °C | 100% | 6 days | BUZ-14 | LC | 30 berries * 3 different days |
| | | | | | S38 | CFS | |
| | | | | | Ser Max® | Cul | |
| | | | | | Amylo-x® | VOCs | |
| Result 2 | VOCs *in vivo* | 22 °C | 100% | 6 days | BUZ-14 | VOCs | 30 berries * 3 different days |
| | | | | | S38 | | |
| | | | | | Amylo-x® | | |
| Result 3 | Temperature | 22 °C | 100% | 6 days | BUZ-14 | LC | 30 berries * 3 different days (pool) |
| | | | | | S38 | CFS | |
| | | 27 °C | | 10 days | Ser Max® | Cul | |
| | | | | | Amylo-x® | VOCs | |
| Result 4 | RH | 22 °C | 85–95 % | 6 days | BUZ-14 | LC | 30 berries * 3 different days (pool) |
| | | | | | S38 | CFS | |
| | | | | | Ser Max® | Cul | |
| | | | | | Amylo-x® | VOCs | |

[a]MoA: Mode of Action; VOCs: Volatile Organic Compounds; RH: Relative Humidity.

[b]LC: Living Cells; CFS: Cell-free Supernatant; Cul: Culture; VOCs: Volatile Organic Compounds

After incubation at 22 °C for 6 days, the following *B. cinerea* severity scale was used for a visual assessment of the percentage of the rotten surface by the pathogen on a per berry basis: 0, 10, 20, 33, 50, 66, 80, 90 and 100 % of berry infected. A first control treatment (positive control) corresponding to non-BCA inoculation was included. Another negative control that lacked *B. cinerea* 213 inoculation was also included in the experiment. The results were expressed as the percentage of disease reduction by comparing the efficacy of BCA treatments with the positive control. Thirty berries per treatment (15 berries per box) were used, and the experiment was performed in triplicate in three separate periods of time (Table 1).

Finally, initial counts (after immersion) and counts after 6 days of incubation at 22 °C were conducted to determine the growth of the biocontrol agent in the berry. Briefly, 10 grams from five random berries previously inoculated with the CUL or LC were selected and intensively agitated (Stomacher 400 Circulator laboratory blender, London, England) for 120 s at 260 rpm. The resulting suspension was diluted in 0.1 % sterile peptone water, plated on TSA (Tryptone Soy Agar, Oxoid Ltd; Basingstoke, Hampshire, UK) plates and counted after 24 h at 30 °C. The results were expressed as CFU $g^{-1}$.

### *5. In vivo* activity of antifungal VOCs: effect of the culture medium

Since volatiles production may depend on the nutrients available and metabolised during BCA bacterial growth and/or multiplication, the influence of the culture medium was investigated on the production of VOC antifungal volatiles and on the antifungal activity of each BCA. Three liquid media, including 863, MOLP and grape juice (section 2.3), were used for the two new BCA candidates, i.e., BUZ-14 and S38, and the commercial bioproduct Amylo-X® was included as the standard of comparison, discarding Ser Max® due to generally worse activity than Amylo-X® and being the latter more recently BP commercially available (Calvo-Garrido *et al.,* 2019). The inoculation procedure, experimental design and efficacy assessment were previously described in section 2.4.2. Berries were incubated at 22 °C for 6 days. Thirty berries were used per agent, and the experiment was conducted in triplicate (Table 1).

## 6. Influence of environmental factors on the biocontrol activity against *B. cinerea* in table grapes

### 6.1 Temperature effects on BCA activity against B. cinerea

The boxes containing the four strains (BUZ-14, S38, Amylo-X® and Serenade Max®) were incubated under two different conditions, namely, 22 °C for 6 days and 27 °C for 10 days, corresponding to optimal pathogen and bacteria growth conditions, respectively. To analyse the results on the influence of the temperature, we pooled the data at 22 °C and all data at 27 °C with no differences between the forms (Cul, LC, CFS or VOCs). The objective was to establish and demonstrate the main effect of the temperature independent of the BCA form.

### 6.2 Optimal and suboptimal RH conditions on BCA activity against B. cinerea

The antifungal activity of the four strains was compared under two different RH conditions using the culture, living cells, CFS and VOCs of the bacterial strains (Table 1). The berries were placed in plastic boxes as described in section 2.4, and the boxes were stored in a controlled climatic chamber at 22 °C. However, a methodological difference was introduced between completely closed boxes (RH at 100%) versus semiclosed boxes where the lid was slightly perforated by using an awl to decrease the RH in the box by approximately 85–95 %. The RH in semiclosed boxes was measured through a HOBO system (HOBO U30 Weather Station, Onset Computer Corporation, USA) by monitoring the temperature and RH during the incubation time (6 days at 22 °C). The results were expressed as the reduction of the infection severity according to the previous scale (section 2.4). Thirty berries per treatment (15 per box) were used, and the experiment was performed in triplicate in three separate periods of time. For the temperature effect (2.6.1), we used all the data per BCA and no differences among the BCA forms (Table 1).

## 7. Statistical analyses

Data were analysed using the SPSS software package for Windows version 26.0 (SPSS Inc., Chicago, IL, USA). Differences in the mean values of parameters were tested by LSD Student's *t*-test and one-way ANOVA. Tukey's honestly significant difference (HSD) post hoc test and Games–Howell test ($P < 0.05$) were used in cases where equal variances and sample sizes were assumed and not assumed, respectively.

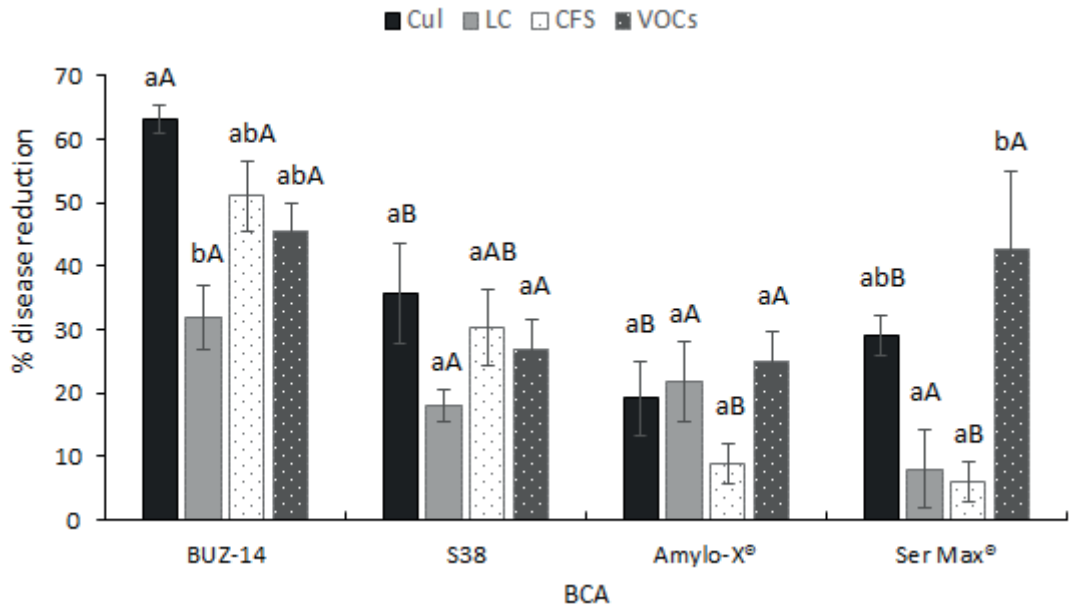


**FIGURE 2.** Percentage of B. cinerea reduction in table grapes (cv. Thomas Seedless) according to the BCA antifungal effect of the culture (Cul), living cells (LC), cell-free supernatant (CFS) or volatile organic compounds (VOCs) produced by *B. velezensis* BUZ-14, *B. ginsengihumi* S38, and the commercial BPs Amylo-X® and Serenade MAX®.

Vertical bars indicate the standard error of the mean. Different lowercase letters indicate significant intrastrain differences (same BCA), whereas uppercase letters indicate significant interstrain differences (among the different BCAs and same fraction) at $P < 0.05$.

## RESULTS AND DISCUSSION

### 1. Modes of action and associated efficacy ranking of the four BCA strains: antifungal activity of culture, living cells, CFS and VOCs

To better characterise and rank the major mode(s) of action (MOAs) of the four bacterial strains tested based on efficacy, the antagonist efficacy was quantified for culture (Cul), living cells (LC), cell-free supernatant (CFS) and volatile organic compounds (VOCs). Particularly, this analysis was necessary to elucidate whether the antifungal activity against *B. cinerea* was mostly due to secondary metabolites produced during the bacteria premultiplication process (subculturing), by living cells when directly interacting with the pathogen at the fruit surface, or both possibilities (Figure 2).

When comparing the strains, the BUZ-14 strain culture reduced the growth of *B. cinerea* up to 60 %, which was similar to that noted for supernatant and volatiles (50 % and 45 %, respectively) without any significant difference (Figure 2). However, a significant difference was noted between living cells and culture since LC reduced the pathogen growth to a lesser extent, i.e., only 30 %. This finding might be attributed to the fact that BUZ-14 exhibited a very low capacity to grow on this specific plant host tissue (Calvo *et al.,* 2019). Regarding the S38 strain, no significant difference was detected among the treatments. However, compared with BUZ-14, a similar trend was confirmed, demonstrating maximal antifungal activity of the culture and minimal antifungal activity due to the living cells. The pathogen reduction was approximately half compared with that due to culture BUZ-14, indicating reduced antifungal activity of the S38 strain against *B. cinerea* in table grapes. Regarding BP Amylo-X®, no significant difference was detected between the BCA forms tested. The metabolite activity (CFS) of the two BPs Amylo-X® and Serenade Max® was very low, i.e., less than 10 % (Figure 2), whereas the volatiles produced by Serenade Max® reduced the disease up to 43 %.

By separately considering every BCA form, the BUZ-14 culture was the most active against *B. cinerea* by significantly reducing the disease by up to 60 % compared with all the other BCA cultures (Figure 2). Overall, the culture form always exhibited minimal antifungal activity of 20 % (minimal in Amylo-X®). Regarding living cells, no significant differences between the BCAs were demonstrated with a relatively low disease reduction, i.e., not exceeding 25 %. This finding clearly indicates that living cells was not the most important fraction and/or mode of action to account for the biocontrol potential of these BCAs. Interestingly, the presence of living cells during the interaction with the pathogen may not be a key factor to assure the overall antagonist efficacy of these BCA strains.

Regarding cell-free supernatant) activity and the associated antibiosis MoA, the lowest disease reduction activity was observed for the two BPs Amylo-X® and Serenade Max® (Figure 2). For both commercial products, the disease reduction was less than 10 %, which differs from the 30–50 % reduction due to the CFS of the two pure strains BUZ-14 and S38 (not formulated). This finding may be attributed to this difference in the formulation process that could diminish the concentration of active antifungal substances (Guijarro *et al.,* 2006; Yánez-Mendizábal *et al.,* 2012). However, the difference could also be related to the bacterial metabolite profiles that may depend on the composition of the culture medium. Finally, the bacterial agents did not exhibit any significant differences in terms of volatiles activity given that the results were very consistent and relatively homogeneous among the four bacterial strains. Quite importantly, the disease reductions were greater than 30 % in all cases, demonstrating the importance of this specific MoA in the overall BCA strain efficiency.

The BCAs were also quantified in the berry to evaluate their developmental capacity on the fruit surface. The results showed that none of the biocontrol strains were able to grow in the berry (data not shown). This notion may account for the poor activity of LC as previously shown (Figure 2). Therefore, the performance of the metabolites produced during the BCA production process before application onto the fruit is important given that the production of antifungal compounds *in planta* should be practically discarded.

This type of assay is typically used to determine and characterise the major MOA(s) underlying BCA efficacy. Our results are consistent with the previously published characterisation of the BUZ-14 strain since it has been described as a lipopeptide and volatile producer (Calvo *et al.,* 2019; Calvo *et al.,* 2020). However, the S38 strain has not been specifically assessed the production of antifungal metabolites, although we consider these studies essential to elucidate more clearly the mode of action of this strain and to enhance the comparison between S38 and BUZ-14.

  

It is thought to carry on this research after the publication of the present results and to achieve a better comparison of strains from a production of hydrosoluble and volatile metabolites term. Furthermore, Calvo-Garrido *et al.* (2014a) showed that the S38 strain significantly reduced grey mould in vineyards up to 72–75 %. In the present laboratory study, the S38 culture exhibited an efficacy of approximately 35 %. However, the relationship between field results and laboratory results remains a very difficult overall and key issue in the biocontrol field of study. For example, we used a virulent *Botrytis* strain 213 (section 2.2) that was quite different from the complex pathogen population structure typically observed in the vineyard (Walker *et al.,* 2015). We inoculated the pathogen in the berry in a manner that significantly favoured its aggressiveness (mycelium plug/wound) compared with other infection pathways, e.g., conidial pathways, potentially leading to an increased BCA efficacy in the vineyard. The recently developed BP Amylo-X® significantly reduced *B. cinerea* in vineyards in approximately 60–70 % of the experiments performed by Calvo-Garrido *et al.* (2019). In the same field study, a similar success frequency was observed for Serenade Max®, which is consistent with our results given that the two BPs were not significantly differentiated in the present laboratory study. It may also be important to consider the difference in the bacteria behaviour according to the precise location and associated microclimate variations in the grapevine cluster zone (bunch) compared with single detached berries as performed in our laboratory bioassays. Furthermore, antifungal substances may not only have a direct antimicrobial effect and also playing a significant role the inducing systemic acquired resistance to confer protection of the fruit (not studied in this investigation). Finally, based on our good efficacy results, novel assays in vineyards with BUZ-14 are underway to confirm such comparisons between the candidate strains using commercial bioproducts as a standard of comparison.

### *2. In vivo* activity of antifungal VOCs: effect of the culture medium

Scientific studies regarding the activity of bacterial VOCs against phytopathogens in cultures are limited. Developing new original protocols and methodologies is crucial to test these gaseous metabolites with great antifungal potential activity as previously demonstrated *in vitro* (Chen *et al.,* 2008; Xie *et al.,* 2018; Zhou *et al.,* 2019)). Moreover, the importance of nutrients has been demonstrated in the production of antifungal compounds (Gotor-Vila *et al.,* 2017a; Raza *et al.,* 2016); therefore, this section focused on identifying a suitable culture medium to improve the development of more powerful VOCs.

When grown in MOLP medium, the S38 strain reduced the development of the pathogen by 88.7 % (Figure 3). Significant differences were demonstrated by comparison with the two other strains on the same medium. For S38, three media led to significantly different disease reduction percentages, which were lower in 863 and grape juice at 31.9 and 14.5 %, respectively. Thus, grape juice was the least favourable medium for antifungal VOC production with this strain. Interestingly, the same result was noted with the other experimental BCA strain BUZ-14, which exhibited significantly increased disease reduction rates on both 863 and MOLP media. The two BCA strains BUZ-14 and Amylo-X® grow in the more favourable MOLP medium at similar rates and achieved similar disease reduction rates of 49.2 % and 54.4 %, respectively. The reduction rate reached by Amylo-X® in grape juice was very similar to that in the two other media, i.e., 863 and MOLP.

The nutrients in the three culture media are different, which might lead to distinct control efficacy of plant diseases (Calvo *et al.,* 2020; Gotor-Vila *et al.,* 2017a; Raza *et al.,* 2016). MOLP is the richest medium; therefore, higher efficacy results are expected. Accordingly, the S38 strain demonstrated a significant difference between MOLP and 863. For BUZ-14, MOLPA (MOLP + agar-agar) was significantly better than 863 in the reduction of *Monilinia* spp. *in vitro*. This finding was also confirmed in biotests using apricots and for *B. amyloliquefaciens* I3 against *B. cinerea* in the same study. Nevertheless, this finding was not significantly confirmed in the present study for both BUZ-14 and Amylo-X®; however, the basic results (Figure 3) tend to confirm the overall superiority of the MOLP medium. However, according to the results reported by Calvo *et al.* (2020) with MOLPA, the difference between solid and liquid media can also represent a significant factor that should be taken into account when developing such bioassays and comparing the outcome. As displayed in Figure 2, the VOCs produced in 863 liquid medium did not show significant differences among the four BCAs, and this notion is also confirmed in this section (Figure 3). Finally, the use of grape juice as a grape-like medium was not favourable to volatiles production.

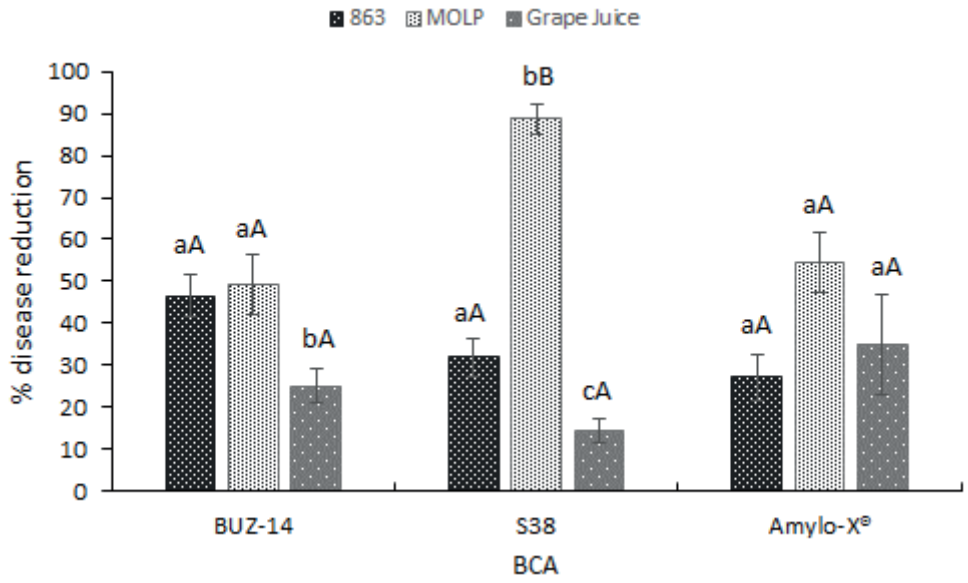


**FIGURE 3.** The percentage of B. cinerea reduction in table grapes (c.v. Thomas Seedless) treated with VOCs produced by the high potential BCAs BUZ-14 and S38 and BP Amylo-X® grown in culture media 863, MOLP and grape juice.

Vertical bars indicate the standard error of the mean. Different lowercase letters indicate significant differences among culture media in the same BCA, whereas uppercase letters represent significant differences among BCAs for the same culture medium at $P < 0.05$.

The three BCA strains grow in the medium; however, likely, the remaining nutrients in the juice were not sufficient to allow the active VOCs to more markedly reduce the growth of *B. cinerea* 213. In contrast to the results in section 3.1 where none of the BCAs were able to proliferate in the berry tissue, the higher pH (5.5) balance due to the addition of NaOH (section 2.3) should have facilitated their proliferation (Calvo *et al.,* 2017).

### 3. Effect of temperature on the *in vivo* overall efficacy of the 4 different BCA *Bacillus* species

In addition to nutrients affecting the production of antifungal metabolites, other key environmental parameters, such as temperature or relative humidity, affect the BCA-pathogen interaction. In these bioassays, the temperature has been investigated as a crucial parameter. Temperature significantly influences both the pathogen, including latent and more or less aggressive pathogens (Ciliberti *et al.,* 2015a; Ciliberti *et al.,* 2015b), and the BCA strain as this parameter affects its multiplication and/or stabilization of its metabolites. The optimal temperature between the pathogen and the BCA is generally not the same, interfering with the antifungal activity of the antagonist (Fedele *et al.,* 2020; Kredics *et al.,* 2003; Mukherjee and Raghu, 1997). Thus, two key temperatures were tested that are optimal for each microorganism, namely 22 °C and 27 °C for the pathogen and the BCA bacterial strains, respectively. Such temperatures also correspond to typical vineyard conditions both in Spain and France during grapevine development (Pieri and Fermaud, 2005). As shown in Figure 4, none of the BCA strains displayed significant differences between temperatures. At 22 °C, BUZ-14 inhibited 46.9 % of the lesion diameter, demonstrating the highest reduction among all BCAs. The S38 strain showed a 32.4 % reduction, whereas the two BPs Amylo-X® and Serenade Max® only achieved approximately 18.0 % reduction. A similar pattern was observed at 27 °C; however, the two BPs Amylo-X® and Serenade Max® exhibited increased inhibition, increasing the reduction of grey mould up to 28.5 and 35.8 %, respectively.

Significant variations in temperature as well as other climatic conditions, such as RH, always occur in vineyards. However, few studies have taken into account BCA behaviour during the season in the context of various unusual climatological conditions, notably abiotic stress, despite the fact that great differences may occur between microorganisms, such as bacteria and fungi (Calvo-Garrido *et al.,* 2014a; Fedele *et al.,* 2020; Haidar *et al.,* 2016b; Longa *et al.,* 2009). Moreover, the expression of typical biocontrol factors may

be affected, e.g., the production of antibiotic compounds by *Pseudomonas fluorescens* CHA0 was significantly reduced at 35 °C compared with 30 °C (Humair *et al.,* 2009). Furthermore, the thermal effect on microorganism survival was also highlighted in the plant tissue or soil (Calvo-Garrido *et al.,* 2014a; Gotor-Vila *et al.,* 2017b; Longa *et al.,* 2009). However, the influence of temperature on the reduction of *B. cinerea* in table grapes was not observed in our study. The temperature typically influences the growth of microorganisms and their survival. Regarding antagonist microorganisms in the *Bacillus* genus (as in the present study), we consider that survival is very important, even under severe abiotic stress conditions, due to the formation of endospores (forms of resistance). As shown in 3.1, these BCAs do not serve as major MOAs on living

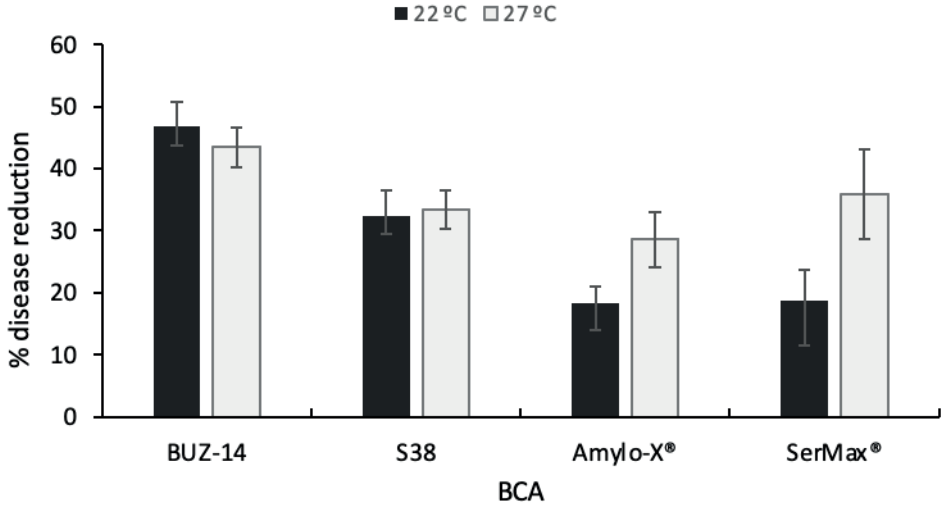


**FIGURE 4.** Percentage of *B. cinerea* reduction in wounded table grapes incubated at 22 °C and 27 °C for the antagonist bacteria strains BUZ-14 and S38 as well as the commercial BPs Amylo-X® and Serenade Max®.

Vertical bars indicate the standard error of the mean (SEM). No letters above the bars indicate no significant differences between the two temperatures for each BCA at $P < 0.05$. The results are pooled data of the reductions obtained with the following four fractions tested at both temperatures: CUL, LC, CFS and VOCs.

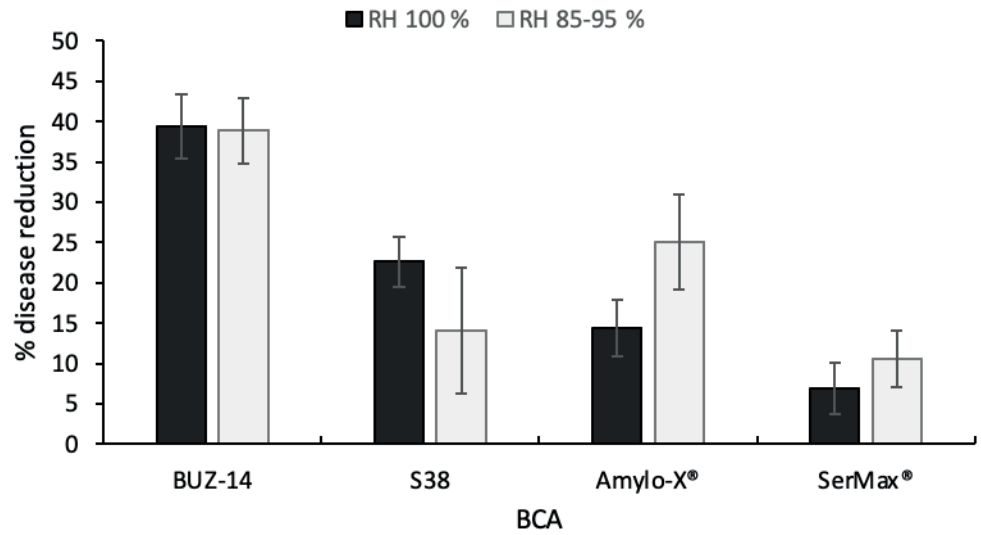


**FIGURE 5:** Percentage of *B. cinerea* reduction in wounded table grapes incubated at 100 % and 85–95 % RH for the antagonist bacteria strains BUZ-14 and S38 as well as the commercial BPs Amylo-X® and Serenade Max®.

Vertical bars indicate the standard error of the mean (SEM). No letters above the bars indicate no significant differences between the two RHs for each BCA at $P < 0.05$. The results are pooled data of the reductions obtained with the four fractions tested at both temperatures: CUL, LC, CFS and VOCs.

  

cells, which is presumably the fraction most affected by temperature. The metabolites display better antifungal activity, and the influence of the temperature on such compounds is quite less significant compared with living cells. It is possible that the difference between the two temperatures assessed (5 °C) should be greater to obtain significant differences between pathogen and BCA optimum temperature.

### 4. Effect of moisture (optimal RH/suboptimal RH) on the *in vivo* overall efficacy of the 4 different *Bacillus* BCA species against *B. cinerea* in table grapes

The efficacy of the different bacteria was further compared under optimal (100 %) *versus* suboptimal (85–95 %) relative humidity conditions for both the pathogen and the bacterial agents. The efficacy of applications in the field depends upon the environmental conditions, notably temperature and moisture, that affect BCA growth and survival as previously mentioned (Fedele *et al.,* 2020). The effect of some extreme climatic conditions has been studied for specific candidates in interaction with the pathogen, including the effect of heavy rain or extreme temperatures on survival, conservation and/or persistence at the plant surface (Calvo-Garrido *et al.,* 2014b; Fedele *et al.,* 2020). However, lower, suboptimal RH conditions have rarely been tested and may interfere with BCA efficacy because lower RH may also limit the growth, infection and sporulation of *B. cinerea* (Ciliberti *et al.,* 2015a; Ciliberti *et al.,* 2015b). Thus, we hypothesised that such suboptimal RH conditions might improve BCA efficacy by delaying/weakening the appearance of the disease (Gotor-Vila *et al.,* 2017c; Lahlali and Jijakli, 2009). However, no significant differences were observed in any of the BCAs tested between optimal and suboptimal relative humidity conditions (Figure 5). Moreover, under optimal and suboptimal RH conditions, the antagonist bacterium BUZ-14 exhibited the highest reduction of the disease (39.4 and 38.9 %, respectively), which confirmed previous results (section 3.1).

## CONCLUSIONS

The main mode of action observed in table grapes against the pathogen involves antibiosis by hydrosoluble and volatile metabolites as well as their combination. The two kinds of antibiosis, caused the greatest inhibition, reducing the disease by more than 60 % in the case of BUZ-14 (culture). Interestingly, the significant influence of the culture medium on VOC production has also been demonstrated. Strain S38 reduced *B. cinerea* growth by 90 % when grown in a MOLP-rich medium. As another major take-home message, for all the *Bacillus* strains tested in the present study, the antifungal activity of living cells, which were separated from their culture medium, was quite limited. Furthermore, we demonstrated that the BCAs were unable to grow and multiply in the berry. Therefore, it may be hypothesised that living cells in the *Bacillus* genus might not be required as a key fraction in the bioproducts for the application of such BCAs against the pathogen to the grapevine. Finally, the environmental conditions tested, including temperature and RH, did not significantly affect the reduction of grey mould in table grapes. The development of new pre- and postharvest trials in berries and/or vineyards based on biofumigation or active packaging will be further studied based on these recent findings and knowledge about the interaction between these biocontrol agents and *B. cinerea*.

## ACKNOWLEDGEMENTS

The authors are grateful to the Bordeaux Wine Council (CIVB) for the recent research projects for the development of new biocontrol agents and strategies against *B. cinerea*, and to the INRAE UMR-1065-SAVE technical staff. H. Calvo has been a beneficiary of the Ibercaja-CAI and Campus Iberus research stay fellowship. Authors also acknowledge to the Bordeaux INRAE-IFV Mixt Technology Unit (UMTseven). Before submission, the present paper was edited by Elsevier Language Editing Services.

## REFERENCES

Ahimou, F., Jacques, P., & Deleu, M. (2000). Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity. *Enzyme Microbial Technology*, *27*(10), 749–754. https://doi.org/10.1016/S0141-0229(00)00295-7

Almenar, E., Catala, R., Hernandez-Muñoz, P., & Gavara, R. (2009). Optimization of an active package for wild strawberries based on the release of 2-nonanone. *LWT - Food Science and Technology*, *42*(2), 587–593. https://doi.org/10.1016/j.lwt.2008.09.009

Altindag, M., Sahin, M., Esitken, A., Ercisli, S., Guleryuz, M., Donmez, M.F., & Sahin, F. (2006). Biological control of brown rot (*Monilinia laxa* Ehr.) on apricot (*Prunus armeniaca* L. cv. Hacihaliloglu) by *Bacillus, Burkholderia,* and *Pseudomonas* application under *in vitro* and *in vivo* conditions. *Biological Control, 38*(3), 369–372. https://doi.org/10.1016/j.biocontrol.2006.04.015

  

Arrebola, E., Sivakumar, D., & Korsten, L. (2010). Effect of volatile compounds produced by *Bacillus* strains on postharvest decay in citrus. *Biological Control, 53*(1), 122–128. https://doi.org/10.1016/j.biocontrol.2009.11.010

Calvo-Garrido, C., Haidar, R., Roudet, J., Gautier, T., & Fermaud, M. (2018). Pre-selection in laboratory tests of survival and competition before field screening of antagonistic bacterial strains against *Botrytis* bunch rot of grapes. *Biological Control, 124,* 100–111. https://doi.org/10.1016/j.biocontrol.2018.05.006

Calvo-Garrido, C., Roudet, J., Aveline, N., Davidou, L., Dupin, S., & Fermaud, M. (2019). Microbial antagonism toward *Botrytis* Bunch Rot of grapes in Multiple Field Tests using one *Bacillus ginsengihumi* strain and formulated Biological Control Products. *Frontiers in Plant Science.* 10:105. https://doi.org/10.3389/fpls.2019.00105

Calvo-Garrido, C., Teixidó, N., Roudet, J., Viñas, I., Usall, J., & Fermaud, M. (2014a). Biological control of *Botrytis* bunch rot in Atlantic climate vineyards with *Candida sake* CPA-1 and its survival under limiting conditions of temperature and humidity. *Biological Control, 79*, 24–35. https://doi.org/10.1016/j.biocontrol.2014.05.011

Calvo-Garrido, C., Viñas, I., Usall, J., Rodríguez-Romera, M., Ramos, M.C., & Teixidó, N. (2014b). Survival of the biological control agent *Candida sake* CPA-1 on grapes under the influence of abiotic factors. *Journal of Applied Microbiology*, *117*(3), 800-811. https://doi.org/10.1111/jam.12570

Calvo, H., Marco, P., Blanco, D., Oria, R., & Venturini, M.E. (2017). Potential of a new strain of *Bacillus amyloliquefaciens* BUZ-14 as a biocontrol agent of postharvest fruit diseases. *Food Microbiology, 63*, 101–110. https://doi.org/10.1016/j.fm.2016.11.004

Calvo, H., Mendiara, I., Arias, E., Blanco, D., & Venturini, M.E. (2019). The role of iturin A from *B. amyloliquefaciens* BUZ-14 in the inhibition of the most common postharvest fruit rots. *Food Microbiology, 82*(10), 62–69. https://doi.org/10.1016/j.fm.2019.01.010

Calvo, H., Mendiara, I., Arias, E., Gracia, A.P., Blanco, D., & Venturini, M.E. (2020). Antifungal activity of the volatile organic compounds produced by *Bacillus velezensis* strains against postharvest fungal pathogens. *Postharvest Biology and Technology, 166*, 111208. https://doi.org/10.1016/j.postharvbio.2020.111208

Chen, H., Xiao, X., Wang, J., Wu, L., Zheng, Z., & Yu, Z. (2008). Antagonistic effects of volatiles generated by *Bacillus subtilis* on spore germination and hyphal growth of the plant pathogen, *Botrytis cinerea*. *Biotechnology Letters, 30*(5), 919–923. https://doi.org/10.1007/s10529-007-9626-9

Chen, X.-H., Vater, J., Piel, J., Franke, P., Scholz, R., Schneider, K., Koumoutsi, A., Hitzeroth, G., Grammel, N., Strittmatter, A.W., Gottschalk, G., Sussmuth, R.D., & Borriss, R. (2006). Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB42. *Journal of Bacteriology, 188*(11)*,* 4024–4036. https://doi.org/10.1128/JB.00052-06

Chowdhury, S.P., Hartmann, A., Gao, X.W., & Borriss, R. (2015). Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42 - A review. *Frontiers in Microbiology, 6*(780):780*,* 1–11. https://doi.org/10.3389/fmicb.2015.00780

Ciliberti, N., Fermaud, M., Languasco, L., & Rossi, V. (2015a). Influence of fungal strain, temperature, and wetness duration on infection of grapevine inflorescences and young berry clusters by *Botrytis cinerea*. *Phytopathology, 105*(3)*,* 325–333. https://doi.org/10.1094/PHYTO-05-14-0152-R.

Ciliberti, N., Fermaud, M., Roudet, J., & Rossi, V. (2015b). Environmental conditions affect *Botrytis cinerea* infection of mature grape berries more than the strain or transposon genotype. *Phytopathology, 105*(8)*,* 1090–1096. https://doi.org/10.1094/PHYTO-10-14-0264-R

Coleman, M.D., O'Neil, J.D., Woehrling, E.K., Ndunge, O.B.A., Hill, E.J., Menache, A., & Reiss, C.J. (2012). A preliminary investigation into the impact of a pesticide combination on human neuronal and glial cell lines *in vitro*. *PLoS One 7*(8), 42768. https://doi.org/10.1371/journal.pone.0042768

Deytieux-Belleau, C., Geny, L., Roudet, J., Mayet, V., Doneche, B., & Fermaud, M. (2009). Grape berry skin features related to ontogenic resistance to *Botrytis cinerea*. *European Journal of Plant Pathology, 125*(555), 551–563. https://doi.org/10.1007/s10658-009-9503-6

Di Francesco, A., Martini, C., & Mari, M. (2016). Biological control of postharvest diseases by microbial antagonists: how many mechanisms of action? *European Journal of Plant Pathology 145,* 711–717. https://doi.org/10.1007/s10658-016-0867-0

Droby, S., Wisniewski, M., Teixidó, N., Spadaro, D., & Jijakli, M.H. (2016). The science, development, and commercialization of postharvest biocontrol products. *Postharvest Biology and Technology, 122,* 22–29. https://doi.org/10.1016/j.postharvbio.2016.04.006

Elad, Y., Vivier, M., & Fillinger, S. (2016a). *Botrytis*, 745 the Good, the Bad and the Ugly. In: Botrytis *746 – the Fungus, the pathogen and its management in agricultural systems*, eds. S. Fillinger & 747 Y. Elad. (Cham: Springer International Publishing), 1-15.

Elad, Y., Pertot, I., Cotes Prado, A., and Stewart, A. (2016b). Plant hosts of *Botrytis* spp.. In: Botrytis *– the Fungus, the pathogen and its management in agricultural systems*. *eds. S. Fillinger & Y. Elad.* (Springer International Publishing), 413-486.

Esteve-Turrillas, F.A., Agulló, C., Abad-Somovilla, A., Mercader, J. V., & Abad-Fuentes, A. (2016). Fungicide multiresidue monitoring in international wines by immunoassays. *Food Chemistry, 196,* 1279–1286. https://doi.org/10.1016/j.foodchem.2015.10.102

Farace, G., Fernandez, O., Jacquens, L., Coutte, F., Krier, F., Jacques, P., Clément, C., Barka, E.A., Jacquard, C., & Dorey, S. (2015). Cyclic lipopeptides from *Bacillus subtilis* activate distinct patterns of defence responses in grapevine. *Molecular Plant Pathology, 16*(2)*,* 177–187. https://doi.org/10.1111/mpp.12170

Fedele, G., Bove, F., González-Domínguez, E., & Rossi, V. A. (2020). Generic model accounting for the interactions among pathogens, host plants, biocontrol agents, and the environment, with parametrization for *Botrytis cinerea* on Grapevines. *Agronomy, 10*(2), 222. https://doi:10.3390/agronomy10020222

Gotor-Vila, A., Teixidó, N., Di Francesco, A., Usall, J., Ugolini, L., Torres, R., & Mari, M. (2017a). Antifungal effect of volatile organic compounds produced by *Bacillus amyloliquefaciens* CPA-8 against fruit pathogen decays of cherry. *Food Microbiology*, *64,* 219–225. https://doi.org/10.1016/j.fm.2017.01.006

Gotor-Vila, A., Teixidó, N., Casals, C., Torres, R., De Cal, A., Guijarro, B., & Usall, J. (2017b). Biological control of brown rot in stone fruit using *Bacillus amyloliquefaciens* CPA-8 under field conditions. *Crop Protection, 102,* 72–80. https://doi.org/10.1016/j.cropro.2017.08.010

Gotor-Vila, A., Usall, J., Torres, R., Ramos, M.C., & Teixidó, N. (2017c). Environmental stress responses of the *Bacillus amyloliquefaciens* CPA-8-formulated products on nectarines and peaches. *Scientia Horticuturae, 225, 359–365.* https://doi.org/10.1016/j.scienta.2017.07.015

Guijarro, B., Larena, I., Melgarejo, P., & De Cal, A. (2006). Effect of drying on conidial viability of *Penicillium frequentans*, a biological control agent against peach brown rot disease caused by *Monilinia* spp. *Biocontrol Science and Technology, 16*(3), 257–269. https://doi.org/10.1080/09583150500335897

Hahn, M. (2014). The rising threat of fungicide resistance in plant pathogenic fungi: *Botrytis* as a case study. *Journal of Chemical Biology, 7*(4), 133–141. https://doi.org/10.1007/s12154-014-0113-1

Haidar, R., Fermaud, M., Calvo-Garrido, C., Roudet, J., & Deschamps, A. (2016a). Modes of action for biological control of *Botrytis cinerea* by antagonistic bacteria. *Phytopathologia Mediterranea, 55*(3), 13−34. https://doi.org/10.14601/Phytopathol_Mediterr-18079

Haidar, R., Deschamps, A., Roudet, J., Calvo-Garrido, C., Bruez, E., Rey, P., & Fermaud, M. (2016b). Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine. *Biological Control, 92,* 5565. https://doi.org/10.1016/j.biocontrol.2015.09.003

Haidar, R., Roudet, J., Bonnard, O., Dufour, M. C., Corio-Costet, M. F., Fert, M., Gautier, T., Deschamps, A., & Fermaud, M. (2016c). Screening and modes of action of antagonistic bacteria to control the fungal pathogen *Phaeomoniella chlamydospora* involved in grapevine trunk diseases. *Microbiological Research, 192,* 172184. https://doi.org/10.1016/j.micres.2016.07.003

Hang, N.T.T., Soon-Ok, O., Gyoung, H.K., Hur, J., & Koh, Y. (2005). *Bacillus subtilis* S1-0210 as a biocontrol agent against *Botrytis cinera* in strawberries. *The Plant Pathology Journal, 21*(1), 59-63. https://doi.org/10.5423/PPJ.2005.21.1.059

Humair, B., González, N., Mossialos, D., Reimmann, C., & Haas, D. (2009). Temperature-responsive sensing regulates biocontrol factor expression in *Pseudomonas fluorescens* CHA0. *The ISME Journal, 3*(8), 955–965. https://doi.org/10.1038/ismej.2009.42

Index Acta Biocontrôle (2018). *ACTA Editions (Les Instituts Techniques Agricoles)*. Paris, ISBN 978 2 8579 4304 4. CS 20016, 61438, Flers cedex, France. 374pp.

Köhl, J., Kolnaar, R., & Ravensberg, W.J. (2019). Mode of action of microbial Biological Control Agents against Plant Diseases: Relevance beyond efficacy. *Frontiers in Plant Science, 10,* 1–19. https://doi.org/10.3389/fpls.2019.00845

Kredics, L., Antal, Z., Manczinger, L., Szekeres, A., Kevei, F., & Nagy, E. (2003). Influence of environmental parameters on *Trichoderma* strains with biocontrol potential. *Food Technology and Biotechnology, 41*, 37–42.

Ky, I., Lorrain, B., Jourdes, M., Pasquier, G., Fermaud, M., & Geny, L. (2012). Assessment of grey mould (*Botrytis cinerea*) impact on phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages. *Australian Journal of Grape and Wine Research, 18,* 21526. https://doi.org/10.1111/j.1755-0238.2012.00191.x

Lahlali, R., & Jijakli, M.H. (2009). Enhancement of the biocontrol agent *Candida oleophila* (strain O) survival and control efficiency under extreme conditions of water activity and relative humidity. *Biological Control, 51,* 403–408. https://doi.org/10.1016/j.biocontrol.2009.07.014

Landa, B.B., Navas-Cortés, J.A., & Jiménez-Díaz, R.M. (2004). Influence of temperature on plant-rhizobacteria interactions related to biocontrol potential for suppression of fusarium wilt of chickpea. *Plant Pathology, 53,* 341–352. https://doi.org/10.1111/j.0032-0862.2004.01008.x

Leroux, P. (2004). "Chemical control of *Botrytis* and its resistance to chemical fungicides," in *Botrytis*: Biology, pathology and control, eds Y. Elad, B. Williamson, P. Tudzynski, and N. Delen (Dordrecht: Kuwer academic publishers), 195–222.

Longa, C.M.O., Savazzini, F., Tosi, S., Elad, Y., & Pertot, I. (2009). Evaluating the survival and environmental fate of the biocontrol agent *Trichoderma atroviride* SC1 in vineyards in northern Italy. *Journal of Applied Microbiology, 106*(5), 1549–1557. https://doi.org/10.1111/j.1365-2672.2008.04117.x

MAPAMA (2018). *Ministerio de Agricultura, Pesca y Alimentación*. Registro de Productos Fitosanitarios.

  

Martinez, F., Blancard, D., Lecomte, P., Levis, C., Dubos, B., & Fermaud, M. (2003). Phenotypic differences between *vacuma* and *transposa* subpopulations of *Botrytis cinerea*. *European Journal of Plant Pathology, 109*(5), 47988.

Mukherjee, P.K., & Raghu, K. (1997). Effect of temperature on antagonistic and biocontrol potential of *Trichoderma* sp. on *Sclerotium rolfsii*. *Mycopathologia, 139*(3), 151–155. https://doi.org/10.1023/A:1006868009184

Ongena, M., & Jacques, P. (2008). *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends in Microbiology, 16*(3), 115–125. https://doi.org/10.1016/j.tim.2007.12.009

Paulitz, T., Nowak-Thompson, B., Gamard, P., Tsang, E., & Loper, J. (2000). A novel antifungal furanone from *Pseudomonas aureofaciens*, a biocontrol agent of fungal plant pathogens. *Journal of Chemical Ecology, 26*(6), 1515–1524. https://doi.org/10.1023/A:1005595927521

Pieri, P., & Fermaud, M. (2005). Effects of defoliation on temperature and wetness of grapevine berries. *Acta Horticulturae, 689,* 109-116. https://doi.org/10.17660/ActaHortic.2005.689.9

Raza, W., Wei, Z., Ling, N., Huang, Q., & Shen, Q. (2016). Effect of organic fertilizers prepared from organic waste materials on the production of antibacterial volatile organic compounds by two biocontrol *Bacillus amyloliquefaciens* strains. *Journal of Biotechnology, 227,* 43–53. https://doi.org/10.1016/j.jbiotec.2016.04.014

Romero, D., de Vicente, A., Rakotoaly, R.H., Dufour, S.E., Veening, J.-W., Arrebola, E., Cazorla, F.M., Kuipers, O.P., Paquot, M., & Pérez-García, A. (2007). The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. *Molecular Plant-Microbe Interaction, 20*(4), 430–440. https://doi.org/10.1094/MPMI-20-4-0430

Sharma, R.R., Singh, D., & Singh, R. (2009). Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. *Biological Control, 50,* 205–221. https://doi.org/10.1016/j.biocontrol.2009.05.001

Spadaro, D., & Droby, S. (2016). Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. *Trends in Food Science and Technology, 47,* 39–49. https://doi.org/10.1016/j.tifs.2015.11.003

Stein, T. (2005). *Bacillus subtilis* antibiotics: Structures, syntheses and specific functions. *Molecular Microbiology, 56*(4), 845–857. https://doi.org/10.1111/j.1365-2958.2005.04587.x

Rahman, S.F., Singh, E., Pieterse, C.M.J., & Schenk, P.M. (2018). Emerging microbial biocontrol strategies for plant pathogens. *Plant Science 267,* 102–111. https://doi.org/10.1016/j.plantsci.2017.11.012

Usall, J., Torres, R., & Teixidó, N. (2016). Biological control of postharvest diseases on fruit: a suitable alternative? *Current Opinion in Food Science 11,* 51–55. https://doi.org/10.1016/j.cofs.2016.09.002

Walker, A.-S., Gladieux, P., Decognet, V., Fermaud, M., Confais, J., Roudet, J., Bardin, M., Bout, A., Nicot, P.C., Poncet, C., & Fournier, E. (2015). Population structure and temporal maintenance of the multihost fungal pathogen *Botrytis cinerea*: causes and implications for disease management. *Environmental Microbiology, 17*(4), 1261–1274. https://doi.org/10.1111/1462-2920.12563

Wu, Y., Zhou, J., Li, C., & Ma, Y. (2019). Antifungal and plant growth promotion activity of volatile organic compounds produced by *Bacillus amyloliquefaciens*. *MicrobiologyOpen, 8*(7), 1–14. https://doi.org/10.1002/mbo3.813

Xie, S., Zang, H., Jun Wu, H., Uddin Rajer, F., & Gao, X. (2018). Antibacterial effects of volatiles produced by *Bacillus* strain D13 against *Xanthomonas oryzae* pv. *oryzae*. *Molecular Plant Pathology, 19*(1), 49–58. https://doi.org/10.1111/mpp.12494

Yánez-Mendizábal, V., Usall, J., Viñas, I., Casals, C., Marín, S., Solsona, C., & Teixidó, N. (2011). Potential of a new strain of *Bacillus subtilis* CPA-8 to control the major postharvest diseases of fruit. *Biocontrol Science and Technology 21*(4), 409–426. https://doi.org/10.1080/09583157.2010.541554

Yánez-Mendizábal, V., Viñas, I., Usall, J., Torres, R., Solsona, C., Abadias, M., & Teixidó, N. (2012b). Formulation development of the biocontrol agent *Bacillus subtilis* strain CPA-8 by spray-drying. *Journal of Applied Microbiology, 112*(5), 954–965. https://doi.org/10.1111/j.1365-2672.2012.05258.x

Yuan, J., Raza, W., Shen, Q., & Huang, Q. (2012). Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 Volatile Compounds against *Fusarium oxysporum* f. sp. cubense. *Applied Environmental Microbiology, 78*(16), 5942–5944. https://doi.org/10.1128/aem.01357-12

Zhang, Y., Li, T., Liu, Y., Li, X., Zhang, C., Feng, Z., Peng, X., Li, Z., Qin, S., & Xing, K. (2019). Volatile Organic Compounds produced by *Pseudomonas chlororaphis* subsp. *aureofaciens* SPS-41 as biological fumigants to control *Ceratocystis fimbriata* in postharvest sweet potatoes. *Journal of Agriculture and Food Chemistry, 67*(13), 3702–3710. https://doi.org/10.1021/acs.jafc.9b00289

Zhou, M., Li, P., Wu, S., Zhao, P., & Gao, H. (2019). *Bacillus subtilis* CF-3 Volatile Organic Compounds inhibit *Monilinia fructicola* growth in peach fruit. *Frontiers in Microbiology, 10,* 1–11. https://doi.org/10.3389/fmicb.2019.01804