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Biocontrol of the wheat pathogen *Zymoseptoria tritici* using cyclic lipopeptides from *Bacillus subtilis*

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Abstract Innovation toward ecofriendly plant protection products compatible with sustainable agriculture and healthy food is today strongly encouraged. Here, we assessed the biocontrol activity of three cyclic lipopeptides from *Bacillus subtilis* (mycosubtilin, M; surfactin, S; fengycin, F) and two mixtures (M + S and M + S + F) on wheat against *Zymoseptoria tritici*, the main pathogen on this crop. Foliar application of these biomolecules at a 100-mg L^{-1} concentration on the wheat cultivars Dinosor and Alixan, 2 days before fungal inoculation, provided significant reductions of disease severity. The best protection levels were recorded with the M-containing formulations (up to 82% disease reduction with M + S on Dinosor), while S and F treatments resulted in lower but significant disease reductions. In vitro and *in planta* investigations revealed that M-based formulations inhibit fungal growth, with half-maximal inhibitory concentrations of 1.4 mg L^{-1} for both M and M + S and 4.5 mg L^{-1} for M + S + F, thus revealing that the observed efficacy of these products may rely mainly on antifungal property. By contrast, S and F had no direct activity on the pathogen, hence suggesting that these lipopeptides act on wheat against *Z. tritici* as resistance inducers rather than as biofungicides. This study highlighted the efficacy of several lipopeptides from *B. subtilis* to biocontrol *Z. tritici* through likely distinct and biomolecule-dependent modes of action.

Keywords Wheat · *Zymoseptoria tritici* · Biocontrol · *Bacillus subtilis* · Lipopeptides · Mycosubtilin

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Introduction

Septoria tritici blotch (STB) caused by *Zymoseptoria tritici*, formerly known as *Mycosphaerella graminicola*, is currently the most damaging foliar disease on wheat crops worldwide, especially in regions with suitable climate conditions such as Western Europe (Jørgensen et al. 2014). Severe disease epidemics can reduce wheat yields by more than 50% (Ponomarenko et al. 2011). Because of its economic importance, experimental amenability, and growing interest within scientist and agronomist communities, *Z. tritici* has been considered as a model for the order Dothideales (Kema et al. 2008) and was recently ranked among the top ten fungal pathogens in the area of molecular plant pathology (Dean et al. 2012). *Z. tritici* is a hemibiotrophic fungus characterized by a symptomless period during which fungal hyphae grow between leaf mesophyll cells without inducing any host necrosis, followed by a short necrotrophic phase (of about 1 week) associated with an increase in fungal biomass, production of cell wall-degrading enzymes, necrosis development, and pycnidium formation (Kema et al. 1996; Siah et al. 2010a). The transition from biotrophy to necrotrophy takes place suddenly around 10 days after the infection process is initiated (Kema et al. 1996), but this time lapse may vary depending both on the cultivar infected and environmental conditions (Lovell

et al. 2004). Since host resistance against *Z. tritici* is not fully effective in most wheat cultivars, disease control relies mainly of the use of conventional fungicides. In Europe, farmers spend every year around 1.3 billion € on synthetic fungicides to protect wheat against diseases, mainly STB (Torriani et al. 2015). However, the use of these products is increasingly controversial because of their potential negative impacts on both the environment and the human health. In addition, the durability of chemical and genetic control strategies is regularly compromised in the field since *Z. tritici* frequently develops resistance to fungicide and circumvent host resistance (Cowger et al. 2000; Cheval et al. 2017). This is due to its high biological fitness degree resulting very likely from its frequent sexual reproduction and genetic recombination in the field (Siah et al. 2010b, 2013; El Chartouni et al. 2011).

The development of environment-friendly and less hazardous phytosanitary products is currently encouraged, especially in Europe, where several national action plans aiming at reducing the use of synthetic conventional fungicides in agriculture were set up (Ravensberg 2015). The use of biocontrol agents, also referred as biofungicides, to control plant pests and diseases is a promising alternative approach for plant protection. Today, biocontrol products hold just 5% of the worldwide crop protection market (approximately $3 billion in value), but this segment of the industry is growing and it is projected to increase by 8.84% annually, reaching more than 7% of the total crop protection market by 2025 (more than $4.5 billion in value) (Olson 2015). Various types of biocontrol agents, including beneficial microbes and their metabolites, plant and microbial cell wall extracts, and even minerals and ions, have been reported to impair attacks by plant bioaggressors (Siah et al. 2017). Among beneficial microbes, *Bacillus* spp., one of the commonly isolated endophytic bacteria, have received considerable attention since several species of this genera confer several positive effects to the plant such as promotion of plant growth and/or enhancement of protection against abiotic and biotic stresses (Perez-Montaño et al. 2014). Biocontrol effect of *Bacillus* species against pathogen attacks is mainly attributed to the production of cyclic lipopeptides, which are amphiphilic molecules consisting of a short peptide chain linked to a lipid tail (Ongena and Jacques 2008; Raaijmakers et al. 2010). Some *Bacillus* species, such as *Bacillus subtilis* and *Bacillus amyloliquefaciens*, may dedicate up to 8% of their genetic equipment to the synthesis of a wide array of antimicrobial compounds, including lipopeptides (Chen et al. 2009; Rückert et al. 2011). Bacterial lipopeptides are synthesized by multi-enzymatic proteins called non-ribosomal peptide synthetases, which confers considerable structural diversity to the molecules and results in the production of linear, branched, or cyclic low toxic compounds (Strieker et al. 2010; Deravel et al. 2014). According to their amino acid sequence, cyclic lipopeptides are divided into three families: iturins (mycosubtilin, iturin A, and bacillomycin), surfactin, and fengycin (Xun-Chao et al. 2013; Guo et al. 2014). Members of the iturin family are heptapeptides with a β-amino fatty acid, while members of the fengycin family, including the related plipastatin, are decapeptides with a β-hydroxy fatty acid (Romero et al. 2007; Jacques 2011). The surfactin family consists of heptapeptides containing a β-hydroxy fatty acid. Cyclic lipopeptides display a broad biological activity against several phytopathogens through direct antagonistic effect and/or the induction of plant immunity system (Ongena and Jacques 2008; Deravel et al. 2014; Farace et al. 2015; Chandler et al. 2015). Members of iturin and fengycin families are mainly known for their antifungal properties, while molecules from surfactin family are powerful biosurfactants also able to induce systemic resistance in plant (Touré et al. 2004; Ongena and Jacques 2008). Although bacterial cyclic lipopeptides have already been tested on a broad spectrum of pathogens, they have never been assessed against *Z. tritici*, despite the economic and environmental importance of this pathogen. In addition, no biocontrol active substance is available to date in Europe as plant protection product against *Z. tritici*, except laminarin (Vacciplant®, Goëmar, France), which is a low-molecular-weight polysaccharide registered as plant resistance inducer toward this pathogen. The aim of the present study was thus to investigate for the first time biocontrol activity on wheat against *Z. tritici* of mycosubtilin, surfactin, and fengycin lipopeptides produced by the *B. subtilis* strains BBG125, BBG131, and Bs2504, respectively, and two mixtures of them (mycosubtilin + surfactin and mycosubtilin + surfactin + fengycin). Furthermore, the mode of action of these lipopeptides was examined by assessing their direct activity toward *Z. tritici* using both in vitro and *in planta* bioassays.

Materials and methods

Lipopeptide production, purification, and analysis

The different lipopeptides used in this study were produced and purified using three different strains of *B. subtilis* (Table 1), according to Coutte et al. (2013). All lipopeptides, including surfactin, fengycin, and mycosubtilin, were produced using a specific corresponding strain. These lipopeptides produced by separate strains were then used to reconstitute lipopeptide mixtures (Table 1). After the production and purification process, the different lipopeptide fractions were lyophilized and the purity of the powder was determined using reversed-phase high-performance liquid chromatography (RP-HPLC) according to Coutte et al. (2010b) and Béchet et al. (2013) in comparison to external standards of these biomolecules supplied by Lipofabrik (Villeneuve d'Acsq, France) and Sigma-Aldrich (Saint-Louis, USA).

Table 1 Different lipopeptides used in this study and their producing *Bacillus subtilis* strains

Lipopeptide(s)	*B. subtilis* strain	Reference
Mycosubtilin	BBG125	Béchet et al. (2013)
Surfactin	BBG131	Coutte et al. (2010a)
Fengycin	Bs2504	Ongena et al. (2007)
Mycosubtilin + surfactin	Mix (50:50 *w*/*w*)	
Mycosubtilin + surfactin + fengycin	Mix (33:33:33 *w*/*w*/*w*)	

Mix lipopeptides were first produced by monoproducer strains and then purified and mixed together

Lipopeptide powders were dissolved in 0.1% dimethyl sulfoxide (DMSO) by taking into account the purity of every lipopeptide. For all further bioassays, equivalent volumes of DMSO (0.1%) were added to control treatments. The characterization of lipopeptide powders was carried out using high-performance liquid chromatography-mass spectrometry (HPLC-MS), according to Hamley et al. (2013).

Plant growth, treatment, and inoculation

Grains of the susceptible wheat cultivars Dinosor (Unisigma, Froissy, France) and Alixan (Limagrain, Saint-Beauzire, France) were pregerminated in Petri dishes (12 × 12 cm) on moist filter paper, according to Siah et al. (2010a). Germinated grains were placed into 3-L pots filled with universal loam (Gamm Vert, France). Six pots of 12 grains were used as replicates for each condition. The pots were placed in the greenhouse at 18 °C ± 2 °C with a day–night cycle of 16/8 h using supplementary illumination. After 3 weeks (third leaves from the base of plants fully expanded), plants of each pot were treated with 30 mL at 100 mg L^{-1} of each lipopeptide or mixture solution supplemented with 0.05% polyoxyethylene-sorbitan monolaurate (Tween 20, Sigma-Aldrich, USA) surfactant using a manual hand sprayer. Plants treated with epoxiconazole (triazole) at 100 mg L^{-1} (Opus®, BASF Agro SAS, France) were used as a fungicide reference control. Two days after treatments, plants of each pot were inoculated using a manual hand sprayer with 30 mL spore suspension (10^6 spores mL^{-1}) supplemented with 0.05% Tween 20. Dinosor and Alixan cultivars were inoculated with the *Z. tritici* strains T01193 and T02596, isolated in 2009 and 2014 from Northern France, respectively. These two strains were chosen because they showed in preliminary experiments a high level of virulence on each corresponding cultivar. Spore suspensions were produced on potato dextrose agar medium (PDA), according to Siah et al. (2010a). Both inoculated and non-inoculated control plants were pretreated with sterile distilled water containing Tween 20 surfactant alone. Immediately after inoculation, each pot was covered with a clear polyethylene bag for 3 days in order to ensure a water-saturated atmosphere compatible with good fungal germination. The disease severity was scored at 21 days post-inoculation (dpi) by assessing the percentage of the third leaf area covered by lesions (chlorosis or necrosis) bearing or not pycnidia.

In vitro antifungal assay

Direct activity of the lipopeptides was assessed using the strain T01193 in clear and sterile flat-bottomed polystyrene 96-well plates (Iwaki, Asahi techno glass, Japan), according to Siah et al. (2010c). Lipopeptides and mixtures were added to the medium at 50 °C following autoclaving. Plate wells were each filled with 150 μL of liquid glucose peptone medium (14.3 g L^{-1} dextrose (VWR), 7.1 g L^{-1} bacto peptone (Difco laboratories), and 1.4 g L^{-1} yeast extract (Merck)) supplemented with lipopeptides at 100, 33.3, 11.1, 3.7, 1.2, 0.4, 0.14, 0.05, 0.015, and 0.005 mg L^{-1} (final concentrations in 200 μL of medium). Aliquots of 50 μL containing 2.10^5 spores mL^{-1} of the *Z. tritici* strain T01193 were added to each plate well, according to Siah et al. (2010c). Eight wells were used as replicates for each condition. In each microplate, non-inoculated medium without lipopeptide, as well as inoculated medium without lipopeptide, were used as experimental controls. Two synthetic fungicides, bixafen (pyrazole-carboxamide) and epoxiconazole (triazole) (Sigma-Aldrich, France), were included in the assay as fungicide references. Plates were incubated for 6 days at 20 °C in the dark while being shaken at 140 rpm, after which fungal growth was measured using a plate reader (MRX, Dynex technologies) at 405 nm.

In planta cytological assay

Monitoring of spore germination and hyphal growth of the strain T02596 on the leaf surface of Alixan cultivar was performed using Fluorescence Brightener 28 (Calcofluor, Sigma-Aldrich), according to Siah et al. (2010a) with few modifications. Briefly, wheat leaf segments (4 cm) were harvested 1 and 5 dpi from control and plants sprayed or not with different lipopeptides and then immersed for 5 min in a solution of 0.1% (*w*/*v*) Calcofluor, 0.1 M Tris–HCl buffer pH 8.5. Three third leaf segments from different plants and different pots were used as replicates for each condition. Leaf segments

were then washed for 1 min with sterile distilled water. After drying in darkness at laboratory temperature, they were placed on a glass slide, covered with a cover slip and observed microscopically (Nikon, Eclipse 80i) under ultraviolet (UV) illumination. Pictures were taken with digital camera (DXM1200C) using image capture software (NIS-ELEMENTS BR). The percentage of germinated spores was calculated at 1 dpi on 100 different fungal spores on each leaf segment. The effect of lipopeptides on fungal hyphal growth was assessed at 5 dpi. Five classes of fungal growth events were recorded from 100 fungal spores on each leaf segment (class 1, non-germinated spore; class 2, germinated spore with small germ tube; class 3, germinated spore with developed germ tube; class 4, germinated spore with a strongly developed germ tube; class 5, germinated spore giving a strong hyphal growth).

Statistical analyses

Comparisons between rates of disease severity, *in planta* spore germination and *in planta* hyphal growth were carried out with the Tukey's test at a significance level of $P = 0.05$ using the XLSTAT software (Addinsoft, France). For in vitro assay, a half-maximal inhibitory concentration (IC_{50}) value was calculated from dose–response curve of each lipopeptide, mixture, or fungicide reference using the GraphPad Prism 7 software (GraphPad Software, Inc., USA). Correlation between protection efficacies found on the cultivar Dinosor inoculated with the strain T01193 (percentages of disease severity reduction compared to the non-treated inoculated control plants) and IC_{50} values obtained in vitro for tested lipopeptides or mixtures using the strain T01193 were assessed with the principal component analysis (PCA) based on the Pearson test using the XLSTAT software. All experiments were repeated twice in two independent assays, except the *in planta* hyphal growth bioassay which was performed in one biological experiment.

Results

Lipopeptide characterization

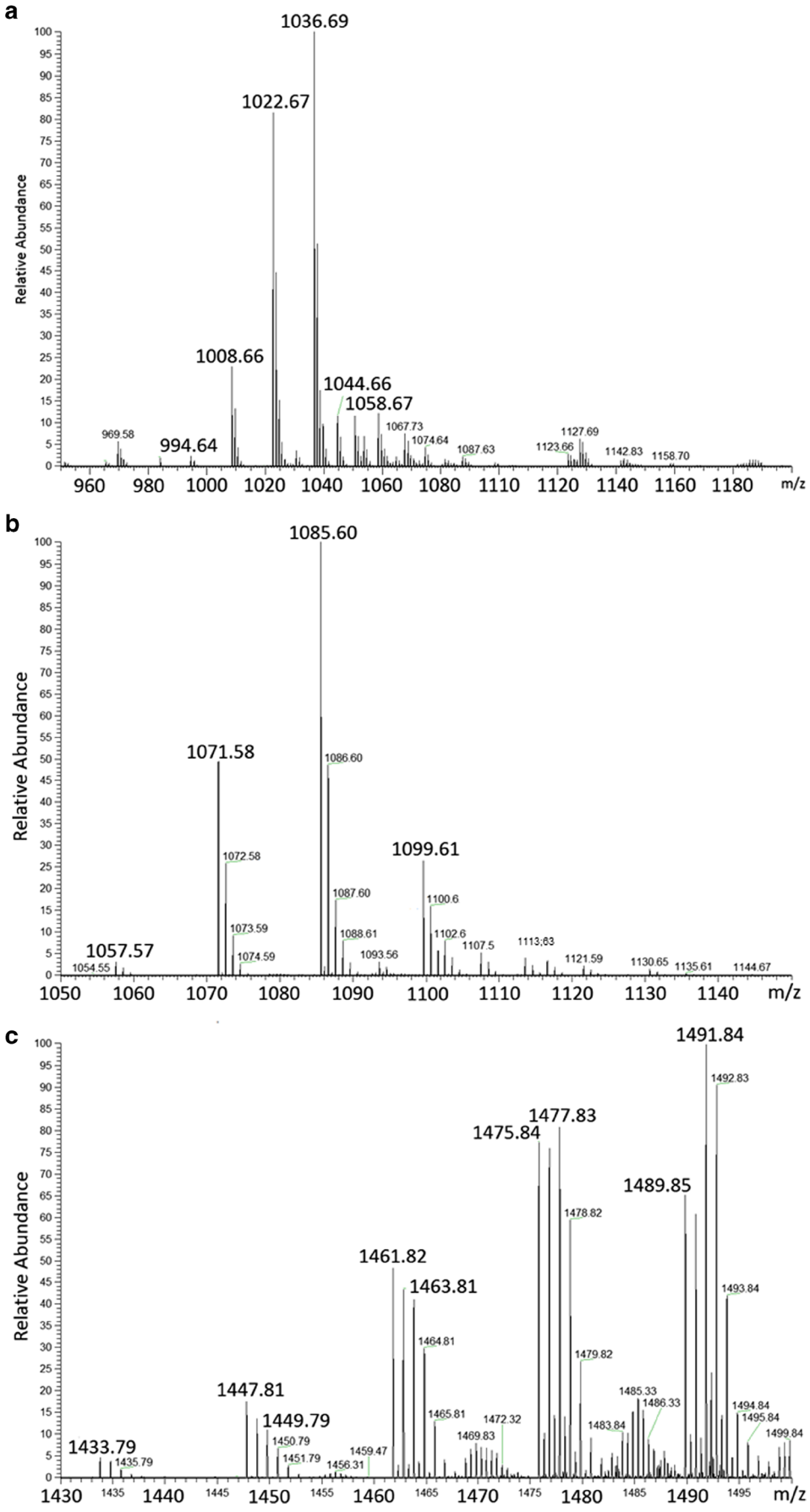
HPLC-MS analysis was carried out on the different samples of lipopeptides used in this study (Fig. 1). Results showed that surfactin is composed of different isoforms with fatty acid chain length from C_{12} to C_{16} carbons ($[M^+H]^+$ 994.64 = C_{12}; $[M^+H]^+$ 1008.66 = C_{13}; $[M^+H]^+$ 1022.67 and $[M^+Na]^+$ 1044.66 = C_{14}; $[M^+H]^+$ 1036.69; and $[M^+Na]^+$ 1058.67 = C_{15}). Mycosubtilin is composed of different isoforms with fatty acid chain from C_{15} to C_{18} carbons ($[M^+H]^+$ 1057.57 = C_{15}; $[M^+H]^+$ 1071.58 = C_{16}; $[M^+H]^+$ 1085.60 = C_{17}; $[M^+H]^+$ 1099.61 = C_{18}). Fengycin is composed of different isoforms of fengycins A and B with saturated fatty acid chain from C_{14} to C_{18} carbons ($[M^+H]^+$ 1435.79 = A-C_{14}; $[M^+H]^+$ 1449.79 = A-C_{15}; $[M^+H]^+$ 1463.81 = A-C_{16} $[M^+H]^+$ 1477.83 = A-C_{17}/B-C_{15}; $[M^+H]^+$ 1491.84 = A-C_{17}/B-C_{16}; $[M^+H]^+$ 1505.84 = B-C_{17}). Fengycin contains also isoforms of fengycins A and B with unsaturated fatty acid chain with one double bound from C_{14} to C_{18} carbons ($[M^+H]^+$ 1433.79 = A-C_{14}; $[M^+H]^+$ 1447.81 = A-C_{15}; $[M^+H]^+$ 1461.82 = A-C_{16}; $[M^+H]^+$ 1475.84 = A-C_{17}/B-C_{15}; $[M^+H]^+$ 1489.85 = A-C_{17}/B-C_{16}). These findings are in accordance with previously published analysis of the lipopeptides isoforms produced by these *B. subtilis* strains (Hamley et al. 2013; Béchet et al. 2013).

B. subtilis lipopeptides confer protection to wheat against *Z. tritici*

Three *B. subtilis* lipopeptides (mycosubtilin, surfactin, and fengycin) and two mixtures (mycosubtilin + surfactin and mycosubtilin + surfactin + fengycin) were tested in the greenhouse for their protection efficacy on two wheat cultivars (Dinosor and Alixan) against *Z. tritici*. A significant disease extent was observed on control plants from both cultivars, although those from Alixan inoculated with the strain T02596 showed overall a slightly higher level of symptoms when compared to those from Dinosor inoculated with the strain T01193 (57.4 and 50.8% of diseased leaf area bearing pycnidia on Alixan and Dinosor, respectively (Fig. 2). Disease scoring at 21 dpi revealed significant protection level by all tested lipopeptides or mixtures (Figs. 2 and 3). Disease reductions conferred by lipopeptides vary depending on the molecule or mixture and the cultivar used (Fig. 2). Disease amounts of lipopeptide-treated plants ranged from 9.4 to 46.3% on Dinosor and from 22.4 to 40.0% on Alixan. Interestingly, the highest disease reductions were obtained with treatments containing mycosubtilin, alone or in mixture with the other lipopeptides, on both cultivars (Figs. 2 and 3; Table 2). Mycosubtilin-based treatments exhibited higher protections on Dinosor (up to 82% disease reduction with the mycosubtilin + surfactin mixture) compared to Alixan (up to 61% disease reduction with the mycosubtilin + surfactin + fengycin mixture) (Table 2). Surfactin and fengycin alone also induced significant protection efficacies, but they did not exceed 35% on Dinosor and 48% on Alixan.

Mycosubtilin-based formulations display an in vitro antifungal effect against *Z. tritici*

All lipopeptides and mixtures, as well as the two reference fungicides epoxiconazole and bixafen, were evaluated for their direct activity against *Z. tritici* using 96-well plate assay.


a
Relative Abundance
1036.69
1022.67
1008.66
1044.66
1058.67
969.58
994.64
1067.73
1074.64
1087.63
1123.66
1127.69
1142.83
1158.70
m/z
b
Relative Abundance
1085.60
1071.58
1086.60
1072.58
1099.61
1087.60
1100.6
1073.59
1088.61
1102.6
1057.57
1054.55
1074.59
1093.56
1107.5
1113.63
1121.59
1130.65
1135.61
1144.67
m/z
c
Relative Abundance
1491.84
1492.83
1477.83
1475.84
1489.85
1478.82
1461.82
1463.81
1493.84
1464.81
1479.82
1447.81
1485.33
1486.33
1449.79
1465.81
1472.32
1494.84
1495.84
1483.84
1469.83
1499.84
1433.79
1435.79
1450.79
1451.79
1459.47
1456.31
m/z

◀ **Fig. 1** HPLC-MS analysis of the lipopeptide powder produced and purified from the *Bacillus subtilis* strains BBG131 for surfactin (**a**); BBG125 for mycosubtilin (**b**); and Bs2504 for fengycin (**c**). Peak at $[M^+H]^+$ 1505.84 was also observed in the total spectrum of the fengycin analysis, but was voluntarily not presented in this figure in order to improve its quality

A dose–response curve was determined for each compound tested; it presents the optical density averages obtained with different concentrations (Fig. 4). As expected, both fungicide references inhibited fungal growth by low concentrations, although bixafen was the most active one in fungal growth inhibition compared to epoxiconazole. The effect of lipopeptides on fungal growth varies depending on the product and the concentration. Mycosubtilin alone or M-containing mixtures were the most active lipopeptides, which completely inhibited fungal growth at the concentration 3.7 mg L^{-1} for mycosubtilin and mycosubtilin + surfactin and at the concentration 11.1 mg $L^{-1}$for mycosubtilin + surfactin + fengycin. Interestingly, surfactin and fengycin did not show any antifungal effect when tested separately. On the other hand, surfactin increased fungal growth at the highest concentration 100 mg L^{-1} (Fig. 4). From the dose–response curves, IC_{50} values were determined for each compound (Table 3). Both fungicide references showed low IC_{50} values. Mycosubtilin-based formulations showed slightly higher but still low IC_{50} values, up to 1.4 mg L^{-1} when tested alone or in mixture with surfactin. This value was 2.33- and 14-fold higher than those of epoxiconazole and bixafen, respectively. The mixture mycosubtilin + surfactin + fengycin displayed relatively higher IC_{50} value, while surfactin and fengycin tested separately showed each a IC_{50} value higher than 100 mg L^{-1} (Table 3).

Mycosubtilin-based formulations reduce *Z. tritici* hyphal growth on the leaf surface

Hyphal growth of *Z. tritici* on the leaf surface of wheat plants treated or not with different lipopeptides was revealed using Calcofluor. Microscopic observations revealed that fungal spores formed germ tubes from their ends (Fig. 5). At 1 dpi, 58.8% of spores germinated in non-treated and inoculated control plants (Table 4). Both surfactin and fengycin did not significantly reduce the rate of germinated spores. All treatments containing mycosubtilin significantly reduced the rates of germinated spores (Table 4), thus agreeing in vitro results. The highest reduction was obtained with mycosubtilin alone

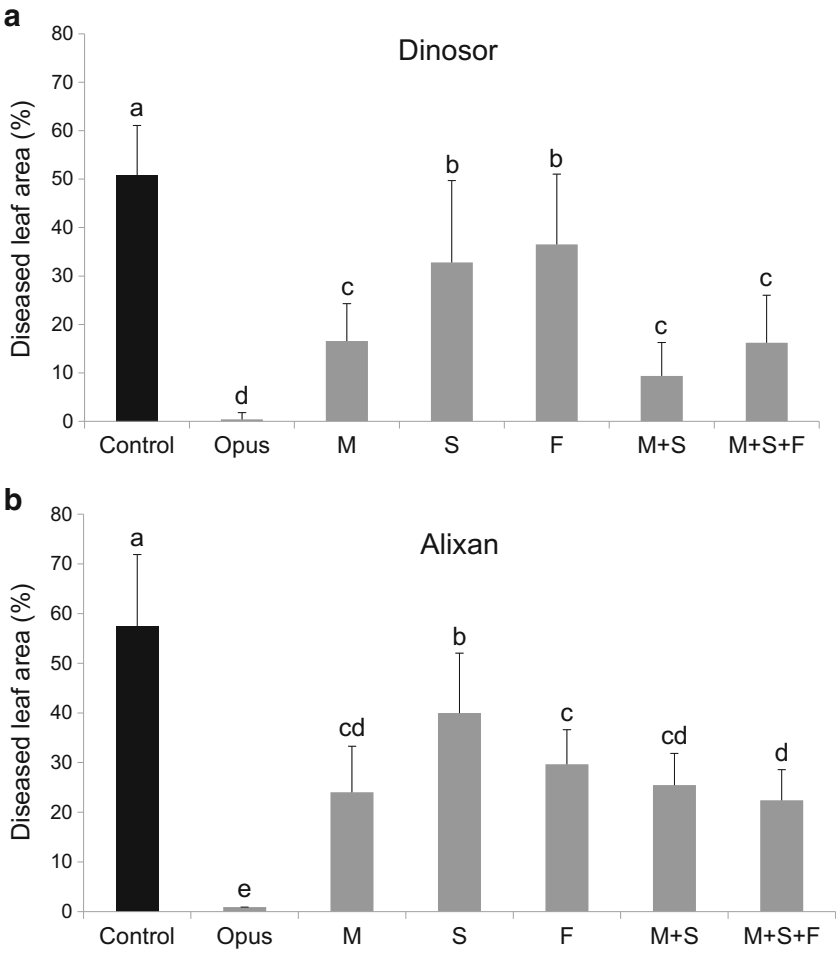


Fig. 2 Disease severity level on wheat plants of the *Dinosor* (**a**) and *Alixan* (**b**) cultivars treated or not with different *Bacillus subtilis* lipopeptides 48 h before challenge inoculation with the *Zymoseptoria tritici* strains T01193 and T02596, respectively. Disease symptoms were recorded 21-day post-inoculation by scoring the percentage of the third leaf area covered with lesions bearing or not pycnidia. Within each cultivar, means tagged with the *same letter* are not significantly different using the Tukey's test at $P = 0.05$. *M* mycosubtilin, *S* surfactin, *F* fengycin, *M* + *S* mycosubtilin + surfactin, *M* + *S* + *F* mycosubtilin + surfactin + fengycin

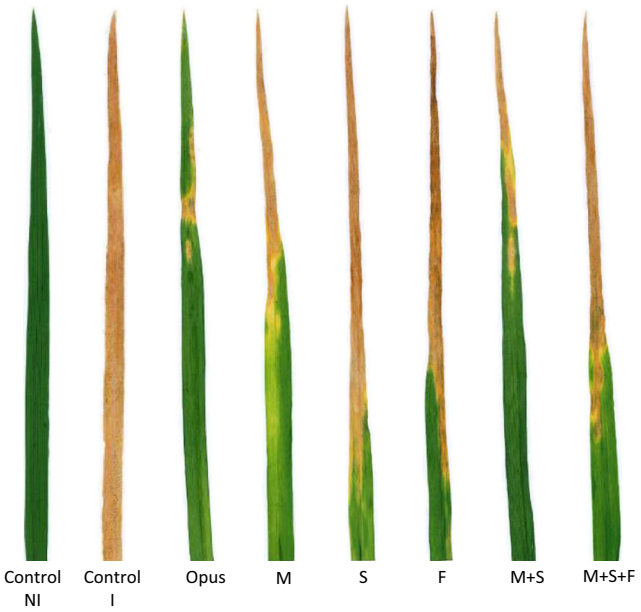


Fig. 3 Representative leaves at 21-day post-inoculation of the Dinosor wheat cultivar treated or not with different *Bacillus subtilis* lipopeptides and the Opus (epoxiconazole) reference fungicide 48 h before challenge inoculation with the *Zymoseptoria tritici* strain T01193. *M* mycosubtilin, *S* surfactin, *F* fengycin, *M* + *S* mycosubtilin + surfactin, *M* + *S* + *F* mycosubtilin + surfactin + fengycin, *NI* non-inoculated, *I* inoculated

with only 19.6% of germinated spores, corresponding to 67% reduction when compared to control plants (Table 4).

At 5 dpi, fungal germ tubes were often branched and oriented randomly on the leaf surface without specific orientation toward stomata and no possible chemotropism or thigmotropism could be noticed. Fungal hyphal growth displayed different patterns on wheat leaves treated or not with the lipopeptides (Fig. 5). On control plants, only 13.7% of spores did not germinate and 72% of spores formed developed or strongly developed germ tubes (classes 3, 4, and 5). Treatments with formulations containing mycosubtilin strongly and significantly reduced fungal growth on the leaf surface, with more than 30% (35.2, 31.6, and 35.1% for mycosubtilin, mycosubtilin + surfactin, and mycosubtilin + surfactin + fengycin, respectively) of spores remained non-geminated on leaves treated with mycosubtilin-based formulations (Fig. 5). Drastic reductions of hyphal growth were also observed, with only 8.4, 15.4, and 11.3% of germ tubes belonging to the four and five classes in plants treated with mycosubtilin, mycosubtilin + surfactin and mycosubtilin + surfactin + fengycin, respectively, when compared to control plants (46.7%) (Fig. 5). In addition, treatments with mycosubtilin-based formulations significantly increased the rates of geminated spores with small germ tube (class 2). The other lipopeptides (surfactin and fengycin) did not show marked effect on the hyphal growth of *Z. tritici* on the leaf surface when compared to control plants.

Correlation between protection efficacy and in vitro antifungal activity

Correlative analysis using PCA revealed strong negative correlation (r = 0.99) between the protection efficacy levels highlighted on the cultivar Dinosor inoculated with the strain T01193 and the IC_{50} values recorded in vitro using this strain (Fig. 6).

Discussion

Z. tritici control strategies rely mainly on chemical fungicide applications and to a lower extent on adequate cultural practices and the use of partially resistant cultivars (Torriani et al. 2015). However, the current controversy on the wide use of conventional pesticides and the frequent adaptation of fungal population, either to applied fungicides or to host resistance, makes urgent the finding of new alternative control strategies compatible with sustainable agriculture and healthy food. Here, we investigated the use of lipopeptides from *B. subtilis* as natural control products on wheat against *Z. tritici*, since such compounds were demonstrated to be much less ecotoxic when compared to chemical fungicides (Deravel et al. 2014). Our results revealed that all tested lipopeptides or mixtures displayed significant disease reduction on the two used cultivars. The best protection levels were obtained with the treatments containing mycosubtilin alone or in mixture with surfactin or with both surfactin and fengycin (up to 82% disease reduction with the mixture mycosubtilin + surfactin on Dinosor), although these efficacies were lower than those found with the Opus® reference fungicide. This protective

Table 2 Protection efficacy of *Bacillus subtilis* lipopeptides at 100 mg L^{-1} on the two wheat cultivars Dinosor and Alixan inoculated with the *Zymoseptoria tritici* strains T01193 and T02596, respectively

Lipopeptide(s)	Protection on Dinosor[a] (%)	Protection on Alixan[a] (%)
Mycosubtilin	67	58
Surfactin	35	30
Fengycin	28	48
Mycosubtilin + surfactin	82	56
Mycosubtilin + surfactin + fengycin	68	61

[a] Percentage of disease reduction compared to the non-treated inoculated control of each cultivar

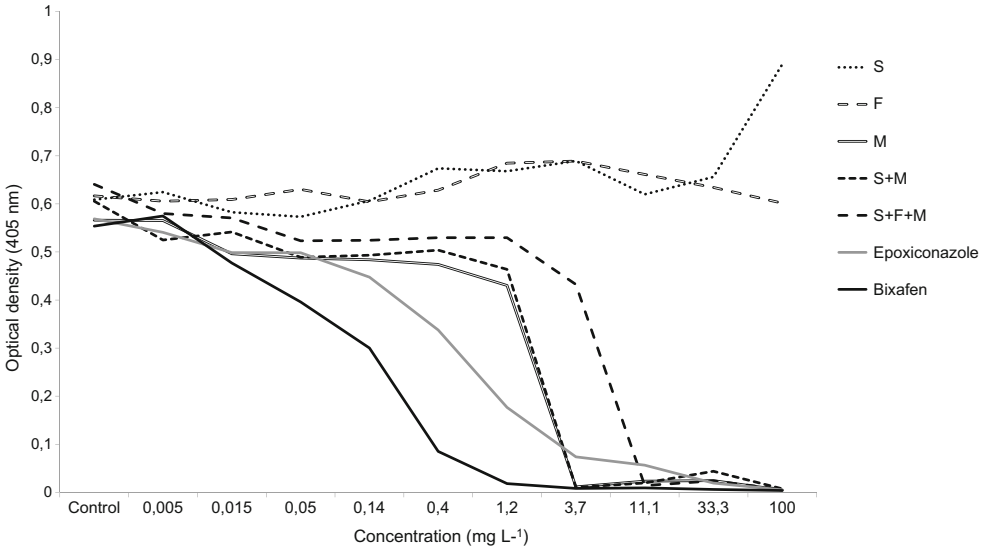


Fig. 4 In vitro dose–response curves obtained with different *Bacillus subtilis* lipopeptides and the bixafen and epoxiconazole reference fungicides using the *Zymoseptoria tritici* strain T01193. *S* surfactin, *M* mycosubtilin, *F* fengycin, *M* + *S* mycosubtilin + surfactin, *M* + *S* + *F* mycosubtilin + surfactin + fengycin

effect of mycosubtilin is likely due to its antifungal activity highlighted in vitro and confirmed *in planta*. Indeed, a strong negative correlation was detected between IC_{50} values and protection efficacies scored on the cultivar Dinosor, indicating that the more the lipopeptide or mixture possesses a direct antifungal effect, the more the biomolecule confers protection against *Z. tritici*, regardless the cultivar. Nevertheless, a contribution of plant resistance induction effect to lipopeptide efficacy cannot be excluded, at least for surfactin and fengycin, for which significant disease reductions were observed without any direct antifungal effect against the pathogen under tested conditions. Mycosubtilin, as other lipopeptides from iturin family, has been shown to confer protection against a broad range of plant pathogens. For instance, treatment of tomato seedlings with a *B. subtilis* mutant strain overproducing mycosubtilin allowed a significant protection against *Pythium aphanidermatum* when compared to wild-type strain (Leclère et al. 2005). Besides, supernatant from this mutant strain containing high amounts of mycosubtilin showed in vitro antagonistic activity against several phytopathogenic fungi, including *P. aphanidermatum*, *Botrytis cinerea*, and *Fusarium oxysporum*, with growth inhibition zones significantly larger than those induced by the wild-type supernatant (Leclère et al. 2005). When applied on lettuce, purified mycosubtilin from *B. subtilis* at 100 mg L^{-1} strongly reduced the incidence of downy mildew, caused by *Bremia lactucae*, by resulting in about seven times less diseased plantlets compared to the control samples (Deravel et al. 2014). Interestingly, Farace et al. (2015) demonstrated in grapevine, for the first time, that mycosubtilin not only has a direct activity on spore germination but also triggers plant defense mechanisms and long-lasting enhanced tolerance to *B. cinerea*. Other iturins have also been reported to be effective against plant pathogens, either via direct antifungal activity, such as iturin A from *B. amyloliquefaciens* against *Rhizoctonia solani* (Yu et al. 2002), iturin A and bacillomycin from *B. subtilis* against *Podosphaera fusca* (Romero et al. 2007), and iturin A from *B. subtilis* against *Colletotrichum gloeosporioides* (Kim et al. 2010), or through the induction of plant resistance such as iturins from *B. amyloliquefaciens* on strawberry against *C. gloeosporioides* (Yamamoto et al. 2015) and on cotton against *Verticillium dahlia* (Han et al. 2015).

Table 3 In vitro half maximal inhibitory concentration (IC_{50}) values obtained with *Bacillus subtilis* lipopeptides using the *Zymoseptoria tritici* strain T01193

Lipopeptide(s) or reference fungicide	IC_{50} (mg L^{-1})
Mycosubtilin	1.4
Surfactin	>100
Fengycin	>100
Mycosubtilin + surfactin	1.4
Mycosubtilin + surfactin + fengycin	4.5
Epoxiconazole	0.6
Bixafen	0.1

Similar levels of protection were obtained with mycosubtilin alone at 100 mg L^{-1} or when tested in mixture with surfactin (50:50 mg L^{-1}) or with surfactin + fengycin (33:33:33 mg L^{-1}) (Fig. 2). Likewise, mixing mycosubtilin with surfactin at 50:50 mg L^{-1} conferred on lettuce the same effect against *B. lactucae* as mycosubtilin alone at 100 mg L^{-1}, while surfactin did not impact *B. lactucae* development and did not reduce disease severity, thus suggesting that the presence of surfactin can improve the biological activities of mycosubtilin (Deravel et al. 2014). Two hypotheses were launched to explain the increased performance of mycosubtilin when it is mixed with surfactin (same performance with a lower concentration), since surfactin is not antifungal per se (Deravel et al. 2014). First, surfactant property of surfactin can enhance foliar penetration,

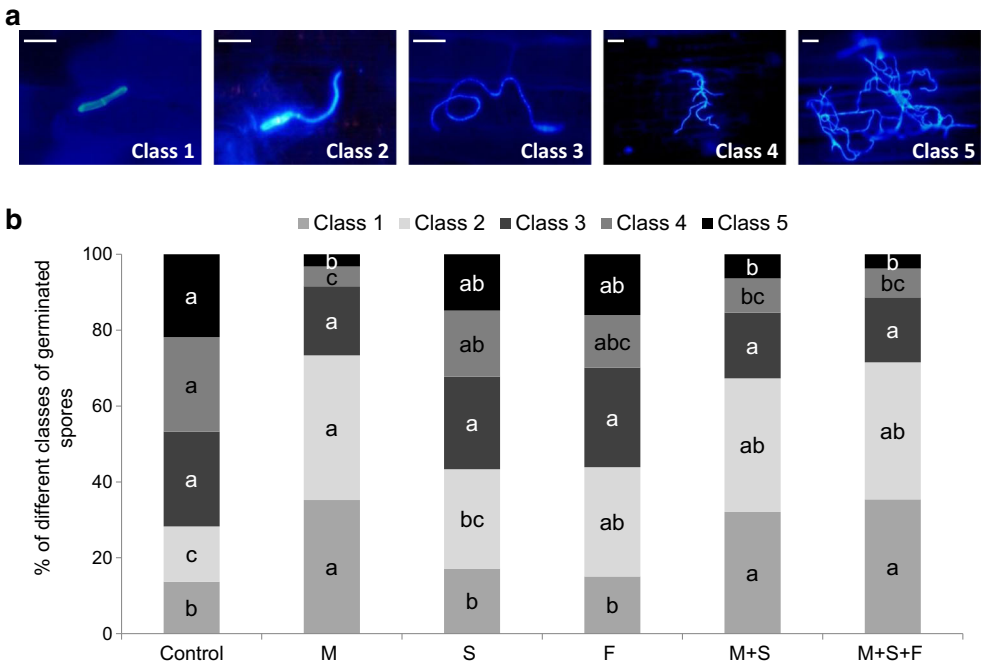


Fig. 5 Effect of different *Bacillus subtilis* lipopeptides on hyphal growth of the *Zymoseptoria tritici* strain T02596 on leaves of the wheat cultivar Alixan pretreated or not with different lipopeptides. Five different classes of Calcofluor-stained germinated spores were assessed 5-day post-inoculation from 100 different spores on each leaf segment for each condition. *Class 1*, non germinated spore; *class 2*, geminated spore with small germ tube; *class 3*, geminated spore with developed germ tube; *class 4*, geminated spore with a strongly developed germ tube; *class 5*, germinated spore giving a strong hyphal growth. *M* mycosubtilin, *S* surfactin, *F* fengycin, *M* + *S* mycosubtilin + surfactin, *M* + *S* + *F* mycosubtilin + surfactin + fengycin. Within each class, *bars with common letters* are not significantly different using the Tukey's test at $P = 0.05$. *Scale bar* = 10 μm

foliar retention, and coverage, as reported for common organic surfactants used in crop protection (Stock and Holloway 1993). Second, synergy between surfactin and mycosubtilin could be due to the formation of mixed micelles, since bioactive single lipopeptides such as surfactin and mycosubtilin may interact together by forming mixed and potentially active or even more active micelles (Jauregi et al. 2013; Deravel et al. 2014). However, these hypotheses should be confirmed by further analyses.

Surfactin and fengycin tested separately significantly reduced disease severity on both wheat cultivars, despite an absence of direct antifungal effect by these biomolecules in both in vitro and *in planta* assays under tested conditions (Figs. 2, 4, and 5). These findings suggest that these lipopeptides confer protection to wheat against *Z. tritici* via the elicitation or priming of plant defense responses rather than by direct antagonism or combined effect (direct and indirect) as previously reported on grapevine for mycosubtilin (Farace et al. 2015). Surfactin and fengycin interact with lipid layers of the plasma membrane and modify cell membrane permeability and structure leading to a cascade of molecular events within the plant cells activating a response of defense (Ongena et al. 2007; Henry et al. 2011). Hence, these two molecules play a key role in the induction of plant immunity driven by beneficial microorganisms, such as induced systemic resistance (ISR) (Raaijmakers et al. 2010). For instance, Farace et al. (2015) found that purified surfactin from *B. subtilis* activates salicylic acid-based defense pathway in grapevine leaves against *B. cinerea*, without any direct antifungal activity against the pathogen. By contrast, they found that plipastatin, belonging to the fengycin family, conferred a low protection efficacy which was assigned to direct antibiosis effect rather than to elicitation properties. In bean, purified *B. subtilis* fengycins and surfactins, applied as soil drenching, provided a significant protective effect against *B. cinerea* and activated similar ISR response to that induced by living cells of the used producing *B. subtilis* strain, thus concluding for the

Table 4 Rates of germinated spores at 1 day after inoculation in plants of the cultivar Alixan treated or not with *Bacillus subtilis* lipopeptides at 100 mg L^{-1} and inoculated with the *Zymoseptoria tritici* strain T02596

Lipopeptide(s)	Rate of germinated spores (%)
Control	58.8 a
Mycosubtilin	19.7 b
Surfactin	68.7 a
Fengycin	60.9 a
Mycosubtilin + surfactin	32.0 b
Mycosubtilin + surfactin + fengycin	32.2 b

Means tagged with the same letter are not significantly different using the Tukey's test at $P = 0.05$

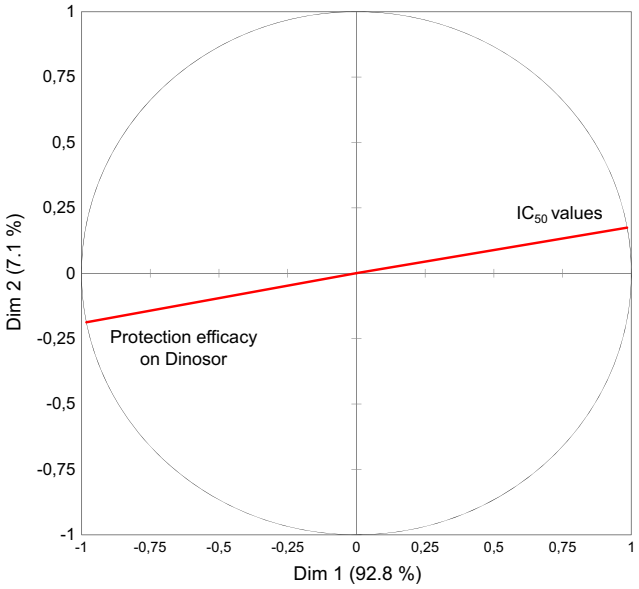


Fig. 6 Principal component analysis (PCA) to assess correlation between protection efficacies obtained with *Bacillus subtilis* lipopeptides on the cultivar Dinosor inoculated with the *Zymoseptoria tritici* strain T01193 (percentages of disease severity reduction compared to the non-treated inoculated control plants) and IC_{50} values found in vitro for the lipopeptides using this strain

first time that fengycins and surfactins are a novel class of compounds from non-pathogenic bacteria that can be perceived by plant cells as signals to initiate defense mechanisms (Ongena et al. 2007). Likewise, surfactin from *B. amyloliquefaciens* triggered ISR on strawberry plants, resulting in the reduction of the severity of anthracnose disease caused by *C. gloeosporioides* (Yamamoto et al. 2015). Addition of surfactin, but not fengycin or iturin, to tobacco cell suspensions, induced a battery of plant defense-related events, including extracellular medium alkalinization coupled with ion fluxes, reactive oxygen species production, stimulation of phenylalanine ammonia-lyase and lipoxygenase activities, and modification of the pattern of phenolics produced by elicited cells (Jourdan et al. 2009). Interestingly, surfactin and fengycin conferred significant protections in rice against *R. solani* and triggered the expression of several defense genes in rice cell suspension cultures, while mycosubtilin did not give any protection, suggesting that the involvement of both fengycin and surfactin in mediating induced resistance in rice against *R. solani* (Chandler et al. 2015).

Conclusion

The present study demonstrated that the potential of lipopeptides from *B. subtilis* to be used as biocontrol agents on wheat against *Z. tritici*. Mycosubtilin, alone or in mixture with surfactin or with both surfactin and fengycin, provided the highest protection levels (up to 82 and 61% disease reduction on the cultivars Dinosor and Alixan, respectively). These strong efficacies may result mainly from the significant biofungicide activity of this biomolecule highlighted both in vitro and *in planta*, thus corroborating previous results on other pathosystems (e.g., Deravel et al. 2014). Nevertheless, the protection efficacy of mycosubtilin and mycosubtilin-containing mixtures was lower than that found for the Opus® reference fungicide. Therefore, the efficacy of mycosubtilin or its mixtures could be improved with formulation by using adapted adjuvants allowing better lipopeptide foliar application. Fengycin and surfactin showed also significant levels of protection, but to a lower extent compared to mycosubtilin. According to the antifungal assay, the mode of action of these two molecules relies on the induction of plant resistance mechanisms rather than on direct activity. Further investigations are needed to confirm this hypothesis and to identify defense pathways involved in such resistance. Such investigations will also be performed with mycosubtilin for which combined direct and indirect (elicitation or priming) activities are suspected.

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