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Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple

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ABSTRACT

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Aim: Test of *Bacillus subtilis* strain GA1 for its potential to control grey mould disease of apple caused by *Botrytis cinerea*.

Methods and Results: GA1 was first tested for its ability to antagonize *in vitro* the growth of a wide variety of plant pathogenic fungi responsible for diseases of economical importance. The potential of strain GA1 to reduce post-harvest infection caused by *B. cinerea* was tested on apples by treating artificially wounded fruits with endospore suspensions. Strain GA1 was very effective at reducing disease incidence during the first 5 days following pathogen inoculation and a 80% protection level was maintained over the next 10 days. Treatment of fruits with an extract of GA1 culture supernatant also exerted a strong preventive effect on the development of grey mould. Further analysis of this extract revealed that strain GA1 produces a wide variety of antifungal lipopeptide isomers from the iturin, fengycin and surfactin families. A strong evidence for the involvement of such compounds in disease reduction arose from the recovery of fengycins from protected fruit sites colonized by bacterial cells.

Conclusions: The results presented here demonstrate that, despite unfavourable pH, *B. subtilis* endospores inoculated on apple pulp can readily germinate allowing significant cell populations to establish and efficient *in vivo* synthesis of lipopeptides which could be related to grey mould reduction.

Significance and Impact of the Study: This work enables for the first time to correlate the strong protective effect of a particular *B. subtilis* strain against grey mould with *in situ* production of fengycins in infected sites of apple fruits.

Keywords: apple, *Bacillus subtilis*, biological control, biopesticide, fengycin, grey mould, lipopeptide.

INTRODUCTION

Treatment with synthetic fungicides is the primary mean of reducing post-harvest losses that have been estimated to 50% worldwide (Wilson *et al.* 1993). However, the development of fungicide-resistant strains of pathogens, the detection of undesirable chemical residues in the food chain and the de-registration of some of the most effective fungicides have intensified the search for safer approaches to efficiently control post-harvest decay caused by microbial infections (Wilson *et al.* 1991; El-Ghaouth 1997). Among the alternatives, biological control through the use of natural antagonistic microorganisms has been extensively studied and some yeast, fungal and bacterial strains have been shown to be

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effective against various post-harvest pathogens (Wisniewski and Wilson 1992; Pusey *et al.* 1993). Several strains belonging to the genus *Bacillus* and particularly to *B. subtilis* and the closely related *B. amyloliquefaciens* species were reported effective for the biocontrol of multiple plant diseases caused by soilborne (Asaka and Shoda 1996; Chen and Wu 1999; Harris and Adkins 1999) or post-harvest pathogens (Ferreira *et al.* 1991; Sholberg *et al.* 1995; Mari *et al.* 1996).

Antibiotic production by some bacteria plays a major role in disease suppression (Raaijmakers *et al.* 2002). So far, Gram-negative bacteria, especially *Pseudomonas* strains, have been intensively investigated with regard to the production of antimicrobial metabolites (Keel *et al.* 1990; Thomashow *et al.* 1990; Howell *et al.* 1993; Whipps 2001). However Gram-positive bacteria and especially strains of *Bacillus subtilis* also produce a variety of antibacterial and antifungal antibiotics such as zwittermicin-A (He *et al.* 1994), kanosamine (Stabb *et al.* 1994) and lipopeptides from the surfactin, iturin and fengycin families. These latter amphiphilic cyclic peptides were already suggested to be involved in plant disease reduction following treatment with *Bacillus* strains (McKeen *et al.* 1986; Gueldner *et al.* 1988; Ferreira *et al.* 1991; Leifert *et al.* 1995; Yoshida *et al.* 2001; Yu *et al.* 2002). They are composed of seven (surfactins and iturins) or 10 α-amino acids (fengycins) linked to one unique β-amino (iturins) or β-hydroxy (surfactins and fengycins) fatty acid. The length of this fatty acid chain may vary from C-13 to C-16 for surfactins, from C-14 to C-17 for iturins and from C-14 to C-18 in the case of fengycins. Different homologous compounds for each lipopeptide family are thus usually co-produced (Jacques *et al.* 1999; Akpa *et al.* 2001). Iturins and fengycins display a strong antifungal activity and are inhibitory for the growth of a wide range of plant pathogens (Peypoux *et al.* 1978; Isogai *et al.* 1982; Loeffler *et al.* 1986; Vanittanakom *et al.* 1986; Phae *et al.* 1990; Klich *et al.* 1994). Surfactins are not fungitoxic by themselves but retain some synergistic effect on the antifungal activity of iturin A (Maget-Dana *et al.* 1992).

Several strains of *B. subtilis* of various origins were isolated in our laboratory on the basis of antibiotic production. When used as seed treatment, some of them were shown to alleviate seedling diseases presumably through direct antibiosis against the soilborne pathogen (unpublished results). In this work, one particular *B. subtilis* strain named GA1 was first tested for its ability to antagonize *in vitro* the growth of a wide variety of plant pathogenic fungi. GA1 was further studied for its potential to reduce grey mould disease of apple caused by the necrotrophic pathogen *Botrytis cinerea* during post-harvest storage and special emphasis was placed on the comprehension of the mechanisms specifically involved in the protection observed through the evaluation of lipopeptide production by the strain.

MATERIALS AND METHODS

Microbial strains

Bacillus subtilis strain GA1 was isolated from strawberry fruits by the Laboratorio Vitrocoop Cesana, Italy. The strain was maintained on Plate Count Agar (PCA) medium (Becton Dickinson and Company, Le pont de Claix, France) at 4°C before experimental use, and stored at −80°C in cryotubes according to the manufacturer recommendations (Microbank, Prolab Diagnostic, Richmond Hill, Canada) for long-term storage. With the exception of *Aspergillus flavus* MUCL14109 (collection of the Catholic University of Louvain, Belgium), all fungal strains were kindly provided by Dr P. Lepoivre from the Phytopathology Unit of the Gembloux Agricultural University, Belgium. Fungi listed in Table 1 were maintained on Potato Dextrose Agar (PDA, Becton Dickinson and Company) at 20°C before use. *B. cinerea* conidia were conserved in glycerol 10% (v/v) at −40°C.

Microbial inocula preparation for *in vivo* assays

Bacterial endospore suspensions used in biocontrol experiments were prepared from 72-h-old cultures of *B. subtilis* GA1 grown at 30°C in agitated flasks (1 l, 180 rev min^{-1}) in 200 ml of a culture medium optimized for lipopeptide production (named Opt medium) and described by Jacques *et al.* (1999). Cultures were centrifuged at 35 000 *g* for 20 min and the biomass pellet was washed twice in sterile

Table 1 Antagonism developed by *Bacillus subtilis* strain GA1 on Potato Dextrose Agar plates against various fungal pathogens. Data are expressed as the percentage of reduction of mycelium expansion compared with control plates without bacteria and represent mean values ± S.D. from four repeats

Fungal pathogens	Mycelium growth inhibition (%)
Soilborne	
Fusarium graminearum	63 ± 3·1
Fusarium oxysporum	58 ± 2·2
Pythium ultimum	45 ± 2·0
Rhizoctonia solani	56 ± 2·8
Rhizopus sp.	45 ± 2·7
Post-harvest, foliar	
Alternaria sp.	55 ± 3·3
Aspergillus flavus MUCL14109	55 ± 2·2
Aspergillus niger	51 ± 1·5
Botrytis cinerea	70 ± 3·5
Gaeumannomyces sp.	30 ± 1·2
Mucor sp.	46 ± 1·8
Penicillium expansum	24 ± 0·7
Trichoderma harzanium	43 ± 2·1
Trichoderma reesei	42 ± 1·7

saline water (0·85% NaCl). Residual vegetative cells were killed by incubation at 80°C for 12 min. Endospores were recovered by centrifugation and their concentration was determined by plate count on PCA medium. They were then resuspended in sterile distilled water to obtain the final desired concentration.

B. cinerea was grown to sporulation on an oat-based medium (oatmeal 45 g l^{-1}; agar 15 g l^{-1}) at 25°C. The pathogen inoculum was prepared by harvesting conidia from 10-day-old cultures in sterile peptone water (Bactopeptone 1 g l^{-1}; NaCl 9 g l^{-1}; Tween 80 0·02%, v/v). After removing mycelial debris by filtration through several layers of cheese cloth, the suspension was centrifuged for 5 min at 5000 *g* and the conidia were resuspended in an adequate volume of sterile distilled water to obtain the desired final concentration determined microscopically by the use of a Burker counting cell.

In vitro antagonism experiments

Strain GA1 was tested for its ability to inhibit the growth of various fungal plant pathogens in Petri dishes on PDA medium supplemented with bromocresol green (used as pH indicator) at a concentration of 40 mg l^{-1}. The bacterium was streaked on the edge of the plates and mycelial plugs (5 mm) of each fungus were deposited in the centre, approx. 3·5 cm from the bacterial colonies. Fungal pathogens with slow growth were introduced 24 h prior to the bacteria. Fungal growth inhibition was evaluated after incubation of the plates for 3 or 5 days at 25°C and was expressed as the percentage of reduction of mycelium expansion compared with control plates without bacteria. Mean values and standard deviations were calculated from five replicate plates used for each fungal strain. The same method was used to test the antagonistic effect of cell-free culture supernatant. In this case, 10-μl aliquots of filter (0·2 μm) sterilized supernatant samples were dispensed in wells (performed with a sterile cork borer, diameter 6 mm) made in the gelified medium 24 h following mycelial plug deposition.

Grey mould disease reduction by strain GA1

Non-mature apple fruits (variety Jonagold) used in all experiments were carefully selected on the basis of their size (70-mm diameter) and on the absence of any disease or wounding symptoms. Fruits were surface disinfected by dipping in sodium hypochlorite 0·016 mol l^{-1} for 3 min, rinsed three times with sterile distilled water and dried under filter-sterilized air flow. Six millimetre wide and 3 mm deep wells were then artificially created with a sterile cork borer. Fruits were treated with the bacterial antagonist by adding 50 μl of cell or endospore suspension containing either 2×10^6, 2×10^7, or 2×10^8 endospores ml^{-1} depending on the experiment, in each wounded site 24 h prior to pathogen challenge. Infection with *B. cinerea* was realized in all cases 24 h after treatment with *Bacillus*, by adding the same volume of a conidial suspension prepared as described above in order to introduce 10^5 conidia per site. Such an inoculum induced a reliable and relatively high infection level on non-treated fruits with mould symptoms visible after 5 days of incubation (data not shown). Two other treatments were also introduced in every independent experiment: healthy controls corresponding to apples that were only inoculated with 50 μl of sterile distilled water and disease control fruits challenged with the pathogen but not treated with the bacterial antagonist.

Five fruits were used for each treatment in every independent assay. Treated fruits were incubated in a laminar air-flow cabinet at 22°C and disease incidence was evaluated 6, 15 and 21 days after pathogen challenge based on the diameter of spreading grey mould lesions that developed around infected sites. Results were expressed as disease reduction percentage $P = (Dt-De)/Dt-K$ where K is the wound diameter (6 mm) and Dt and De are lesion diameters measured on disease controls and on treated fruits, respectively. Disease reduction percentages are mean values calculated from four independent repeats and thus on a total of 40 infection sites per treatment. The software MINITAB was used for statistical analyses. The homogeneity of variances was tested by ANOVA and data from experiments with the same set-up were pooled for analysis when interaction between experiment and treatment was not significant at $P = 0{\cdot}05$. Means from the different treatments were compared by the method of Newman and Keuls (least significant difference at $\alpha = 0{\cdot}05$).

Disease reduction by heat-killed cells and lipopeptide extract

Biocontrol experiments were also carried out with heat-killed *Bacillus* cells and with a lipopeptide-enriched extract. In the first case, a bacterial suspension containing both vegetative cells and endospores was obtained after growth of strain GA1 for 48 h at 30°C in the Opt medium. This suspension was washed as described above, adjusted to reach a final concentration similar to the one used to test living endospores and autoclaved at 121°C for 30 min. The absence of growth was checked on PCA medium before use as described for endospore suspension. The lipopeptide-enriched extract was obtained from 20 ml of the corresponding cell-free supernatant after purification on a ISOLUTE C-18 CE type cartridge (International Sorbent Technology Ltd., Hengoed, UK) following the method described by Razafindralambo *et al.* (1993). Lipopeptides were resolubilized in $NaHCO_3$ 0·1 mol l^{-1}. Five fruits were treated with 25 μl of this solution added in wells 24 h before

pathogen challenge. Healthy and disease controls were treated with the same volume of $NaHCO_3$ 0·1 mol l^{-1}. Disease reduction assessments were realized as described for treatment with endospore suspensions.

Characterization of lipopeptides produced *in vitro* by strain GA1

The various homologous compounds and isoforms were characterized by reverse phase high performance liquid chromatography (HPLC) as described by Akpa and collaborators (2001) using specific elution gradients for each lipopeptide family. Individual compounds were thus first identified on the basis of their retention times compared with those of pure products. Purified fengycins, iturins and surfactins were available in our laboratory as they were previously obtained from large-scale purification of products synthesized by other *B. subtilis* strains by applying the method described by Razafindralambo and associates (1993). The identity of these pure individual homologues and isoforms was obtained by comparing their M_r deduced from the m/z ratio of $[M+H]^+$ ions detected in electrospray ionisation mass spectrometry with data from the literature (Hbid 1996; Leenders *et al.* 1999; Schneider *et al.* 1999). HPLC peak areas were also used to quantify the different lipopeptides produced by strain GA1 on the basis of values obtained for standards.

Evaluation of *Bacillus* cell populations on fruits

Apple pulp samples were collected every 24 h during the first 5 days after inoculation of the pathogen. Samples were harvested with a sterile scalpel in a 1-cm zone around inoculated sites (1·5-cm depth) and homogenized with a pre-cooled mortar and pestle. Samples were resuspended in sterile peptone water, serially diluted in peptone water, plated onto PCA medium and incubated for 36 h at 30°C. This incubation time was necessary for a reliable detection and counting of *Bacillus* colonies identified on the basis of their morphological aspect. These typical colonies were in the large majority among the total CFUs. Data are expressed as total *Bacillus* cell number per inoculated site and results presented for each time point are mean values obtained from the analyses of five different sites on different fruits.

Identification and quantification of lipopeptides recovered from fruits

Six days after challenge with the pathogen, 2-g samples of tissues were collected around the wells that served or not (controls) for the inoculation of *B. subtilis*. Samples were homogenized in a mortar and resuspended in 50 ml of $NaHCO_3$ 0·1 mol l^{-1}, pH 7. This suspension was stirred for 30 min at 4°C and centrifuged 20 min at 35 000 *g*. The resulting supernatant was submitted to solid-phase extraction on ISOLUTE C-18 cartridge and further analysed for its lipopeptide content by HPLC as described above for the characterization of compounds produced *in vitro*. Lower limit for detection of fengycins and iturins were about 0·3 μg g^{-1}. Mean values of lipopeptide amounts and standard deviations were calculated from analyses of five samples collected from different fruits and this quantification was repeated once in another independent experiment with similar results.

RESULTS

In vitro antagonism developed by *B. subtilis* GA1

Bacillus subtilis strain GA1 was first tested for its ability to antagonize the growth of a wide variety of plant pathogenic fungi responsible for diseases of economical importance. Following *in vitro* assays on solid medium, the grey mould causing agent *Bo. cinerea* appeared to be one of the most sensitive species with a 70% relative inhibition of mycelial growth (Table 1). As illustrated in Fig. 1a, the antagonism developed by *B. subtilis* GA1 against *Botrytis* was associated with the formation of a white precipitate surrounding bacterial colonies and in front of the mycelium inhibition zone. This suggested the excretion of fungitoxic compounds which precipitate in contact with the acidified medium induced by the *B. cinerea* growth. This acidification is visualized by colour change of PDA supplemented with bromocresol green which turned to yellow in the zone colonized by the fungus. The synthesis of compounds inhibitory to the growth of *Botrytis* by strain GA1 was further exemplified by testing filter-sterilized crude supernatant obtained from culture in the Opt medium. Twenty microlitres of this solution was sufficient to clearly inhibit mycelium expansion (Fig. 1b).

Reduction of grey mould of apple and evidence for the production of antifungal compounds

Treatment of fruits with endospores of strain GA1 was very effective at reducing disease incidence during the first 5 days following pathogen inoculation (Fig. 2a) compared with non-bacterized but infected fruits. A 80% protection level was maintained over the next 10 days and a disease reduction of about 40% was conserved after 21 days of incubation. As an example, disease control observed 15 days after pathogen challenge is illustrated in Fig. 3. Our results also showed that the endospore concentration in the bacterial inoculum must be at least two hundred times higher than the concentration of pathogen conidia used for infection to guarantee a high protection level during the first

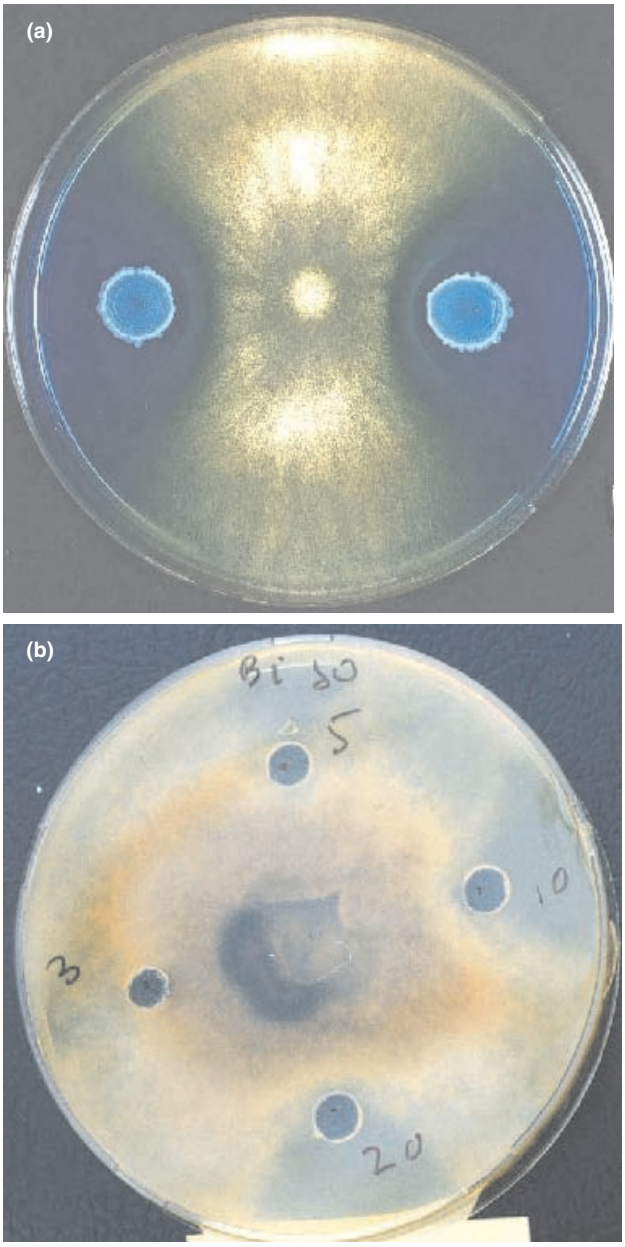

Fig. 1 *In vitro* growth inhibition of the pathogen *Botrytis cinerea* caused by *Bacillus subtilis* GA1 on Potato Dextrose Agar (PDA) medium supplemented with bromocresol green as pH indicator (a) and by cell-free culture supernatant (b) added in increasing quantities from 3 to 20 μl on PDA. The bacterial/supernatant sample and the fungus were inoculated at the same time and the antagonism was scored after incubation of the plates for 4 days at 25°C

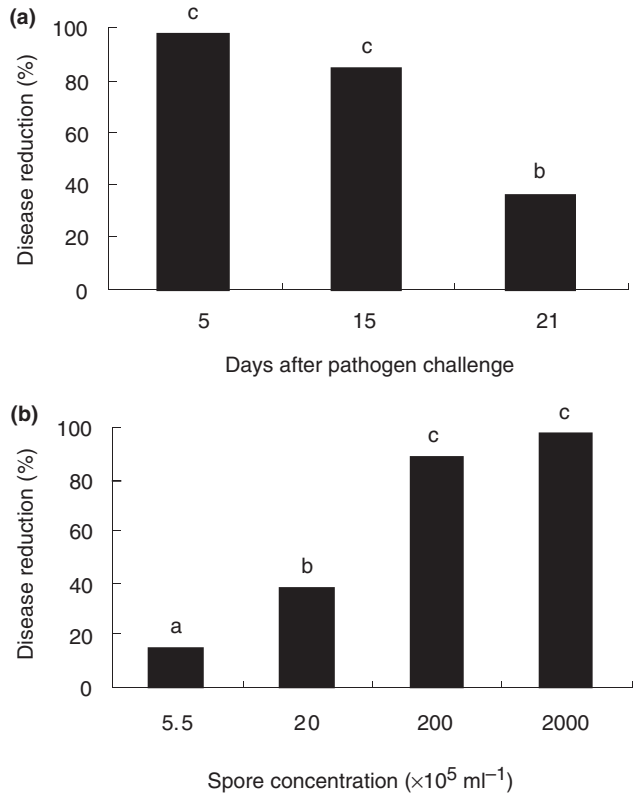

Fig. 2 Protection obtained by treatment of apple fruits with endospore suspensions of *Bacillus subtilis* GA1as a function of time (a) and as function of the concentration of the antagonist inoculum after 6 days (b). In all experiments, the pathogen was introduced at 10^5 conidia ml^{-1} in wounded sites pre-inoculated (24 h before) with the antagonist. The homogeneity of variances was tested by ANOVA and data from experiments with the same set-up were pooled for analysis as interactions between experiment and treatment were not significant at $P = 0{\cdot}05$. Means from the different treatments were compared by the method of Newman and Keuls (least significant difference at $\alpha = 0{\cdot}05$). Values represented by bars with different letters are statistically different. Letters other than 'a' mean that disease reduction is statistically significant

few days after infection (Fig. 2b). When tested under the same conditions, vegetative cells were slightly but not significantly less effective than endospores at protecting fruits as 91 ± 4 and 64 ± 6% disease reductions were observed after 6 and 15 days, respectively.

Results from three independent experiments also showed a strong protective effect of a methanolic extract obtained after solid-phase extraction of the GA1 culture supernatant (25 μl corresponding to 1·6 ml of crude supernatant). This protection level was similar to the one obtained after treatment with live bacteria over the 21-day sampling period (Fig. 4). Virtually no grey mould symptom was indeed visible during 15 days following pathogen inoculation and a significant 60% reduction of disease incidence could still be conserved after 21 days. By contrast, heat-killed *Bacillus* cells inoculated at the same concentration as living cells were not effective at protecting fruits.

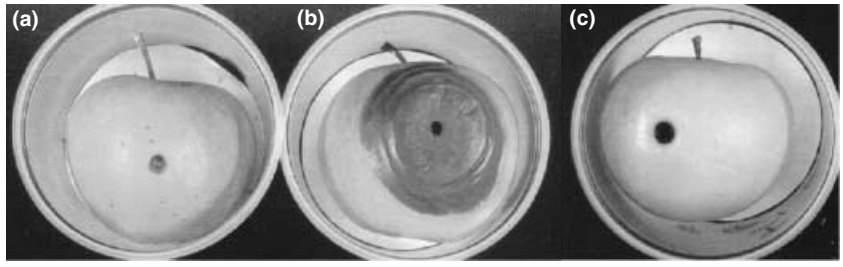

Fig. 3 Examples of fruits observed 15 days after treatment with sterile distilled water (healthy control, a), with sterile distilled water and challenged with *Botrytis cinerea* (disease control, b) and with *B. subtilis* GA1 endospores (2×10^8 endospores ml^{-1}) 24 h prior to pathogen challenge (c)

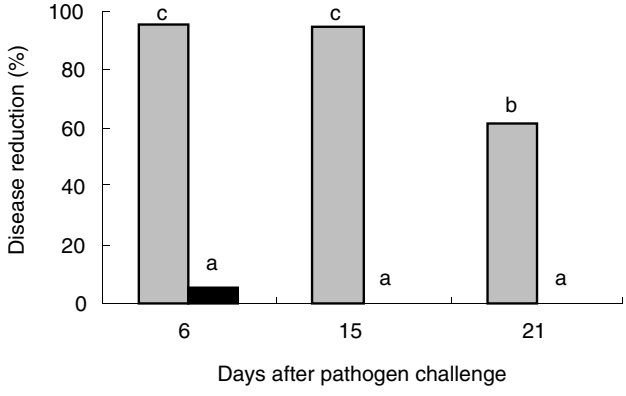

Fig. 4 Protection obtained by treatment of apple fruits with supernatant extract (grey bars) and heat-killed cell suspension (dark bars) obtained after growth of *Bacillus subtilis* GA1 for 48 h in the Opt culture medium. The homogeneity of variances was tested by ANOVA and data from experiments with the same set-up were pooled for analysis as interactions between experiment and treatment were not significant at $P = 0{\cdot}05$. Means from the different treatments were compared by the method of Newman and Keuls (least significant difference at $\alpha = 0{\cdot}05$). Values represented by bars with different letters are statistically different. Letters other than 'a' mean that disease reduction is statistically significant

In vitro synthesis of lipopeptides

As lipopeptides of the surfactin, iturin and fengycin groups are among the most bioactives non-polar antibiotics produced by many strains of the *Bacillus* genus, their production by *B. subtilis* GA1 in the Opt medium was studied. Members of the different families were detected in the methanolic extract obtained from the culture supernatant. They were identified and quantified by HPLC on the basis of their retention times and corresponding peak areas compared with purified molecules used as standards. A wide variety of homologous compounds were detected within each group i.e. fengycins A C-14 to C-18 and B C-17, iturins A C-14 to C-17 and surfactins C-13 to C-15 (Table 2). Total amounts of fengycins, iturins and surfactins produced per litre after 72 h of growth were 520 mg, 460 mg and 340 mg, respectively (mean values calculated from four independent cultures).

Table 2 Lipopeptide homologues and isoforms produced by *Bacillus subtilis* GA1 after growth for 72 h in Opt medium. Compounds were identified and quantified by reverse-phase HPLC analyses using elution gradient specific for the separation of the three families on the basis of their retention times compared with purified standards. The relative proportions of the various homologues in each family were calculated on the basis of their corresponding peak areas

Lipopeptides	Retention times (min)	Relative proportions (%)
Fengycins		
A C_{14}	6·0	3
A C_{15}	7·8	12
A C_{16}	10·4	31
A C_{16}	14·1	10
A C_{17}	14·5	21
B C_{17}	17·7	4
B C_{17}	20·5	9
A C_{18}	24·1	10
Iturins		
A nC_{14}	8·8	44
A aiC_{15}	11·8	16
A $isoC_{15}$	12·5	24
A $isoC_{16}$	20·4	4
A nC_{16}	23·2	8
A aiC_{17}	32·5	4
Surfactins		
C_{13}	10·5	11
iC_{14}	13·2	8
nC_{14}	13·9	32
iC_{15}+ nC_{15}	16·9	49

Time-course accumulation studies showed that surfactins were mainly produced during exponential growth with the higher value for cell productivity observed after 12 h (Fig. 5). By contrast, iturins and fengycins were mostly synthesized once the culture entered the stationary phase to reach optimal production rate after 72 h.

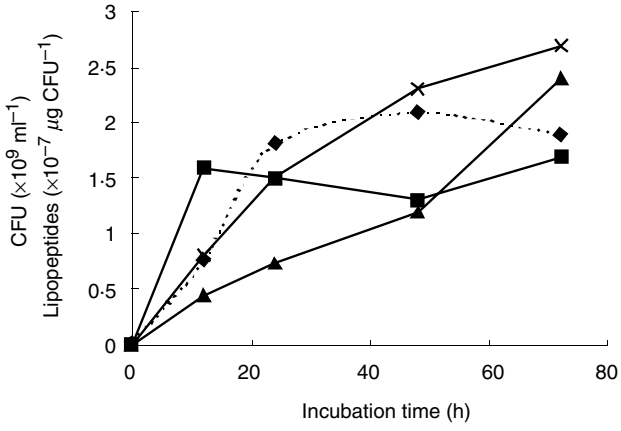

Fig. 5 Evolution of biomass (--◆--) and lipopeptide production by *Bacillus subtilis* GA1 cells during growth for 72 h in the Opt medium. Total amounts of fengycins (–×–), iturins (–▲–) and surfactins (–■–) were calculated by summing HPLC peak areas corresponding to the various homologues in each family and subsequent comparison with areas obtained for purified molecules used as standards

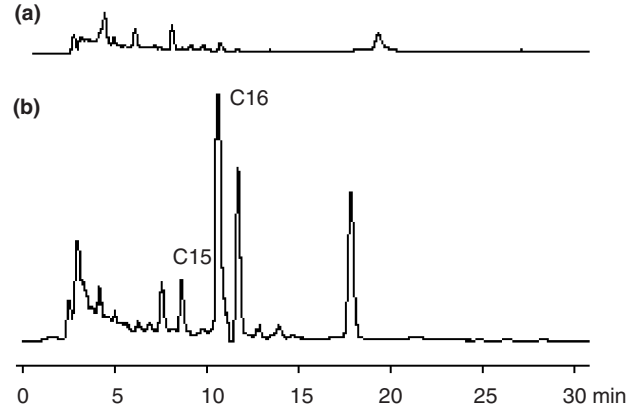

Fig. 6 HPLC chromatograms of methanolic extracts and obtained from apple tissues collected around inoculation sites 6 days after challenge with the pathogen on control fruits (a) and on protected fruits previously treated with GA1 endospores (b). Samples were analysed with elution conditions specific for fengycins and retention times of peaks identified as C15 and C16 in B corresponded to those obtained for fengycins A C_{15} and A C_{16} produced by the strain *in vitro* (Table 2)

Bacterial growth and lipopeptide production on fruits

The low pH value of approx. 3 (measured with a pH surface electrode) of apple pulp could be unfavourable for *in situ* endospore germination and growth of the GA1 strain. However, plate counts realized on samples from inoculated sites showed a significant development of *Bacillus* cells during the first 5 days following inoculation. *In situ* growth was characterized by a 24-h lag-phase followed by a constant increase of cell population from $1{\cdot}25 \times 10^6$ CFU to values up to 9×10^7 CFU per inoculation site observed after 120 h (mean values from five independent samples).

In an attempt to correlate pathogen growth inhibition with *in situ* production of antifungal compounds, concentrated lipopeptide extracts were prepared from 2-g samples of fruit tissue collected around the inoculation site after 120 h of incubation. HPLC analyses of samples from 10 fruits in two independent experiments showed significant amounts of fengycins at concentrations of $1{\cdot}5 \pm 0{\cdot}2$ $\mu g\ g^{-1}$ FW from fruits inoculated with the bacterium and challenged with the pathogen. In each case, fengycins A C-15 and A C-16 were the main homologues detected (Fig. 6). Iturins were only measured in very low quantities below 70 ng g^{-1} FW and surfactins were not detected.

DISCUSSION

Results presented in this paper describe the ability of *B. subtilis* strain GA1 to protect wounded apple fruits against grey mould disease caused by *B. cinerea*. Treatment with endospores or vegetative cells from strain GA1 allowed a very effective disease control over the first 15 days after pathogen challenge. These results confirm the potential of *B. subtilis* species for the control of post-harvest diseases already reported for other fruits (Pusey *et al.* 1988; Ferreira *et al.* 1991). From a technological point of view, the efficacy of endospores is interesting as they are more stable than vegetative cells and maintain viability for years under appropriate storage conditions of the product. Endospores are also far more resistant to drying processes for powder formulation and are relatively easy to produce with industrial fermentation technology (Brannen and Kenney 1997). However, additional experiments are needed to evaluate whether the loss of efficiency observed after 21 days is associated with a decrease of the beneficial strain population and/or with the spontaneous or fungal-induced degradation of the active bacterial metabolites involved in pathogen inhibition. Such results are required to assess the number and timing of treatments leading to an optimal long-term control of the disease.

The biocontrol potential of GA1 was related to the strong antagonism developed *in vitro* by the strain and by its cell-free culture filtrate against *B. cinerea*. Moreover, the protection level provided by the bacterium increased proportionally with the number of bacterial cells used for treatment. This suggested that antibiosis because of the production of fungitoxic compounds by strain GA1 could play a major role in the inhibition of the disease. Although competition for nutrients cannot obviously be ruled out, other mechanisms of biological control such as host-resistance induction were probably of minor importance as

this later phenomenon is independent of bacterial population size above a defined threshold level (van Loon *et al.* 1998). In that way, further analysis of culture broth extracts revealed that GA1 produces a wide variety of lipopeptides. Iturins are a group of antifungal lipopeptides that consist of iturin A-E, bacillomycin D, F and L and mycosubtilin (Besson *et al.* 1976; Peypoux *et al.* 1976; Besson *et al.* 1977; Peypoux *et al.* 1978). Strain GA1 mainly synthesizes isomers of the iturin A sub-group that were differentiated on the basis of the n-, iso- or anteiso- form of their fatty acid side chain. Both A- and B-types of fengycins varying by the substitution of an alanine residue by valine were detected with various lengths of the acyl side-chain from C-14 to C-18. Quantitatively, fengycins are the main fungitoxic lipopeptides produced by GA1 in the Opt medium. As surfactins (C-13 to C-15) are also synthesized, GA1 can be added to the limited number of *B. subtilis* strains reported to co-produce the three families and is, as far as we know, the first isolate described to synthesize so many different lipopeptides including isomers with long aliphatic chains that are potentially more bioactive (Jacques *et al.* 1999; Leenders *et al.* 1999).

Treatment of fruits with a lipopeptide-enriched extract of GA1 culture supernatant also provided a strong protective effect that was similar to the one observed with live cells. This gave a first indication for a role of these lipopeptides in the biocontrol activity of the strain. Further evidence arose from the detection of significant amounts of fengycins in fruit tissues colonized by bacterial cells. Total fengycin amounts recovered per inoculation site were about 3 μg. On the basis of bacterial populations present in inoculated sites, fengycin production per cell unit calculated after 5 days was thus approx. 3×10^{-8} μg CFU^{-1}. This productivity level is 8-fold lower than the one calculated after *in vitro* growth in the optimal Opt medium ($2{\cdot}5 \times 10^{-7}$ μg CFU^{-1}) but do reflect an effective *in vivo* synthesis of such compound. Moreover, the fengycin quantities recovered from fruits are in the range of those previously reported (from 0·4 to 9 μg) to be inhibitory for *in vitro* growth of *Botrytis* and other fungal pathogens (Loeffler *et al.* 1986; Vanittanakom *et al.* 1986; Hbid 1996). As the antifungal compounds were extracted from pathogen infected sites that did not develop grey mould symptoms, this *in situ* production of fengycins could be associated with inhibition of conidial germination or germ tube extension. Iturins were also detected but in lower amounts that did obviously not correspond to local concentrations sufficient to inhibit fungal development. Although the involvement of another antimicrobial compound cannot be excluded, our results suggest a crucial role for lipopeptides in disease reduction. The use of fengycin non-producing mutants of strain GA1 should confirm this hypothesis. Such a role of *Bacillus* lipopeptides in the reduction of various fungal plant diseases was already suggested on the basis of fungitoxic activity of the compounds isolated from *in vitro* culture broth (McKeen *et al.* 1986; Gueldner *et al.* 1988; Ferreira *et al.* 1991; Leifert *et al.* 1995; Yoshida *et al.* 2001; Yu *et al.* 2002). However, very few investigations reporting *in vivo* production of significant amounts of lipopeptides are available (Asaka and Shoda 1996). In this work, we associate the protective effect of a particular *B. subtilis* strain with *in situ* production of inhibitory amounts of fengycins in infected sites of fruits. It thereby reinforces the interest for these particular lipopeptides as active metabolites involved in the expression of *B. subtilis* biocontrol potential.

Results also showed that *in situ* bacterial populations readily increased during the first few days following inoculation. It means that the low pH value (around three) and the presence of particular carbohydrates and amino acids as carbon and nitrogenous sources specific for apple pulp did not represent an unfavorable medium for endospore germination and cell growth. This was supported by positive growth tests realized on a gelified medium that exclusively contained apple pulp juice (data not shown). However, the evolution of *Bacillus* cell populations suggested that inoculated endospores must adapt to the specific environment prior to germination and that further growth rate of vegetative cells was somewhat limited compared with that observed in an artificial medium. Such a low cell growth rate could be related to a more efficient production of fengycins compared with iturins and surfactins that were not detected. Indeed, production kinetic studies showed that fengycins were preferably synthesized by GA1 in the late logarithmic or early stationary phase corresponding to low cell growth rate in contrast with the two other lipopeptide groups that are clearly produced during exponential growth (surfactins) or later in the stationary phase (iturins). Our results are in agreement with investigations reported by Lin *et al.* (1999) showing that the transcription of fengycin synthetase genes was optimal at the end of the exponential phase.

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