Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Diversity of food-borne *Bacillus* volatile compounds and influence on fungal growth

C. Chaves-López[1], A. Serio[1], A. Gianotti[2], G. Sacchetti[1], M. Ndagijimana[3], C. Ciccarone[4], A. Stellarini[1], A. Corsetti[1] and A. Paparella[1]

1 Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Mosciano Stazione, Italy
2 Department of Agri-Food Science and Technology (DISTAL), University of Bologna, Bologna, Italy
3 Department of Agricultural, Food and Nutritional Science (AFNS), Faculty of Agricultural, Life and Environmental Sciences (ALES), 4-10 Agriculture/Forestry Centre, University of Alberta, Edmonton, AB, Canada
4 Department of Agro-Environmental, Chemistry and Crop-Protection, Faculty of Agricultural Science, University of Foggia, Foggia, Italy

Keywords
antifungal compounds, *Bacillus*, *Fusarium oxyporum* f. sp. *lactucae*, *Moniliopthora perniciosa*, volatile organic compounds.

Correspondence
Clemencia Chaves-López, Via C.R. Lerici 1, 64023 Mosciano Stazione (TE), Italy.
E-mail: chaves@unite.it

2015/0251: received 5 February 2015, revised 2 April 2015 and accepted 4 May 2015

doi:10.1111/jam.12847

Abstract

Aims: To evaluate the antifungal activity of the volatile organic compounds (VOCs) produced by 75 different food-borne *Bacillus* species against *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus clavatus*, *Fusarium oxysporum* f. sp. *lactucae* and *Moniliophthora perniciosa* and to determine the VOCs responsible for the inhibition.

Methods and Results: *Bacillus* strains inhibited fungal growth, although with different inhibition grades, with *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus cereus* strains as the best antifungal VOCs producers. While *M. perniciosa* DM4B and *F. oxysporum* f.sp. *lactucae* MA28 were the most sensitive fungi, *A. parasiticus* MG51 showed the greatest resistance to *Bacillus* VOCs exposure. Thirty-seven compounds were detected by SPME-GC-MS analysis, although similar patterns in volatile compounds were evidenced within the species, interspecific VOCs differences determined different effects on fungal growth. Multiple partial least regression (MPLRS) and antifungal activity of the individual VOCs revealed that only propanone, 1-butanol, 3-methyl-1-butanol, acetic acid, 2-methylpropanoic acid, carbon disulphide, 3-methylbutanoic acid and ethyl acetate were responsible for mycelia inhibition of *M. perniciosa* DM4B and *F. oxysporum* f.sp. *lactucae* MA28.

Conclusions: The antagonistic activity of the *Bacillus* VOCs was demonstrated, although it cannot easily be explained through the action of a single molecule, thus a holistic approach could be more appropriate to estimate the fungal growth inhibition.

Significance and Impact of the Study: VOCs produced by *Bacillus* from cooked food can be considered as promising antifungal compounds useful in the control of fungal plant pathogens. This study investigates for the first time the correlation between mycelia inhibition of *M. perniciosa* and *F. oxysporum* f. sp. *lactucae* and the VOCs emitted by the *Bacillus* species.

Introduction

Microbial volatile organic compounds (MVOCs) are a variety of molecules originating from different metabolic pathways during the growth of fungi and bacteria, and their production may represent a major pathway by which carbon is moved from terrestrial to atmospheric pools. It has been suggested that MVOCs have played an important role during the evolution of micro-organisms in the context of their communities whereby they can mediate microbial interaction with some VOCs acting as 'infochemicals'. Microbial volatile organic compounds

can have either harmful or positive effects on the organisms perceiving the signal, by stimulating or inhibiting the growth of specific microbial populations (Wheatley 2002). In nature, the regulatory effects of these volatiles might require modification to the active derivative by cell metabolism, which may result from combined actions of several biologically active molecules, and may depend on dose. On the other hand, the composition of these volatile profiles depends on species, growing medium, abiotic factors as well as on population and function dynamics (Börjesson *et al.* 1990; Wheatley 2002).

Kai *et al.* (2008) underlined the importance of bacterial volatiles that represent a source for new natural compounds that can be interestingly used, for example, to improve human health, to increase the productivity of agricultural products, to protect the plants from disease or to promote plant growth. Species of genus *Streptomyces, Pseudomonas, Serratia, Xanthomonas, Alcaligenes*, *Bacillus* and *Agrobacterium* have been reported as the more frequent bioactive VOC producers. In particular VOCs emitted by *Bacillus* species showed to be a potential to inhibit fungal growth (Chaurasia *et al.* 2005; Yuan *et al.* 2012; Baysal *et al.* 2013); this antagonistic action could reflect a competitive pressure in the natural environments which leads to a selection of the microbiota.

To prevent fungal growth, several synthetic antifungal compounds have been used; however, there is some concern due to the environmental issues such as residual toxicity, safety, development of resistant races of the pests, resurgence of secondary pests and ecological imbalance (Shukla *et al.* 2009). Some information is available concerning the antagonisms of the VOCs from soil bacteria towards the species of phyto-pathogenic moulds, belonging to the genera *Alternaria, Fusarium, Paecilomyces, Pythium, Rhizoctonia Aspergillus, Geotrichum, Sclerotinia, Trichoderma, Botrytis, Verticillium dahliae* and *Colletotrichum* (Chaurasia *et al.* 2005; Kai *et al.* 2007; Zheng *et al.* 2013). However, there are no reports regarding *Moniliophthora perniciosa* and *Fusarium oxysporum* f. sp. *lactucae.* It is well known that *M. perniciosa* species is associated with Witches' broom disease (WBD), and is one of the three main fungal diseases of *Theobroma cacao* (cacao), that in South America has caused significant economic losses. In addition to causing WBD, *M. perniciosa* is also capable of causing disease on several members of the *Solanaceae* and other unrelated tropical hosts (Griffith *et al.* 2003). *Fusarium oxysporum* f. sp. *lactucae* is the causal agent of root of lettuce (*Lactuca sativa* L.), and has been associated also to the root vascular stele of cauliflower plants and spinach plants (Scott *et al.* 2014).

Species of *Bacillus subtilis, and Bacillus cereus* group are normal inhabitants of soil and rhizosphera and they are found in foods through contamination, hence they have been isolated. *Bacillus* genus is known to produce compounds which contribute considerably to the biocontrol of plant diseases (Chaurasia *et al.* 2005) and in preventing decay and senescence in the postharvest phase (Chunmei *et al.* 2014). In particular, a great variety of antifungal compounds, among which peptides either from ribosomal and nonribosomal origin, (Munimbazi and Bullerman 1998; Timmusk *et al.* 2005) are produced. In addition, *Bacillus* genus shows peculiar characteristics, such as the capability to colonize plants, spores and antibiotics production as well as to induce the synthesis of plant hormones (Turner and Backman 1991), biofilm production (Bais *et al.* 2004) all which make the species belonging to *Bacillus* genus suitable to be used in biocontrol.

Since technological processes which foods undergo select bacteria with particular traits, the aim of this study was to evaluate the antifungal activity of the VOCs produced by *Bacillus* species isolated from cooked foods against some phyto-pathogenic fungi and to determine the VOCs responsible for the inhibition.

Materials and methods

Micro-organisms

The 75 strains belonging to *Bacillus* genus used in this study were previously isolated from different cooked food sources (meat and vegetables) and were used to screen potential antagonists toward *Aspergillus niger* MG43, *Aspergillus flavus* MG09, *Aspergillus parasiticus* MG51, *Fusarium oxysporum* f.sp. *lactucae* MA28, *Aspergillus clavatus* MG103 and *Moniliophthora perniciosa* DM4B. All the strains belong to the Faculty of Bioscience and Technologies for Food, Agriculture and Environment of the University of Teramo (Italy). Fungal strains were grown in Potato Dextrose Agar (PDA- OXOID-Basingstoke, UK) slants at 28°C for 4 days and stored at 4°C. Bacteria were grown on plate count agar (PCA- OXOID) at 28°C for 24 h and used for the different assays.

Effects of VOCs on radial mycelial growth and hyphae morphology

Fungal species were grown in PDA medium for 5 days at 28°C. Antagonism assay was performed in sealed divided plates as reported by Fernando *et al.* (2005); 5-mm mycelial disk of actively growing culture of the fungus was placed in one compartment of the plates containing PDA with chloramphenicol 150 ppm, while the other compartment, containing PCA medium (OXOID), was used to inoculate the *Bacillus* strains (by swab) that were previously grown in PCA medium, as reported above. Plates

with only fungal disk served as control. Plates were sealed with parafilm and incubated at 28–30°C for up to 7 days.

The percent of inhibition (I %) was calculated as I % = [A–B/A]*100, where A is the radial growth of the pathogen in control and B is the radial growth of fungi in the presence of VOCs head space with a single *Bacillus* strain. The experiment was run in three different days. After 7 days of incubation, an agar plug was removed from the edge of inhibited fungal colonies and placed onto fresh PDA plates. The viability of the transferred fungal subculture was measured up to 25 days.

After exposure to VOCs, fungal hyphae were taken from the areas showing inhibition and fixed in lactophenol-cotton blue and examined under microscope (Nikon ECLIPSE E 200, Nikon, Melville, NY) for structural abnormalities. Photographs were taken with Samsung COLOR CAMERA SAC-410 PA interfaced with a PC.

Identification of volatile compounds

For the strains that showed the highest antagonistic activity against the tested fungal species, VOCs were identified. Thus, 100 μl of a 24 h preculture of *Bacillus* in liquid (containing 8 Log CFU) and grown in nutrient broth (OXOID) was inoculated in vials of 20 ml containing 5 ml of PCA. Samples were hermetically closed and incubated at 28°C for 5 days. To provide a repeatability of the experiment, four samples for each strain were prepared and noninoculated vials containing only PCA were used as control.

VOCs were analysed using solid phase microextraction (SPME) coupled with gas-chromatography tandem mass spectrometry analysis according to the method previously described by Chaves-López *et al.* (2011). Briefly a SPME fibre (75 mm, carboxen/polydimethylsiloxane (CAR/PDMS) was exposed to the headspace of the vials while maintaining the sample at 35°C for 40 min. Compounds were then desorbed for 10 min in the injection port of the gas chromatograph (GC HP 5890 series II) at 220°C for 10 min with the purge valve off (split-less mode).

An Agilent Hewlett-Packard 6890 GC gas-chromatograph equipped with a MS detector 5970 MSD (Hewlett-Packard, Geneva, Switzerland) was used for peak separation and detection. A fused silica capillary column was a CP-Wax 52 CB (50 m × 0·32 mm – Chrompack – Middelburg, the Netherlands), coated with polyethyleneglycol (film thickness 1·2 μm) as stationary phase was used. The injector and FID temperature was 250°C; detector temperature 220°C; carrier gas (He) flow rate, 1 ml min^{-1}. The GC oven temperature program began when the fibre was inserted and was held at 40°C for 2 min, ramped to 200°C at 10°C per min, then ramped from 200 to 250°C at 15°C per min and finally held at 250°C for 5 min.

Volatile peak identification was carried out by computer matching of mass spectral data with those of the compounds contained in the Agilent Hewlett-Packard NIST 98 and Wiley ver. 6 MASS SPECTRAL DATABASE (probability set at >90%). Positive identification of each chemical constituent was performed whenever possible by comparison with that of authentic standards (Sigma–Aldrich, Taufkirchen, Germany).

The volatile compounds quantities were expressed as relative area concentrations, and corrected taking into consideration the area of the compounds detected in noninoculated controls.

Effect of selected organic volatile compounds produced by *Bacillus subtilis* SV75-1 against *Moniliophthora perniciosa* DM4B and *Fusarium oxysporum* f.sp. *lactucae* MA28 growth

Selected compounds, such as ethyl acetate, 2-propanone, 2-butanone, 3-hydroxy-2-butanone, 1-butanol, 3-methyl-1-butanol, 2-methyl propanoic acid, 3-methyl butanoic acid and carbon disulphide, were tested for their antifungal activity towards *M. perniciosa* DM4B and *F. oxysporum* MA28. This experiment was performed using Petri plate (8·5 mm) with two compartments. Sterile filters of paper were added of 5, 10,15, 20, 25 and 50 μl of each single compound and placed in the PCA side of the plate, while a section of mycelium was placed on PDA in the other side. The plates were sealed and incubated for 5 days at 28°C and the diameter of the mycelia grown was measured. The results are given as percentage of inhibition. Tests were performed in triplicate.

Production of volatile compounds and antimicrobial activity of *Bacillus subtilis* SV75-1, during incubation time

The strong antagonistic activity showed by *B. subtilis* SV75-1 was tested on *M. perniciosa* DM4B and *F. oxysporum* f.sp. *lactucae* MA28 mycelia, exposing the moulds to head space of *B. subtilis* culture grown for 2 and 5 days in dual plates, to evaluate the effect of the culture age. The experiment was confirmed in sealed plates as reported by Chaurasia *et al.* (2005). The percentage of inhibition was calculated after 7 days of exposure as described above.

Statistical analysis

All the experiments were performed in triplicate and all the analyses were carried out at least in duplicate. The average and standard deviations were calculated for the

experimental data. Data were submitted to variance analysis (One-way ANOVA), to test the significance of the effects. Differences between mean values were tested using the Fisher LSD test.

The relationship between the peak area of VOCs of the *Bacillus* strains and the mycelia inhibition of *M. perniciosa* DM4B and *F. oxysporum* f.sp. *lactucae* MA28 was evaluated by multiple partial least regression MPLSR, which was applied to study cause-effect relationship between the area of individual peaks (independent variables) and the percentage of mycelia inhibition (response variable). The PLS model was validated by leave-one-out cross-validation, which uses all but 1 sample to build a calibration model and repeats for each sample in the data set (Martens and Naes 1989). The number of PLS extracted factors was optimized based on the lowest root mean square error of prediction (RMSEP) values to avoid overfitting of data. The adequacy of the model was expressed by the optimum number of extracted factors, the determination of coefficient R^2 and the RMSEP. Data were processed using the STATISTICA for Windows (StatSoft^{TM}, Tulsa, OK) package.

Results

Preliminary screening of antifungal VOC

Only 16 of 75 *Bacillus* strains produced VOCs able to significantly inhibit ($P < 0{\cdot}05$) the fungal growth, although with different inhibition extent (Table 1). In general, the pattern of the VOCs produced by *Bacillus* was species and strain-dependent, whereas their antifungal activity was fungal species-dependent.

A wide inhibition spectrum was showed by VOCs produced by the strains of *B. subtilis*, (SV75-1 and SV44-2), *Bacillus amyloliquefaciens* (M49 and SV20-2) and *B. cereus* (SV40), even though the mycelium reduction percentage was low for some fungal species. In this context, it is worthy to note the action of *B. subtilis* SV75-1 that reduced the mycelia growth of *M. perniciosa* DM4B by about 84% and that of *Fusarium oxysporum* f.sp. *lactucae* MA28 by about 50%. Whereas, VOCs emitted by the strains of *B. amyloliquefaciens* SV68 and SV103-2, by *B. subtilis* M45 and by *B. stearothermophylus* SV25 showed both a less complex VOCs profile and a lower antagonistic activity than other strains and reduced the growth of only one of the fungal species under investigation.

The pecking order of the fungal sensitivity to *Bacillus* VOCs was *M. perniciosa* DM4B > *A. niger* MG4 > *F. oxysporum* f.sp. *lactucae* MA28 and *A. flavus* MG09 > *A. clavatus MG103* > *A. parasiticus* MG51.

Effects of volatile compounds on the morphology of the colonies, mycelium microstructure and the production of spores

The diversity of *Bacillus* VOCs led not only to a reduction in the mycelia growth but also to changes in morphology of the colonies, the production of spores as well as in the microstructure of the hyphae. Four different effects were observed: (i) reduction in sporification as observed in *A. flavus* MG09 in presence of *B. cereus* SV40, *B. subtilis* SV36/2 and *Bacillus coagulans* SV95 VOCs (Fig. 1a); (ii) strong reduction in mycelia growth, as observed in *M. perniciosa* DM4B when exposed to the VOCs produced by *B. cereus* SV75-1 (Fig. 1b); (iii) change in mycelia growth and reduction in the pink pigment production, as evidenced in *F. oxysporum* f.sp. *lactucae* MA28 in the presence of VOCs produced by *B. amyloliquefaciens* M49 (Fig. 1c); (iv) promotion of mycelia growth and increase in the conidiation, as showed for *A. clavatus* MG103 and *A. parasiticus* MG51, subjected to *B. cereus* SV40, and *B. amyloliquefaciens* SV20-2 VOCs respectively (data not shown).

Microscopic observations of fungal species exposed to the bacterial VOCs showed degenerative changes in the hyphal morphology in comparison to control ones. In Fig. 2, some of the effects of the bacterial VOCs on hyphae morphology are shown. As evidenced, the presence of VOCs from *B. subtilis* SV75-1 highly influenced the *M. perniciosa* BM4B mycelium in that untreated hyphae presented some parts of the mycelium coiled (Fig. 2a) in contrast to treated mycelium which appears with a more flacced hyphae, presenting a retracted protoplasm and the formation of empty segments and a thinner wall (Fig 2b). In *A. parasiticus* MG51, the exposure to VOCs produced by *B. amyloliquefaciens* SV20-2, led to a shortened and swollen somatic hyphae (Figs 2c,d), while exposure to *B. cereus* SV40 lead to a protoplasma retraction. Moreover a reduction in conidia number and granulation of the mycelia was detected in *F. oxysporum* f. sp. *lactucae* MA284 when exposed to head space of SV75-1(data not shown).

The subculture of *M. perniciosa* DM4B mycelia, inhibited by *B. subtilis* SV75-1 and *B. amyloliquefaciens* M49 VOCs, did not grow during 25 incubation days, indicating a fungicide action; whereas the mycelia of all the other fungi inhibited by VOCs produced by other *Bacillus* species grew within this period, indicating a fungistatic action of these VOCs towards the tested fungal species.

Identification of volatile compounds produced by *Bacillus* species

The strains of *B. amyloliquefaciens* (SV56-2, SV20/2, BT33, M49), *B. cereus* (SV40) and *B. subtilis* (SV75-1,

Table 1 Spectrum of antifungal activity showed by *Bacillus* spp. '*in vitro*' after 5 days of incubation at 28°C

	Moniliophthora perniciosa DM4B	*Aspergillus flavus* MG09	*Aspergillus parasiticus* MG51	*Aspergillus niger* MG43	*Fusarium oxysporum* f. sp. *lactucae* MA28	*Aspergillus clavatus* MG103
Bacillus amyloliquefaciens						
SV20-2	43·6 ± 2·6	n.r	n.r	39·6 ± 4·6	10 ± 2·3	47·2 ± 9·0
SV56-2	34·3 ± 4·0a	20·4 ± 3·8b	n.d	18·5 ± 2·5b	n.d	n.d
SV68	n.d	n.d	n.d	25·9 ± 5·7	n.d	n.d
SV74-2	22·0 ± 2·1	n.d	n.d	n.d	n.d	n.d
SV103-1	10·2 ± 2·1a	n.d	n.d	n.d	n.d	30 ± 2·4b
SV103-2	n.d	n.d	n.d	14·8 ± 2·0	n.d	n.d
SV104-1	53·3 ± 5·6a	17·6 ± 4·1b	n.d	n.d	n.d	n.d
BT33	65·6 ± 4·2a	n.d	n.d	18·5 ± 4·3b	n.d	n.d
M49	33·6 ± 4·5a	n.d	n.d	22·7 ± 2·6b	48·3 ± 8·9c	28·5 ± 4·2a
Bacillus subtilis						
SV36-2	32·5 ± 2·5a	n.d	n.d	n.d	n.d	36·8 ± 9·8a
SV75-1	84·0 ± 4·3a	11·3 ± 1·5b	n.d	22·2 ± 4·6c	50·2 ± 16·2d	n.d
SV44-2	11·7 ± 3·4a	n.d	n.d	45·1 ± 5·8b	22·0 ± 2·1c	32·1 ± 5·9d
M45	n.d	n.d	16·3 ± 1·3a	n.d	n.d	36·0 ± 7·1b
Bacillus cereus						
SV40	26·6 ± 1·6a	27·3 ± 7·2a	n.d	n.d	40·6 ± 4·0b	47·3 ± 7·8b
Geobacillus stearothermophilus						
SV25	n.d	8·6 ± 2·0	n.d	n.d	n.d	n.d
Bacillus coagulans						
SV95	n.d	33·3 ± 5·2a	14·2 ± 2·8b	n.d	n.d	n.d

Mean (expressed as inhibition percentage) and standard deviation of 3 repetitions; n.d, nondetectable. Different letters in the same row mean significant differences ($P < 0.05$) among the age of the culture.

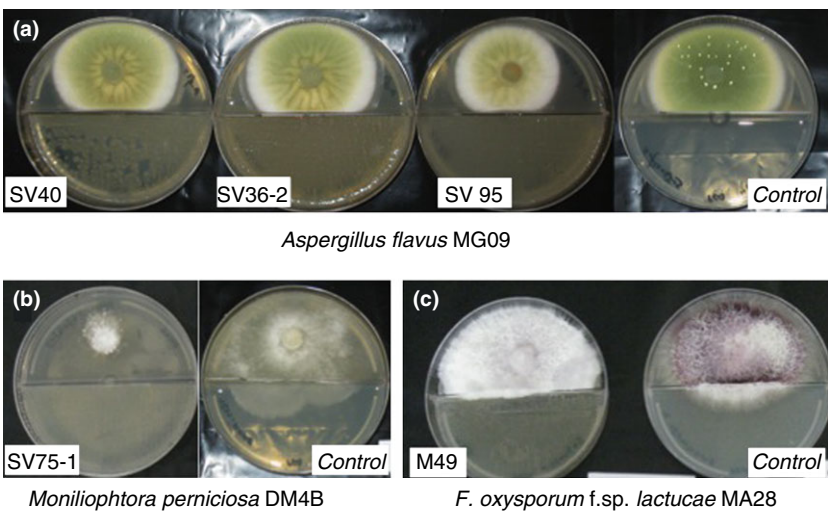

Figure 1 Effects of volatile organic compounds produced by (a) *Bacillus cereus* SV40, *Bacillus subtilis* SV36-2 and *Bacillus coagulans* SV95; (b) *B. subtilis* SV75-1; (c) *Bacillus amyloliquefaciens* M49, grown in plate count agar medium, against the mycelia growth of *Aspergillus flavus* MG09, *Moniliophthora perniciosa* DMB4 and *Fusarium oxysporum* f. sp. lactucae MA28 incubated at 28°C for 7 days on potato dextrose agar.

SV44/2, SV36-2) that showed the highest antagonistic activity against the tested fungal species were studied for their VOCs production. Overall, 37 different volatile compounds were identified (Table 2), five other unidentified compounds were detected, but they were considered negligible because the relative abundance was $<10^2$ of arbitrary unit (AU). As reported in Table 2, there was a significant difference ($P < 0{\cdot}05$) in the concentration of the single VOCs among the different *Bacillus* strains, although some compounds were in common. Most of the compounds has previously been reported to be produced by *Bacillus* species, however, as far we know the alcohols 1-octanol, furfurol and phenylethanol, hydrocarbons such as 1,3 pentadiene, 2-methyl propane and 1-octene as well as other compounds as 5,6-dihydro-6-pentyl-2H-pyran-2-one have not been previously reported.

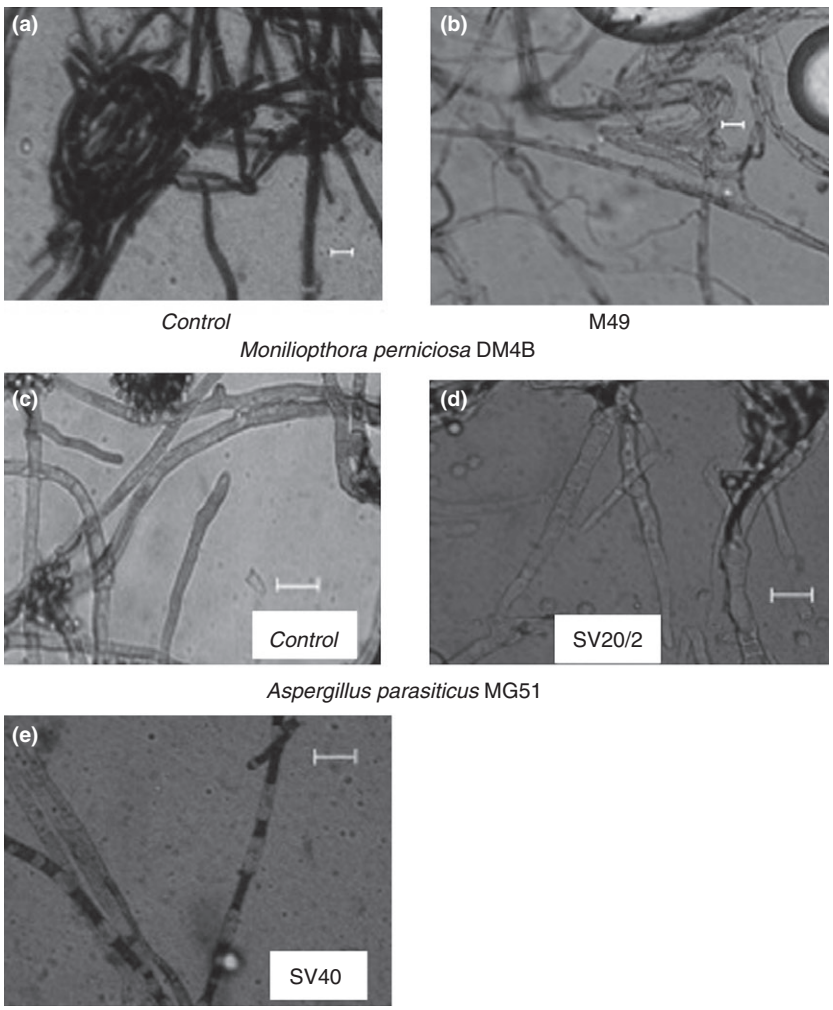

Figure 2 Microscopic observations (100X) of *Moniliophthora perniciosa* DM4B hyphae incubated at 28°C for 7 days (a) mycelia control (b) mycelia exposed to *Bacillus amyloliquefaciens* M49 volatile organic compounds (VOCs); and *Aspergillus parasiticus* MG51 hyphae (c) control, (d) exposed to *B. amyloliquefaciens* SV20-2 VOCs, (e) exposed to *Bacillus cereus* SV40 VOCs.

Bacillus amyloliquefaciens strains were the major producers of VOCs. Alcohols, in particular, represented approx. 25% of VOCs produced by *B. amyloliquefaciens* and *B. cereus* strains and nearly 18% of those produced by *B. subtilis* strains in PCA medium. In addition, the content of ketones was higher in the headspace of *B. amyloliquefaciens* with 2,3 butanedione (diacetile) and 3-hydroxy-2-butanone (acetoin) being the most compounds present. As far as esters are concerned, their diversity was very low, with ethyl acetate being detected in all the strains of the three species under study, whereas ethyl 2-methylpropanoate (ethyl isobutyrate) was detected only in the headspace of *B. amyloliquefaciens.*

High relative amounts of propanone and 1-butanol were detected in the headspace of the strain *B. subtilis* SV75-1, which drastically reduced the mycelia growth of both *M. perniciosa* BM4B and *F. oxysporum* f.sp. *lactucae* MA28. In addition, the strain *B. subtilis* SV36-2, that reduced mycelia and conidiation in *A. flavus* MG09, produced high quantities of carbon disulphide and 1,3 pentadiene. Moreover, *B. amyloliquefaciens* M49, that caused the inhibition of the growth of *A. niger* MG43 and the reduction in pink pigment production in *F. oxysporum* f.sp. *lactucae* MA28, emitted high quantities of 2,3-butanedione and tetramethyl pyrazine and lower quantities of 2-butanone, and carbon disulphide in the head space.

Correlation between volatile compounds produced by *Bacillus* species and mycelia inhibition of *Moniliophthora perniciosa* DM4B and *Fusarium oxysporum* f.sp. *lactucae* MA28

The relationships between the peak area of volatile compounds and mycelia inhibition of *M. perniciosa* DM4B and *F. oxysporum* f. sp. *lactucae* MA28, that showed major sensitivity to the VOCs, were studied by MPLSR. Ten of 37 VOCs present in heterogeneous mixtures within the headspace of *Bacillus* resulted responsible for the growth inhibition of both fungi when studied using

Table 2 Volatile compounds (Area relative × 10^6) detected in the head space of the *Bacillus* cultures inoculated in plate count agar at 28°C for 5 days

	Bacillus amyloliquefaciens				*Bacillus cereus*	*Bacillus subtilis*		
	SV56-2	SV20-2	BT33	M49	SV40	SV75-1	SV44-2	SV36-2
Aldehydes								
Benzaldehyde	n.d	n.d	n.d	n.d	n.d	n.d	4·26a	2·90a
Pentanal	77·15a	101·41b	n.d	n.d	n.d	n.d	145·16b	1·25c
Ketones								
Methyl isobutyl ketone	9·08a	15·42b	12·90b	n.d	n.d	n.d	n.d	n.d
Propanone	7·02a	19·57b	n.d	n.d	0·71c	12·56d	28·73b	3·51a
2-butanone	19·81a	36·61a	27·03a	2·24b	0·75c	0·93c	5·84b	1·73b
2-heptanone	n.d	n.d	n.d	n.d	0·47a	0·58a	n.d	n.d
2,3 butanedione	95·66a	49·75a	247·67b	287·14b	n.d	n.d	n.d	n.d
3-hydroxy-2-butanone	120·07a	181·80b	198·80a	180·95a	0·28c	0·37c	7·84d	10·72d
2,3-pentanedione	n.d	n.d	n.d	n.d	0·33a	0·39a	n.d	n.d
5-methyl-3-hexanone	22·30a	n.d	n.d	n.d	n.d	n.d	n.d	n.d
3-octanone	n.d	n.d	n.d	n.d	0·43	n.d	n.d	n.d
Esters								
Ethyl acetate	20·08a	5·37b	49·05b	n.r	0·60c	1·14ab	n.d	n.d
Ethyl isobutyrate	34·55a	3·86b	n.d	n.d	n.d	n.d	n.d	n.d
Alcohols								
Ethanol	21·38a	11·00a	67·21a	n.r	0·05b	n.d	n.d	n.d
1-butanol	73·80a	43·36a	98·41b	67·14a	0·21c	76·20d	65·66a	55·94a
1-hexanol	n.d	n.d	n.d	n.d	0·52	n.d	n.d	n.d
2-hexanol	6·61a	4·64a	4·52a	4·73a	n.d	n.d	8·38a	6·65a
1-octanol	2·09	n.d	n.d	n.d	n.r	n.d	n.d	n.d
3-methyl-1-butanol	14·38a	8·97b	53·90a	45·67a	0·43c	19·7a	15·54a	11·90a
Furfurol	2·58a	4·54a	3·98a	5·08a	n.d	1·23a	8·33a	4·94a
1-pentanol	n.d	n.d	n.d	n.d	0·14	n.d	n.d	n.d
Phenylethanol	9·13a	6·40a	5·08a	4·14a	n.d	0·05b	6·32a	5·02a
1-octen-3-ol	n.d	n.d	n.d	n.d	0·38	n.d	n.d	n.d
Hydrocarbons								
1,3-pentadiene	72·80a	91·94a	110·83ab	36·66a	n.d	n.d	124·45b	115·68b
2-methyl propane	n.d	14·04a	30·25a	15·19a	n.d	n.d	4·75b	3·97b
1-octene	2·64a	n.d	3·83a	5·07a	n.d	n.d	2·79a	4·27a
Acids								
2-methyl propanoic acid	54·38a	63·47a	10·44a	15·71a	1·16b	6·71b	0·75c	0·28c
Acetic acid	21·84a	19·19a	2·67b	2·05b	2·24b	1·73b	1·66b	2·73b
Butanoic acid	n.d	n.d	n.d	n.d	0·58	n.d	n.d	n.d
3-methyl butanoic acid	51·56a	58·02a	n.d	n.d	n.d	5·84b	n.d	n.d
Pyrazine								
2,6-dimethyl pyrazine	14·83a	3·23b	37·01a	4·46b	n.d	n.d	10·75a	5·06b
2,3,5-trimethyl pyrazine	n.d	n.d	8·59a	n.d	n.d	n.d	n.d	n.d
2-ethyl-3,5-dimethyl pyrazine	n.d	n.d	15·60a	2·13b	n.d	0·24c	1·15b	n.d
Tetramethyl pyrazine	4·44a	n.d	62·61b	40·22b	n.d	n.d	1·09a	0·93a
Other compounds								
Carbon disulphide	60·46a	68·82a	71·56a	3·75b	n.d	39·91b	102·13a	98·12b
5,6-dihydro-6-pentyl-2H-pyran-2-one	n.d	1·71a	n.d	n.d	n.d	n.d	8·72b	3·05b
2-acetylthiazole	n.d	n.d	n.d	n.d	1·88	n.d	n.d	n.d

Mean of 3 repetitions; n.d: nondetectable with the method used.
Different letters in the same row mean significant differences ($P < 0·05$) among the strains.

MPLSR. The coefficient of determination of the calibration regressions for *M. perniciosa* DM4B was of 0·98 and the coefficient of determination of the validation regression was of 0·90, indicating that the PLS models have a good predictive ability with a root mean square error of prediction (RMSEP) of 8·3. Instead, the coefficient of determination of the calibration regressions for *F. oxysporum* was of 0·66 and the coefficient of determination of

the validation regression was of 0·64, indicating that, in this case, the PLS models have a limited predictive ability with a RMSEP of 12·6.

In the PLS models, some compounds which were highly present in the control sample showed a negative correlation with mycelia inhibition, whilst other neo-formation compounds showed a positive correlation with the dependent variable and are likely to be responsible for mycelia inhibition. As reported in Table 3, the compounds correlated with the inhibition of *M. perniciosa* DM4B and *F. oxysporum* f.sp. *lactucae* MA28 growth were the same for both fungi, with propanone and 1-butanol being the most effective inhibitory compounds on the basis of the regression coefficient.

Table 4 shows the effect on the mycelia inhibition and hyphae microstructure, of the single compounds that positively influenced the mycelia inhibition, as determined by MPLSR. Moreover, other VOCs inhibited the fungal growth even though MPLSR did not evidence their effect on growth inhibition; this could be likely due to their low concentration in the headspace, which cannot be quantified through the analytical approach used in this study.

We also tested 3-hydroxy-2-butanone and 2 butanone, which showed negative influence on mycelia inhibition by MPLSR analysis. While 2-butanone lead to mycelia inhibition in both of the species studied, 3-hydroxy-2-butanone stimulates the *M. perniciosa* DM4B growth mycelia and inhibited *F. oxysporum* mycelia and the number and size of conidia. Thus, the amounts present in the head-space of the plates were probably lower than those used in this test.

Our investigation evidenced that the antimicrobial activity on *M. perniciosa* DM4B and *F. oxysporum* f. sp. *lactucae* MA28 mycelia growth, caused by the single molecules studied here was different between these species. While the most suppressive effect of *M. perniciosa* DM4B was associated to the presence of the following molecules in order: carbon disulphide > 2-butanone > ethyl acetate > 3-methyl butanoic acid and 2-methyl propanoic acid > 3methyl- 1-butanol > 2-propanone > 1-butanol > 3-hydroxy-2-butanone; for *F. oxysporum* f. sp. *lactucae* MA28 were carbon disulphide > 3-methyl butanoic acid > 2-methyl propanoic acid > 3-methyl-1-butanol > 1-butanol and 2-butanone > ethyl acetate > 2-propanone and 3-hydroxy-2-butanone.

Production of volatile compounds and antimicrobial activity of *Bacillus subtilis* SV75-1 during incubation time

Qualitative and quantitative significant differences ($P < 0·05$) were detected among the VOCs collected from

Table 3 Results of partial least squares regression analysis between micelia inhibition and the peak area of the compounds produced by *Bacillus subtilis* SV75-1

Micelia inhibition (%)	Compounds	Calibration R^2	Validation R^2	Validation RMSEP	F_{opt}
Moniliophthora perniciosa	Propanone*	0·98	0·90	8·31	6
	1-butanol				
	3-methyl-1-butanol				
	Acetic acid				
	2-methylpropanoic acid				
	2-ethyl-3,5-dimethyl pyrazine				
	Carbon disulphide				
	3-methylbutanoic acid				
	Ethyl acetate				
	2,3,5,6-tetramethylpyrazine				
Fusarium oxysporum	Propanone	0·66	0·64	12·55	6
	1-butanol				
	2-methylpropanoic acid				
	3-methyl-1-butanol				
	3-methylbutanoic acid				
	2-ethyl-3,5-dimethyl pyrazine				
	Carbon disulphide				
	Acetic acid				
	Ethyl acetate				
	2,3,5,6-tetramethylpyrazine				

RMSEP: root mean square error of prediction; F_{opt}: optimum number of extracted factors.
*Compounds listed in descending order according to their standardized regression coefficient.

Table 4 Antifungal activity of individual volatile compounds produced by *Bacillus subtilis* SV75-1 on the diameter of inhibition of mycelia growth of *Moniliophthora perniciosa* DM4B and *Fusarium oxysporum* f. sp. *lactucae* MA28

	Moniliophthora perniciosa DM4B			*Fusarium oxysporum* f. sp. *lactucae* MA28		
	% of mycelia inhibition			% of mycelia inhibition		
Compound	25 μl	50 μl	Hyphae microstructure	25 μl	50 μl	Hyphae microstructure
1- butanol	3·1 ± 0·5*	5·0 ± 1·1	Vacuolization and hyphae slightly swelled	18·8 ± 2·8	33·3 ± 3·5	Supercoil of the hyphae, several hyphae abnormal and reduction in the micro and macroconidia size
3-methyl -1 -butanol	18·3 ± 1·5	40·3 ± 3·0	Thinning of the hyphae	40·5 ± 3·5	45·12 ± 1·8	Supercoil of the hyphae and reduction in the conidia number
2-propanone	7·4 ± 1·3	18·4 ± 3·6	No effect	9·7 ± 2·0	11·1 ± 2·1	Thinning of the hyphae, reduction on number and size of visible conidia
2-butanone	35·4 ± 7·0	66·7 ± 3·5	Thinning of the hyphae and protoplasm retracted	18·6 ± 2·8	33·3 ± 4·1	Swelling of the hyphae, supercoil of the hyphae, and reduction in the conidia size
Ethyl acetate	28·1 ± 3·0	41·7 ± 4·7	Protoplasm retracted and granulation	9·72 ± 1·2	13·9 ± 3·1	Several hyphae abnormal, reduction in the conidia size
2-methyl propanoic acid	18·2 ± 4·2	35·1 ± 3·5	Thinning of the hyphae	44·7 ± 2·5	46·2 ± 2·8	Thinning of the hyphae, reduction in conidia number
3-methyl butanoic acid	20·1 ± 1·1	38·7 ± 1·3	Thinning of the hyphae	61·2 ± 3·0	71·4 ± 3·0	Thinning of the hyphae, reduction in conidia production
Carbon disulphide	54·3 ± 3·0	61·7 ± 6·1	Vacuolization as well as thinning of the hyphae	100	100	Not growth
3-hydroxy-2-butanone	Stimulation of mycelia growth		No effect	9·72 ± 3·5	12·50 ± 2·0	Supercoil of the hyphae, several hyphae with protoplasma retractile. Reduction in the conidia size

*Mean and standard deviation of three repetitions.

B. subtilis SV75-1 at 2 days of incubation and those collected at 5 days, in which alcohols were the most abundant group of VOCs (Table 5). It was evident that the VOCs pattern produced after 2 days of the inoculum was most effective against the mycelia inhibition, in fact both *M. perniciosa* DM4 and *F. oxysporum* f.sp. *lactucae* MA28 species were inhibited by up to 80% and 52·8% respectively.

Discussion

In this study, the diversity of VOCs produced by foodborne *Bacillus* species and strains was studied and their activity against some phyto-pathogenic fungi was determined. As evidenced, the fungal response to *Bacillus* VOCs appears to be species-specific, probably indicating an additional regulatory effect on the physiology and development of the fungal organism. Although the relative amounts of each single compound depends on the extraction method, the use of the SPME technique allowed the comparison of the VOCs produced by the different *Bacillus* strains on a semi-quantitative basis.

Our findings revealed that none of the bacterial isolates was effective against all of the fungi and this is in line with the results obtained by Wheatley (2002) with soil bacteria. The antifungal action of the VOCs produced by the *Bacillus* studied here, largely depended not only on the growth phase as previously suggested by other authors (Arrebola *et al.* 2010; Trefz *et al.* 2013) but also on the nature of the pathogenic fungi against which the VOCs were tested. This points out to the fact that the observed variations in fungal responses may reflect differences in the site of action or in the ability of fungi to detoxify the metabolites.

Bacillus VOCs were particularly active in reducing *M. perniciosa* DM4B and *F. oxysporum* f. sp. *lactucae* MA28 mycelia. Previous studies (Melnick *et al.* 2011; Falcao *et al.* 2014) reported the *in vitro* antagonistic activity of endophytic *B. subtilis, B. amyloliquefaciens, B. mycoides* and *B. cereus* towards *M. perniciosa*, however, they did not identify the compounds responsible for the inhibition. To the best of our knowledge, this is the first report on the antifungal activity of *Bacillus* VOCs against *M. perniciosa*. On the other hand, as evidenced by the

Table 5 Volatile organic compounds (VOCs) (relative percentage area) detected in head space of *Bacillus subtilis* SV75-1 after 2 and 5 days of incubation in plate count agar at 28°C

	Age of *Bacillus* culture (days)	
	2	5
Mycelia inhibition*		
Moniliophthora perniciosa	82·0 ± 5·2%	54·1 ± 7·2%
Fusarium oxysporum	52·9 ± 8·4%	38·5 ± 4·1%
Volatile compounds		
Esters		
Ethyl acetate	8·7 ± 1·4a**	5·9 ± 0·4b
Ketones		
2-propanone	7·4 ± 1·5a	18·9 ± 0·5b
2-butanone	7·1 ± 2·1a	6·4 ± 4·6a
2-heptanone	n.d	2·2 ± 0·2
3-Hydroxy-2-butanone	28·9 ± 3·0a	5·1 ± 1·6b
Alcohols		
1-butanol	9·4 ± 1·2a	30·8 ± 3·3b
3-Methyl-1-butanol	3·9 ± 0·4a	3·3 ± 1·7a
phenylethanol	1·2 ± 0·4a	2·5 ± 0·2b
Pyrazine		
Tetramethyl pyrazine	3·4 ± 0·1a	4·5 ± 0·6b
2,6-dimethyl pyrazine	1·3 ± 0·2a	3·5 ± 0·3b
Acids		
Acetic acid	n.d	1·9 ± 0·7
3-methylbutanoic	4·4 ± 1·3a	3·9 ± 0·5a
Sulphure compounds		
Carbon disulphide	5·0 ± 0·8a	4·5 ± 0·8a
Dimethyl sulphide	n.d	2·2 ± 0·9
Other compounds		
pentanal	4·0 ± 1·2a	1·28 ± 1·2b
1,3-pentadiene	8·4 ± 2·3a	2·2 ± 0·4b
2-methyl propane	0·4 ± 0·02	n.d

Mean and standard deviation of 3 repetitions; n.d: nondetectable during the experiment.

*Measured after 5 days of exposure to VOC.

**Different letters in the same row mean significant differences ($P < 0·05$) among the age of the culture.

results of this study, the high antagonistic activity of *B. subtilis* SV75-1 VOCs against *M. perniciosa* BM4B growth seemed to be related to the high relative amounts of 2-methyl-propanoic acid, 3-methyl-butanoic acid and 3-methyl-1-butanol (isoamyl alcohol) as well as to the low abundance of 3-hydroxy-2-butanone that showed a growth promoting effect on this species, when tested singularly. Although Chaurasia *et al.* (2005) reported the inhibition of about 34·40% of *F. oxysporum* when subjected to the volatile compounds produced by *B. subtilis*, there are no reports regarding the activity of bacterial VOCs against the subspecies *lactucae*. The growth of *F. oxysporum* appeared to be limited by the same compounds which inhibited the growth of *M. perniciosa*, despite these fungi belonging to different taxonomical phyla. Among the 10 single VOCs correlated with the fungal inhibition, only carbon disulphide prevented the growth of *F. oxysporum* f.sp. *lactucae* MA28 completely.

As proposed by Maruzzella (1961), the activities of antifungal VOCs decrease in the following order: organic acids > aldehydes > alcohols > ethers > ketones > esters > lactones. However, in this work, when single molecules were tested on *M. perniciosa* DM4B and *F. oxysporum* f. sp. *lactucae* MA28, this order was not observed. Antifungal activity of VOCs has been reported to be associated with the functional group (Wei-wei *et al.* 2008) and it is dependent on the hydrophobicity of the solute, which affects the depth of penetration into the bilayer, and the induced changes in the physico–chemical properties (Urbanek *et al.* 2012). It is well known that the effect that a lipophilic compound has on the integrity of a membrane depends on its location in the membrane where it has been accumulated, resulting in a lower membrane integrity and an increase in the proton passive flux-across the membrane; this could be the case of carbon disulphide that dramatically reduced the growth of *M. perniciosa* DM4B and *F. oxysporum* f. sp. *lactucae* MA28 when tested as a single molecule.

On the other hand, fatty acids with short chains are highly active compounds against fungi probably due to their higher solubility in water (Urbanek *et al.* 2012). In this study, 2-methyl propanoic acid was produced by the selected *Bacillus* studied and demonstrated very effective antifungal activity and in particular, against *F. oxysporum* f. sp. *lactucae* MA28. In contrast to fatty acids, alcohols are not selectively adsorbed and are mainly accumulated in the cell membrane determining membrane functions inhibition which, in turn, is responsible for their antimicrobial activity (Ingram and Buttke 1984). In particular 3-methyl-1-butanol resulted to be more effective against fungal growth in both *M. perniciosa* DM4B and *F. oxysporum* f. sp. *lactucae* MA28. Moreover, some alcohols, such as isoamyl alcohol, seem to be adsorbed on the spores' surface and therefore adhere to it for a long time, resulting in spore germination inhibition (Ando *et al.* 2012).

Some article report analyses of the antifungal activity of selected molecules produced by *Bacillus* species tested singularly (Fernando *et al.* 2005; Arrebola *et al.* 2010; Yuan *et al.* 2012; Zhang *et al.* 2013) and the activity of ketones has been negatively correlated with the number of the carbon atoms, and thus 2-nonanone and 2-decanone, in particular, showed strong inhibition activity (Yuan *et al.* 2012). However, in this study, the analysis of the headspace of *Bacillus* species did not reveal any ketone with a high number of carbons but, rather ketones at low number of carbons; in particular 2-butanone was more efficient than 2-propanone against both *M. perniciosa* DM4B and *F. oxysporum* f.sp. *lactucae* MA28. Other volatile molecules produced by *Bacillus* species have been tested as single

compounds towards fungal growth of other species showing a significant antifungal activity, for example: aldehydes, like o-anysaldehyde, nonanal and n-decanal, which reportedly inhibit germ tube formation of *Alternaria alternata* (Andersen *et al.* 1994), or mycelia growth of *B. cinerea* and *S. sclerotiorum* (Fernando *et al.* 2005; Zhang *et al.* 2013). Furthemore, alcohols like 1-hexanol, cyclohexanol, 2-ethyl, benzothiazole and dimethyl trisulphide showed high fungicidal activity against *S. sclerotiorum* (Fernando *et al.* 2005). In addition, 2-methylpirazyne and *β*-benzeneethanamine showed antifungal activity against *Colletotrichum gloesporoides* (Zheng *et al.* 2013). In this study, we reported that also 3-methyl-1-butanol, 2-methyl propanoic acid, 3-methyl butanoic acid and carbon disulphide produced by *B. subtilis* SV75-1, determined the reduction in mycelia growth and lead to the modification of the fungal hyphae of both *M. perniciosa* DM4B and *F. oxysporum* f. sp. *lactucae* MA28.

The modification on hyphae structure such as lysis of fungal hyphae, vacuolization and granulation in mycelium structures by the *Bacillus* spp. VOCs, suggest that *Bacillus* VOCs of the species here studied act in multiple modes of action during the interaction with fungal species and the effect could be direct or indirect, as previously suggested by Zhang *et al.* (2013). In this context, it has been suggested that the negative effects observed on the hyphae growth and morphology may be indicative of toxin(s) accumulation, with the physiological response of the fungus being compartmentalization and removal of the toxin from the growing cells (Fiddaman and Rossall 1993). In particular, the increase in vacuole formation in the hyphae of *M. perniciosa* DM4B in presence of 1-butanol could be regarded as a stress response since vacuoles serve as compartments either for storage of resources or even for detoxification purposes (Bloemberg and Camacho Carvajal 2006). On the another hand, coiling of the hyphae observed in *M. perniciosa* DM4B under head space of *B. subtilis* SV75-1, and on *F. oxysporum* f. sp. *lactucae* MA28 in presence of 1-butanol and 3-methyl-1-butanol tested singularly, probably reflects a general adaptation to stress conditions. In addition, volatiles act by changing protein expression and the activity of specific enzymes (Wheatley 2002) or by increasing the cellular membrane permeability, thus reducing the activity of membrane-associated enzymes and impairing nutrient uptake (Fialho *et al.* 2011).

Although when *Bacillus* is grown on the planta or in the rhizosphere their metabolism may be modified which may lead to a difference in the VOCs produced, the results here obtained also evidenced the contribution of the VOCs of some *Bacillus* strains to induce the growth of specific fungal species. We hypothesize that these strains produced a pattern of VOCs that could act as a chemical signal in these fungal species, probably interfering with the mechanism involved in the switch between vegetative and reproductive phases of fungal development. These findings stress the necessity to make an accurate selection of the strain to be used in fungal control.

Moreover, although MPLSR allow the correlation of the area of selected compounds to the mycelia growth inhibition with certain accuracy, it is important to stress that the statistical method used in this study does not permit an evaluation of the synergistic or antagonistic effect of volatiles towards fungal growth.

In conclusion, the findings of this study indicate that food-borne *Bacillus* strains emitted promising antifungal VOCs patterns, exerting fungal inhibition that was mould species-specific. In particular, *B. subtilis* SV75-1, which showed a broad spectrum of antagonistic capability, was able to produce a strong and stable inhibitory activity against the mycelium growth of *M. perniciosa* DM4B.

Our results suggest the overall antifungal activity of *Bacillus* VOCs, which are a heterogeneous mixture, and cannot be easily explained through the action of a single or few molecules. In this study, a holistic approach was attempted to identify the bacterial VOCs that mostly affect the inhibition of fungal growth. Even though some studies report the results of the analysis of VOCs produced by *Bacillus* species with fungal antagonistic activity, this study investigates for the first time the correlation between mycelia inhibition of *M. perniciosa* and *F. oxysporum* f. sp. *lactucae* and the VOCs emitted by *Bacillus* species. Further studies are needed to explore this antifungal potential when inoculated *in situ*, as well as to unravel the antifungal mechanism of the most important VOCs with antagonistic activity.

Conflict of Interest

No conflict of interest declared.

References

Andersen, R.A., Hamilton-Kem, T.R., Hidelbrand, D.F., McCraken, C.T. Jr, Collins, R.W. and Fleming, P.D. (1994) Structure-antifungal activity relationship among C6 and C9 aliphatic aldehydes, ketones and alcohols. *J Agric Food Chem* **42**, 1563–1568.

Ando, H., Hatanaka, K., Ohata, I., Yamashita-Kitaguchi, Y., Kurata, A. and Kishimoto, N. (2012) Antifungal activities of volatile substances generated by yeast isolated from Iranian commercial cheese. *Food Control* **26**, 472–478.

Arrebola, E., Sivakumar, D. and Korsten, L. (2010) Effect of volatile compounds produced by *Bacillus* strains on postharvest decay in citrus. *Biol Control* **53**, 122–128.

Bais, H.P., Fall, R. and Vivanco, J.M. (2004) Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by Pseudomonas syringae is facilitated by biofilm formation and surfactin production. *Plant Physiol* **134**, 307–319.

Baysal, O., Lai, D., Xu, H.H., Siragusa, M.C., Alıskan, M., Carimi, F., da Silva, J.A. and Tör, M. (2013) A proteomic approach provides new insights into the control of soil-borne plant pathogens by *Bacillus* species. *PLoS ONE* **8**, e53182.

Bloemberg, G.V. and Camacho Carvajal, M.M. (2006) Microbial interactions with plants: A hidden world? In *Soil Biology, Vol. 9 Microbial Root Endophytes* ed. Schulz, B., Boyle, C. and Sieber, T.N. pp. 321–336. Heidelberg, Germany: Springer-Verlag Berlin.

Börjesson, T., Stöllman, U. and Schnürer, J. (1990) Volatile metabolites and other indicators of *Penicillium aurantiogriseum* growth on different substrates. *Appl Environ Microbiol* **56**, 3705–3710.

Chaurasia, B., Pandey, A., Palni, L.M.S., Trivedi, P., Kumar, B. and Colvin, N. (2005) Diffusible and volatile compounds produced by an antagonistic *Bacillus subtilis* strain cause structural deformations in pathogenic fungi *in vitro*. *Microbiol Res* **160**, 75–81.

Chaves-López, C., Paparella, A., Tofalo, R. and Suzzi, G. (2011) Proteolytic activity of *Saccharomyces cerevisiae* strains associated with Italian dry-fermented sausages in a model system. *Int J Food Microbiol* **150**, 50–58.

Chunmei, J., Junling, S., Yanlin, L. and Chengyong, Z. (2014) Inhibition of *Aspergillus carbonarius* and fungal contamination in table grapes using *Bacillus subtilis*. *Food Control* **35**, 41–48.

Falcao, L.L., Silva-Werneck, J.O., Vilarinho, B.R., da Silva, J.P., Pomella, A.W.V. and Marcellino, L.H. (2014) Antimicrobial and plant growth-promoting properties of the cacao endophyte *Bacillus subtilis* ALB629. *J Appl Microbiol* **116**, 1584–1592.

Fernando, D.W.G., Ramarathnama, R., Krishnamoorthy, A.S. and Savchuka, S.C. (2005) Identification and use of potential bacterial organic antifungal volatiles in biocontrol. *Soil Biol Biochem* **37**, 955–964.

Fialho, M.B., Duarte de Moraes, M.H., Tremocoldi, A.R. and Pascholati, S.F. (2011) Potential of antimicrobial volatile organic compounds to control *Sclerotinia sclerotiorum* in bean seeds. *Pesqui Agropecu Bras* **46**, 137–142.

Fiddaman, P.J. and Rossall, S. (1993) The production of antifungal volatiles by *Bacillus subtilis*. *J Appl Bacteriol* **74**, 119–126.

Griffith, W.G., Nicholson, J., Nenninger, A., Birch, N.R. and Hedfer, N.J. (2003) Witches' brooms and frosty pods: two major pathogens of cacao. *N Z J Bot* **41**, 423–435.

Ingram, L.O. and Buttke, T.M. (1984) Effect of alcohols on microorganisms. *Adv Microb Physiol* **25**, 253–300.

Kai, M., Effmert, U., Berg, G. and Piechulla, B. (2007) Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Arch Microbiol* **187**, 351–360.

Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B. and Piechulla, B. (2008) Bacterial volatiles and their action potential. *Appl Microbiol Biotechnol* **81**, 1001–1012.

Martens, H. and Naes, T. (1989) *Multivariate Calibration*. New York, NY: John Wiley and Sons.

Maruzzella, J.C. (1961) Antimicrobial substances from ferns. *Nature* **191**, 518–519.

Melnick, R.L., Suárez, C., Bailey, B.A. and Backman, P.A. (2011) Isolation of endophytic endospore-forming bacteria from *Theobroma cacao as* potential biological control agents of cacao diseases. *Biol Control* **57**, 236–245.

Munimbazi, C. and Bullerman, L.B. (1998) Isolation and partial characterization of antifungal metabolites of *Bacillus pumilus*. *J Appl Microbiol* **84**, 959–968.

Scott, J.C., McRoberts, D.N. and Gordon, T.R. (2014) Colonization of lettuce cultivars and rotation crops by *Fusarium oxysporum* f. sp. *lactucae,* the cause of fusarium wilt of lettuce. *Plant Pathol* **63**, 548–553.

Shukla, R., Kumar, A., Singh, P. and Dubey, N.K. (2009) Efficacy of *Lippia alba* (Mill.) N.E. Brown essential oil and its monoterpene aldehyde constituents against fungi isolated from some edible legume seeds and aflatoxin B1 production. *Int J Food Microbiol* **135**, 165–170.

Timmusk, S., Grantcharova, N. and Wagner, E.G.H. (2005) *Paenibacillus polymyxa* invades plant roots and forms biofilms. *Appl Environ Microbiol* **71**, 7292–7300.

Trefz, P., Koehler, H., Klepik, K., Moebius, P., Reinhold, P., Schubert, J.K. and Miekisch, W. (2013) Volatile emissions from *Mycobacterium avium* subsp. *paratuberculosis* mirror bacterial growth and enable distinction of different strains. *PLoS ONE* **8**, e76868.

Turner, J.T. and Backman, P.A. (1991) Factors relating to peanut yield increases after seed treatment with *Bacillus subtilis*. *Plant Dis* **75**, 347–353.

Urbanek, A., Szadziewski, R., Stepnowski, P., Boros-Majewska, J., Gabriel, I., Dawgul, M., Kamysz, W., Sosnowska, D. *et al.* (2012) Composition and antimicrobial activity of fatty acids detected in the hygroscopic secretion collected from the secretory setae of larvae of the biting midge *Forcipomyia nigra* (Diptera: Ceratopogonidae). *J Insect Physiol* **58**, 1265–1276.

Wei-wei, L., Wei, M., Bing-yu, Z., You-chen, D. and Liu, F. (2008) Antagonistic activities of volatiles from four strains of *Bacillus* spp. and *Paenibacillus* spp. against soil-borne plant pathogens. *Agric Sci China* **7**, 1104–1114.

Wheatley, R.E. (2002) The consequences of volatile organic compound mediated bacterial and fungal interactions. *Antonie Van Leeuwenhoek* **81**, 357–364.

Yuan, J., Raza, W., Shen, Q. and Huang, Q. (2012) Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. *Cubense*. *Appl Environ Microbiol* **78**, 5942–5944.

Zhang, X., Li, B., Wang, Y., Guo, Q., Lu, X., Li, S. and Ma, P. (2013) Lipopeptides, a novel protein, and volatile compounds contribute to the antifungal activity of the

biocontrol agent *Bacillus atrophaeus* CAB-1. *Appl Microbiol Biotechnol* **97**, 9525–9534.

Zheng, M., Shi, J., Shi, J., Wanga, Q. and Li, Y. (2013) Antimicrobial effects of volatiles produced by two antagonistic *Bacillus* strains on the anthracnose pathogen in postharvest mangoses. *Biol Control* **65**, 200–206.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. UPGMA-based dendrogram from RAPD-PCR profiles obtained with M13 primer and REP-PCR profiles obtained with $(GTG)_5$ primer, of 46 studied isolates, selected as producers of mould inhibition percentage greater than 8%, at least for one fungal strain.

Table S1. Strains of *Bacillus* spp. and correlated species used for the antifungal activity screening.