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Heterobasidion-growth inhibiting *Bacillus subtilis* A18 exhibits medium- and age-dependent production of lipopeptides

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ABSTRACT

Heterobasidion annosum s.s. and *H. parviporum* are severe pathogens of conifers causing butt rot and root rot thus reducing the economic value of timber. Here, the antifungal activity of *Bacillus subtilis* isolate A18 against these two *Heterobasidion* species was investigated. Five different culture media with different culture age were investigated to study the effect of substrate composition and culture age for metabolite production. Bacterial cultures and cell-free culture filtrates were tested for antifungal activity. Inhibition of fungal growth was analysed using the agar disc-diffusion method. MALDI-TOF and LC-HRMS analyses were used to identify the antifungal metabolites. Substrate composition and age of culture were found to be active variables with direct effect on the antifungal activity of bacterial culture extracts. High anti-fungal activity was observed when *B. subtilis* was cultured in PDB, SGB and LB media for four days. Mass-spectrometry analysis showed the presence of lipopeptides in culture filtrates identified as members of the surfactins, polymixins, kurstakins and fengycins. A culture filtrate containing fengycin-type lipopeptides showed the highest bioactivity against *Heterobasidion* species. Bacterial cultures had higher bioactivity compared to their respective cell free culture filtrates. The results of the present study suggest that *B. subtilis* A18 is a powerful biocontrol agent against *Heterobasidion* infections of tree wounds and stumps.

1. Introduction

Conifers constitute the dominant group of plants in the Northern-hemisphere boreal forests. They play an important role in the economy of many countries by providing raw materials for a number of industries. Being long-lived organisms, conifers are attacked by a number of pests including pathogenic fungi; however, they possess powerful dynamic defence mechanisms against herbivores, insects and pathogens by producing a wide variety of defense compounds such as phenolics, tannins and terpenoids (Franceschi et al., 2005). *Heterobasidion* species belong to the most important fungal pathogens due to their ability to cause severe damage to heartwood and reduce the economic value of timber. The annual economic loss to the European forest industry related to *Heterobasidion* damage has been estimated to about 800 million euro (Asiegbu et al., 2005; Zeng et al., 2018).

The most common source of *Heterobasidion* infection for healthy trees is through root connection with infected trees or stumps (Asiegbu et al., 2005). *Heterobasidion* species produce basidiospores that infect freshly cut stumps, colonize, establish and further infect healthy trees through root interactions (Morrison and Redfern, 1994; Asiegbu et al., 2005). These fungal species can cause root rot in conifers without showing any apparent symptom of infection for many years (Rönnberg

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and Vollbrecht, 1999). The ability to actively spread itself without being detected makes *Heterobasidion* one of the most important pathogenic agents of economically important conifers.

Suggested preventive measures include winter cuttings and mixed stands; however, these methods may not be feasible due to the industry supply demands, hence finding alternate methods are necessary to control *Heterobasidion* infections (Oliva et al., 2008). A number of methods, such as chemical treatment, silvicultural practices and biocontrol have been adopted to prevent *Heterobasidion* infection of fresh stumps during the thinning process of managed forests. The chemical method involves treating stumps or wounds with urea or borate solutions, which protects them from pathogenic fungi belonging to *Zygomycetes* and *Basidiomycetes* (Vasiliauskas et al., 2004). However, bryophytes and some vascular plants located close to the stumps are adversely affected (Westlund and Nohrstedt, 2000) leading to ecological disturbance in the forest surroundings.

Presently, the fungus *Phlebiopsis gigantea* is used as a biocontrol agent that functions through competing with *Heterobasidion* species (Korhonen et al., 1994). For complete prevention from *Heterobasidion* infection, full coverage of stumps with *P. gigantea* suspension is necessary as this fungus does not have any antagonistic activity against *Heterobasidion* (Thor and Stenlid, 2005). Other fungal strains having antagonistic activity or resource-competition ability are *Bjerkandera adusta* (Kallio and Hallarsela, 1979), *Penicillium adametzii* (Szwajkowska-Michałek et al., 2012), *Resinicium bicolor* (Holmer and Stenlid, 1997) and *Trichoderma* sp. (Nicolotti and Varese, 1996). Recently, Terhonen et al. (2016) reported the antagonistic activity of the endophytic fungus *Cryptosporiopsis* sp. against *H. parviporum*. Many investigations have been reported where fungal strains were studied for the management of *Heterobasidion* species. However, there are only a few reports where bacterial strains were studied for their antagonistic or competitive activity (Hallaksela, 1993; Murray and Woodward, 2003; Lehr et al., 2007; Mesanza et al., 2016).

Among the biocontrol agents, *Bacillus* species are well known biological control agents and are used to suppress soil borne pathogens. Their biocontrol mechanisms are competition for space and nutrients, induction of systemic resistance and production of antibiotics (Li et al., 2014). Yamamoto et al. (2015) reported that *Bacillus amyloliquefaciens* strain S13-3 has an antagonistic activity against *Colletotrichum gloeosporioides* in strawberry by producing lipopeptide antibiotics. Lipopeptides have been explored as antimicrobial compounds against various plant pathogens and also used in other industrial applications (Batool et al., 2011; Zhao et al., 2017). *Bacillus* species are considered to be potent bacteria in producing active molecules inhibiting the growth of pathogenic microorganisms (Meena and Kanwar, 2015; Zhao et al., 2017). However, the antifungal effect of *Bacillus* species against *Heterobasidion* is poorly understood. To this end, the present study investigates the potential of a *Bacillus subtilis* isolate to inhibit the growth of *H. annosum* and *H. parviporum*.

2. Materials and methods

2.1. Isolation and maintenance of bacterial culture

A bacterial isolate (A18) was recovered as a contaminant on a PDA Petri plate during an antifungal bioassay against *Heterobasidion parviporum* in the Ecological Chemistry laboratory, KTH, Sweden. The possible contamination source could be *Hylobius abietis* frass that was being investigated for the isolation of pine-weevil associated microbes (Azeem et al., 2015). Serial dilutions were performed to obtain single-cell colonies and to isolate a pure bacterial culture. The isolated culture was maintained on nutrient agar (NA) till identification as *Bacillus subtilissubtilis* (see below) and then used for anti-fungal experiments. Bacteria were incubated overnight at 30 °C and maintained at 4 °C.

2.2. Bacterial isolate identification

The pure colony of the isolate A18 was mixed in 100 µL of water and incubated at 100 °C for 5 min before being vortexed. From this solution, 1 µL was used for PCR in 25 µL reactions with AccuPrime SuperMixII (Invitrogen). For 16S rRNA gene amplification, we used the primers 8f (5′AGAGTTTGATIITGGCTCAG- 3′) and 1501 r (5′-CGGITACCTTGTTACGAC- 3′) and the program 94 °C for 3 min, 30 cycles of [94 °C for 30 s, 58 °C-48 °C for 30 s (the temperature was decreased by 1 °C every cycle for 10 cycles and then held at 48 °C for 20 cycles), 72 °C for 1 min 30 s], followed by a final extension step at 72 °C for 10 min (Lindh et al., 2005).

The 16S rRNA showed that A18 belonged to the *Bacillus subtilis* group and for subspecies determination to *B.* subtilis subtilis the *phoR* gene sequence was amplified according to Guo et al. (2012) with the primers Bacillus-phoR-F 5′-TTY ARY TCA TGR GAV ACA TT-3′ and Bacillus-phoR-R 5′-GGI TAY AAA IAR GAG GAG CC-3′.

The PCR products were sequenced by Macrogen, South Korea and analyzed with BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) and the sequences for isolate A18 were deposited with the GenBank accession numbers KU729674 (16S rRNA) and KU729673 (phoR).

2.3. Fungal culture media and growth conditions

The fungal cultures of *Heterobasidion annosum* and *H. parviporum* used in this study were obtained from Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden. Fungi were grown for 10 days and maintained at room temperature (22 ± 2 °C) in yeast extract peptone dextrose agar (YEPD) medium. *H. parviporum* had a higher growth rate than *H. annosum*. Fully grown fungal cultures were stored at 4 °C.

2.4. Co-culturing fungal-inhibition bioassay

Antagonistic activity of *B. subtilis* A18 was tested against both fungal species through co-culturing on PDA plates by modifying and combining methods described by (Barbieri et al., 2005; Leelasuphakul et al., 2008). Briefly, a 50 µL bacterial suspension with 10^8 CFU/mL was evenly applied on a small portion of a PDA plate along one side of a Petri plate. Then a plug of fully grown fungus (5 mm diameter) was inoculated at the center of the remaining area of the plate immediately after applying bacterial suspension. Five replicates of each fungus were co-cultured with *B. subtilis* A18 in the same way as described above and five replicates of each fungus were cultured without bacteria as control. All the Petri plates were incubated at room temperature (22 ± 2 °C). Fungal mycelial growth on control and test Petri plates were monitored and measured for mycelia growing in the direction towards the bacteria after 6, 8, 10, and 12 days of inoculation. The growth of fungus on control and test plates was determined by measuring the straight distance from the fungus-inoculation point to the mycelial tips.

2.5. Extraction of bacterial secondary metabolites by solvent extraction and solid phase extraction

Two different methods of bacterial metabolite extraction were employed: solid phase extraction (SPE) and sequential solvent extraction using a separating funnel. For sequential solvent extraction, *B. subtilis* A18 was cultivated in 100 mL potato dextrose broth (PDB) under shaking conditions (180 rpm) at 30 °C for 4 days. Supernatant was collected after centrifugation at 5000 × g for 10 min and cell free medium was first extracted with 3 × 50 mL ethyl acetate (EtOAc) and then subsequently with 3 × 50 mL *n*-butanol. Excess solvent from EtOAc and *n*-butanol extracts was evaporated on a rotary evaporator under reduced pressure and the resulting residues were re-dissolved in EtOAc and methanol, respectively, with a concentration of 10 mg/mL. The extracts were stored at -20 °C until used for fungal inhibition

bioassay.

For extraction of bacterial metabolites on solid phase extraction (SPE) cartridge, *B. subtilis* A18 was cultivated in 100 mL nutrient broth (NB) under shaking condition for 4 days. The cells were removed by centrifugation at 5000 × g for 10 min and the resulting supernatant was extracted using C_{18} solid phase extraction (SPE, Sigma-Aldrich, Sweden) cartridge (3 × 500 mg). The SPE adsorbent was activated, sample loaded, washed and desorbed as described by the manufacturer. Briefly, the SPE cartridge was first flushed with 5 mL methanol then equilibrated with 10 mL extra pure water and then the sample was loaded. The sample-loaded adsorbent was washed with at least 3 mL water before eluted in 5 mL of 95% acetonitrile (MeCN)/water. The flow rate was 2–3 mL/min throughout the elution process. The MeCN extract obtained was freeze dried, weighed and re-constituted as 10 mg/mL in 80% methanol/water and stored at 20 °C till chemical analysis and test of anti-fungal activity.

2.6. Anti-fungal activity of solvent extract and SPE extract of B. subtilis

A 5 mm plug from a fully grown fungal culture was inoculated at the center of a PDA plate and grown for 3 days at room temperature. Five replicates of each fungal species were inoculated in the same way. Three paper discs were placed on the PDA plate at a maximum distance from the fungal plug, Petri plate wall as well as from each other. Each paper disc was treated with an aliquot of 5 µL SPE extract of *B. subtilis* A18 or SPE extract of control medium. All the plates were incubated at room temperature till the fungal mycelium crossed the paper discs on control Petri plates. The clearing zone was observed and fungal growth was determined by measuring the distance from the fungal plug origin to that of mycelium tips on the plate. A similar experiment setup was used for determining the anti-fungal activity of EtOAc and butanol extracts of bacteria grown in PDB medium.

2.7. LC-HRMS analysis of SPE-extracted bacterial metabolites

An SPE extract of a bacterial culture was analyzed by LC-HRMS on a reversed phase HPLC column (3.0 × 50 mm, 2.6 µm, Accucore RP-MS, Thermo Scientific, Waltham, MA, USA) connected to a maXis Impact Q-TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) using an electrospray ionization ion-source. The mass spectrometer was operated in positive mode, scanning *m/z* 50–1500, and the mass spectra were calibrated against sodium formate ion clusters. The column was eluted with a gradient of MeCN in water, both with 0.2% formic acid (10–95% MeCN in 3 min, 95% MeCN for 4 min, at 0.8 mL min^{-1}; injection volume: 3 µL). Tentative identification of compounds was achieved by comparison of *m/z* of separated compounds with values in the database Antibase (Wiley).

2.8. Effect of growth media and the age of bacterial cultures on the production of antifungal metabolites

In our previous experiments, two different media and extraction methods were used to test the antagonistic activity of the bacterial culture extracts. Now, the effect of nutrients in the media and the age of culture on the antagonistic activity of bacterial cells and their culture filtrates were investigated. *B. subtilis* A18 was grown in five different media; nutrient broth (NB), potato dextrose broth (PDB), trypticase soy broth (TSB), Sabourad's glucose broth (SGB) and Luria Bertani (LB) kept at 30 °C and 180 rpm on a rotary shaker. Six replicates of each type of media broths (20 mL) were seeded with 100 µL of *B. subtilis* A18 suspension made in saline with an optical density of 0.5 at OD_{600}. The *B. subtilis* A18 suspensions were removed from incubation after 1, 4 and 7 days of bacterial growth. From each bacterial culture, 1 mL was used to determine the OD_{600} by using a spectrophotometer (Biowave DNA, UK). One mL of each liquid bacterial culture was stored at −20 °C to test their antifungal activity against fungi, and the remaining bacterial culture was centrifuged at 2000 rpm for 5 min, the supernatant filtered through a membrane (Millipore, 0.22 µm), concentrated (10×) on a rotatory evaporator and stored at −20 °C till used for antifungal bioassays.

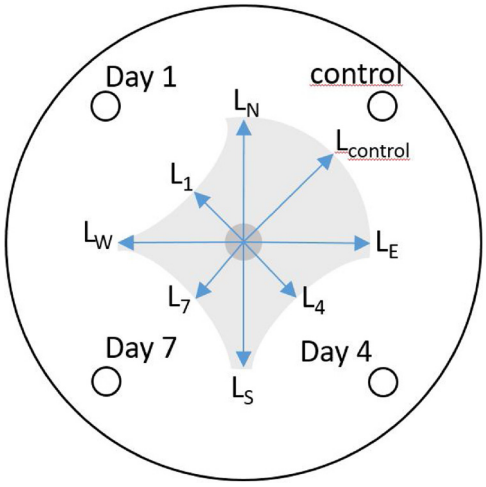

Fig. 1. Setup of antifungal-activity bioassay.

2.9. Antifungal activities of bacterial cultures and culture filtrates grown on different media

The agar disc-diffusion method was used to test the antifungal activity of bacterial cultures and the cell-free culture filtrates against the test fungi. A 5 mm plug taken from fully grown *H. annosum* or *H. parviporum* cultures was placed at the centre of a YEPD agar plate. Four filter paper discs of 5 mm diameter were placed on the agar surface, at a maximum distance from each other and at 30 mm distance from the fungal plug (Fig. 1). Three paper discs located along L_1, L_4 and L_7 were treated with 5 µL of bacterial cultures or bacterial culture filtrates harvested after 1, 4 or 7 days whereas on the fourth paper disc ($L_{control}$) 5 µL saline or control media was applied. Since the optical density of bacterial cultures grown on different media and of different age was different, the volumes were adjusted in order to have approximately the same bacterial content in each sample. Three replicates of each fungal species versus bacteria grown on each type of previously described culture or culture filtrate were employed. Fungal growth was monitored on each Petri plate by measuring the growth of fungal mycelia along lines $L_{control}$, L_1, L_4 and L_7 (Fig. 1) after 6, 8, 10, 15 and 20 days. The maximum fungal growth (L_{max}) was measured along $L_{control}$ and the antifungal activity of bacterial culture or culture filtrate was expressed as percentage of growth inhibition (PGI_i), which was calculated using the formula

$$PGI_i = \frac{L_{control} - L_i}{L_{control}} \times 100$$

where i = 1, 4, 7 days.

2.10. Fungal growth in the presence of well-established B. subtilis A18

The saline suspension of *B. subtilis* A18 was evenly spread on a YEPD agar plate and incubated for 24 h. When the bacterium covered the whole agar surface, a plug of fully grown *H. annosum* or *H. parviporum* was placed over the bacterial culture surface. The plates were observed for fungal growth/inhibition for a period of two months. Two replicates of each fungus were grown in the same way.

2.11. Chemical analysis of bacterial culture filtrates by MALDI-TOF

The bacterial cultures and culture filtrates grown on different media

for 4 days showed higher anti-fungal activity compared to 1 and 7 days. Therefore, the culture filtrates of *B. subtilis* A18 grown on different media for four days were analysed by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The peptides were separated from bacterial culture filtrates by gel filtration chromatography on a Sephadex^{TM} G-25 PD-10 desalting column (Amersham Biosciences). The separation was made for the removal of salts and the recovery of low molecular weight substances (Mr < 1000), by desalting and buffer exchange (Zhao et al., 2013). The peptide fraction of each media type was collected, concentrated in a rotatory evaporator and re-suspended in ammonium acetate buffer. For MALDI-TOF MS analysis the sample solution was mixed with matrix (α-cyano-4-hydroxycinnamic acid solution in 70% MeCN/H_2O containing 0.1% trifluoroacetic acid) and 1 μL of the resultant mixture was spotted on a 100-well steel plate. The mass spectra were recorded in positive ion linear mode in the range from 500 to 2500 Da with a Voyager time-of-flight mass spectrometer (Applied Biosystems, USA). The pure compound surfactin (purchased from Sigma Aldrich, Sweden) was also analysed under the same conditions to be used as positive control.

2.12. Statistical analysis

The statistical analyses were conducted by using SPSS (IBM, USA) computer software. *T*-test was used to find the statistical differences between the antifungal activity of bacterial cultures and culture filtrates on two *Heterobasidion* species. One way ANOVA with Bonferroni *post-hoc* test (Albabtain et al., 2017) was used to determine if two or more different treatments (e.g. age of culture and different culture media) showed significant differences on growth inhibition.

3. Results

3.1. Co-culturing of bacteria and fungi

The mycelial growth rate of *H. parviporum* was greater than that of *H. annosum* throughout the observation period on control Petri plates (Fig. 2). *B. subtilis* A18 inhibited both fungi in a similar fashion. On the 6th day after inoculation, a slight fungal growth inhibition was observed on the side facing towards bacteria; however, the difference was not significant. A significant growth inhibition was observed on the 8th day and continued subsequent days for both test fungi (Fig. 2). The inhibition effect was only found on the side facing bacteria (Fig. S1).

3.2. Fungal inhibition activity of SPE and solvent extracts

The butanol extract of *B. subtilis* A18 showed higher inhibition activity for both fungi compared to EtOAc extract, but the effect was significantly higher against *H. parviporum* as compared to *H. annosum* (Fig. 3). The antifungal activity of the SPE extract was similar to that of the butanol extract against *H. parviporum*. However, this extract was more active than the butanol extract against *H. annosum*. Overall, *B. subtilis* A18 media extracts exhibited higher inhibitory activities against *H. parviporum* compared to *H. annosum* (Fig. 3). The solvent or media control did not show any effect on the fungal growth. EtOAc extracts of *B. subtilis* A18 showed minor inhibition against *H. parviporum* and *H. annosum* (Fig. 3).

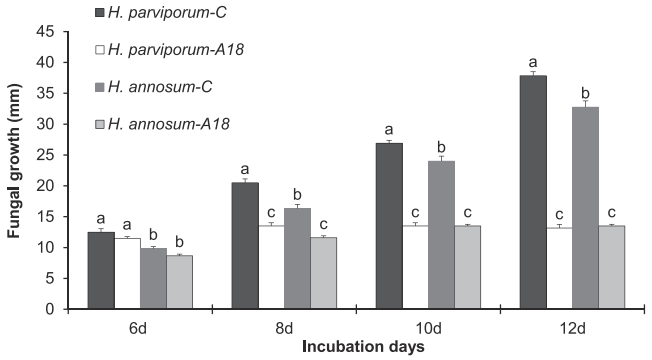

Fig. 2. Growth of *H. parviporum* and *H. annosum* in the absence and presence of *B. subtilis* A18. Columns with different small letters are significantly different from each other (p < 0.05) when comparison was made between treatments on the same day of observation (one way ANOVA *post-hoc* Bonferroni test). Error bars stand for SEM (n = 5).

3.3. LC-HRMS analysis of bacterial metabolites

LC-HRMS analysis of the *B. subtilis* NB culture filtrate extracted by SPE, along with the corresponding medium as control, suggested the presence of the lipopeptides plipastatin A2, plipastatin B1, plipastatin B2 and surfactin C1 or C2. These lipopeptides were not found in the control sample (Fig. 4, Table S1).

3.4. Effect of bacterial culture age and nutrient composition on antifungal activity

Overall, 4-day-old bacterial cultures grown on all five different media showed higher activity against *H. parviporum* compared to 7 days except TSB and SGB. In the case of TSB, 4-day-old and 7-day-old cultures showed similar activity, whereas SGB-grown cultures of different age did not show any difference (Fig. 5A). Bacterial culture grown for 1 day on NB and SGB media exhibited significantly higher activity compared to the same age of bacteria cultured on other media. Bacteria cultured in LB media for 4 days showed significantly higher activity than bacterial culture grown on other media for the same period of time (Fig. 5A). The culture filtrate from 1-day-old bacteria grown on all media showed lower activity compared to their respective bacterial culture (Fig. 5A, B). The bacterial culture filtrates of NB and SGB incubated for 4 and 7 days exhibited similar fungal inhibition (p > 0.05) (Fig. 5B).

B. subtilis A18 cultured on different media exhibited less activity against *H. annosum* compared to *H. parviporum*. In all cases, bacteria grown for 4 or 7 days showed significantly more activity compared to day 1 (Fig. 6A). A18 grown on different media for 4 days showed similar activity except LB-grown bacterial culture that showed significantly lower activity than PDB, TSB and SGB bacterial cultures (Fig. 6A).

In contrast to the activity against *H. parviporum*, the culture filtrate of LB media showed strong fungal inhibition against *H. annosum* and the bioactivity of the 1-day-old culture filtrate was significantly higher than other media of the same age (Fig. 6B). Overall, the fungal inhibition activity of 4-day-old bacterial culture filtrates was higher in all media except NB, which in turn showed similar result to that of TSB media (Fig. 6B).

On comparing the anti-fungal activity of 4-day-old *B. subtilis* cultures and cell-free culture filtrates, it was observed that *H. parviporum* was more susceptible towards both bacterial cultures and their cell-free filtrates compared to *H. annosum* when the bacteria was grown on NB, PDB, SGB and LB media (Fig. 7).

3.5. Effect of glucose on antifungal activity

The cell free culture filtrates of *B. subtilis* A18 cultured on SGB with and without glucose showed different activity against *H. parviporum* and *H. annosum* (Fig. S2). The antifungal activity of cell-free culture filtrates against *H. annosum* was significantly higher in the presence of glucose than in absence of it. However, no noticeable difference was observed in case of *H. parviporum* (Fig. S2).

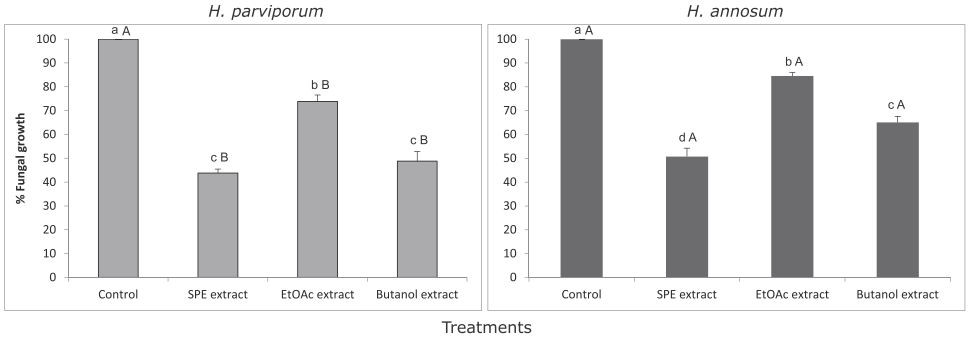

Fig. 3. Growth of *H. parviporum* and *H. annosum* in the presence of control media and *B. subtilis* A18 broth culture filtrate extracted by SPE, EtOAc and butanol, respectively. Columns with different small letters are significantly different ($p < 0.05$) from each other when the comparison was made between the effect of different extracts on the growth of *H. parviporum* and *H. annosum* independently (one way ANOVA *post-hoc* Bonferroni test). Different capital letters on columns indicate that the inhibitory effect of an extract was different ($p < 0.05$) for the different fungal species (*t-test*). Error bars stand for SEM (n = 5).

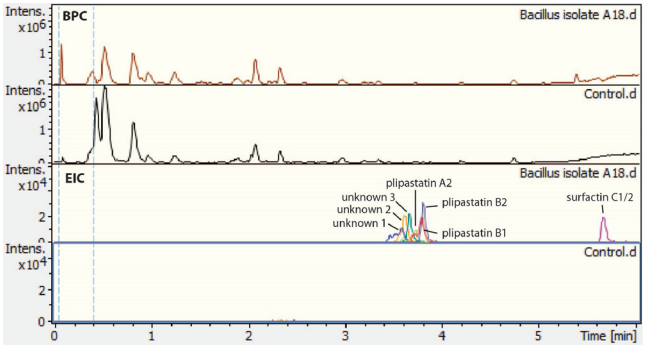

Fig. 4. Base-peak chromatograms (BPC) and extracted-ion chromatograms (EIC) from LC-HRMS analysis of SPE extracts of *Bacillus subtilis* A18 bacterial broth and control medium. Annotated peptides were tentatively identified by the database Antibase.

3.6. Lipopeptide production of *B. subtilis* A18 when different media were employed

B. subtilis A18 produced different kinds of lipopeptides when cultured in different media. Fengycin was found to be produced in the culture filtrate of *B. subtilis* grown in NB, SGB and TSB for 7 days. The relative amount of this compound was higher in SGB than in NB media (Table 1). Kurstakin was found in the culture filtrate of *B. subtilis* A18 grown in PDB and LB media. *B. subtilis* A18 produced polymixin when TSB and SGB were employed as culturing media (Table 1).

3.7. Absence of fungal growth on well-established *B. subtilis* A18

A fully grown *B. subtilis* A18 culture completely inhibited the growth of *Heterobasidion* on YEPD agar plates for more than two months (Fig. 8).

4. Discussion

This study describes the antagonistic activity of a *Bacillus subtilis* strain against conifer pathogenic fungi. A co-culturing bioassay revealed a complete inhibition of mycelial growth of the fungi *Heterobasidion annosum* and *H. parviporum* by an isolate (A18) of *B. subtilis*. Previously, it has been reported that *B. subtilis* inhibits the mycelial growth of *Pythium ultimum* and seed treatment with isolates of this bacteria reduces the sugar-beet damping-off disease under greenhouse conditions (Abo-Elnaga, 2006). Montealegre et al. (2003) reported mycelial cytoplasmic leakage of *Rhizoctonia solani* when exposed to *B. subtilis*.

In order to understand the factors behind the inhibiting effect of *B. subtilis* A18, we investigated a number of properties. The secondary metabolites of *B. subtilis* A18 extracted by SPE and butanol exhibited

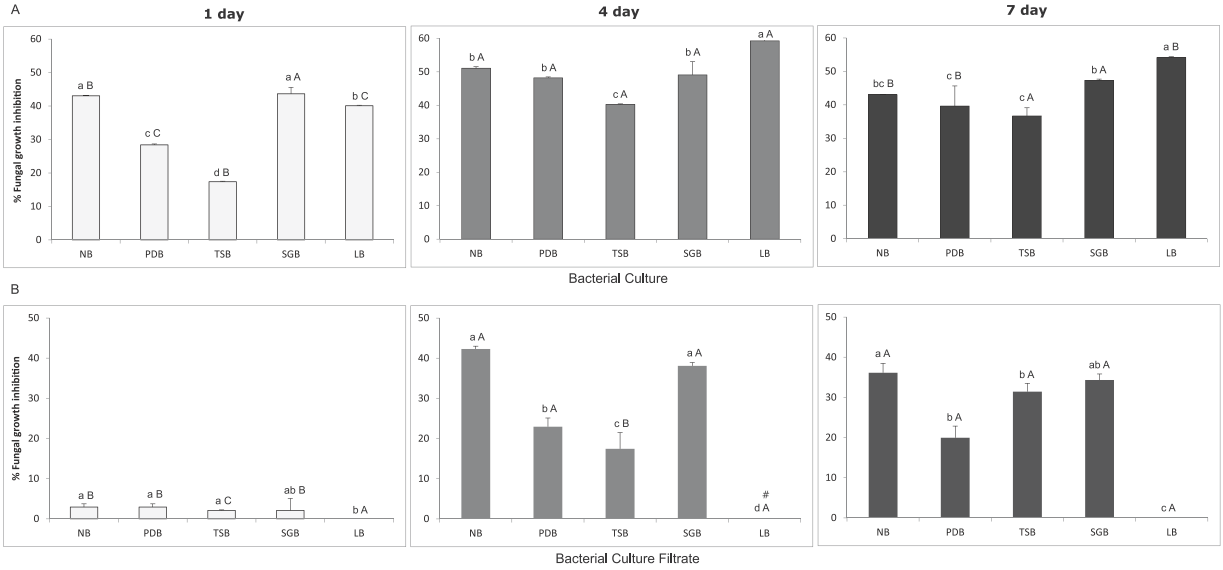

Fig. 5. Percent growth inhibition of *H. parviporum* by *B. subtilis* A18 cultures (A) and by cell-free culture filtrates (B). Different small letters on columns are showing significant difference ($p < 0.05$) between the fungal inhibition activity of bacterial cultures or bacterial culture filtrates grown on different media for 1 day, 4 day and 7 day independently (one way ANOVA *post-hoc* Bonferroni test). Different capital letters on bars indicate significant difference ($p < 0.5$) of bacterial cultures or cell free filtrates when the comparison was made between the fungal inhibitory activity of cultures grown on a specific medium for 1, 4 and 7 days (one way ANOVA *post-hoc* Bonferroni test). Error bars are expressing SD.

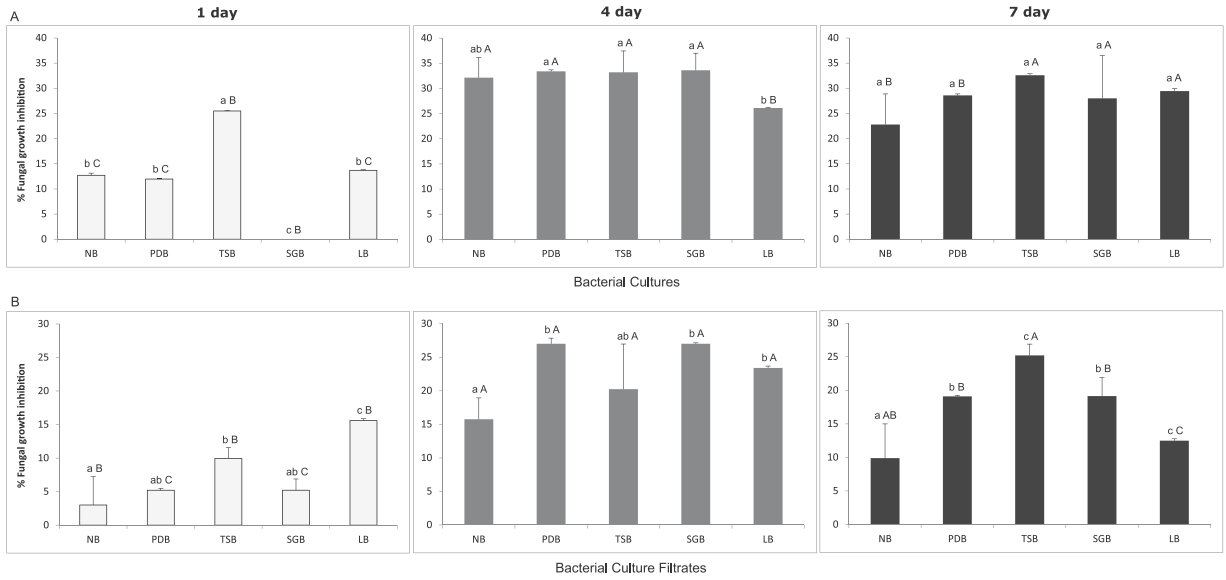

Fig. 6. Percent growth inhibition of *H. annosum* by *B. subtilis* A18 cultures (A) and by cell free culture filtrates (B). Different small letters on columns show significant difference ($p < 0.05$) between the anti-fungal activity of bacterial cultures or bacterial culture filtrates grown on different media for 1 day, 4 day and 7 day independently (one way ANOVA *post-hoc* Bonferroni test). Different capital letters on bars indicate significant difference ($p < 0.05$) of bacterial cultures or cell free filtrates when the comparison was made between the fungal inhibitory activity of cultures grown on a specific medium for 1, 4 and 7 days (one way ANOVA *post-hoc* Bonferroni test). Error bars are expressing SD.

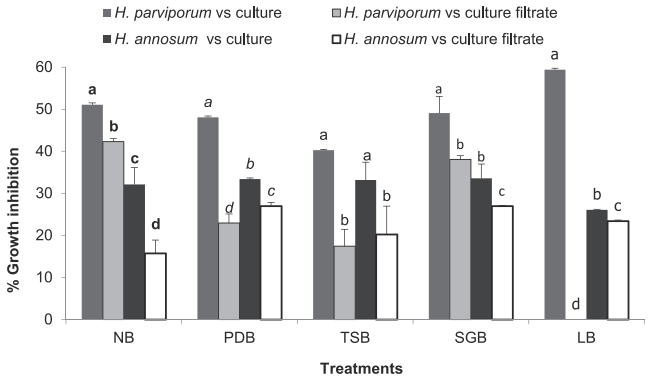

Fig. 7. Percent growth inhibition of *H. parviporum* and *H. annosum* by 4-day-old *B. subtilis* A18 cultures and culture filtrates. Different letters on columns show significant difference ($p < 0.05$) between the anti-fungal activity of bacterial culture and bacterial culture filtrate grown on a specific medium (one way ANOVA *post-hoc* Bonferroni test). Error bars are expressing SD.

significant effect on the fungal growth of both species; however, *H. parviporum* was more susceptible to butanol and SPE extracts. LC-HRMS analysis of SPE extracts revealed the presence of plipastatins and surfactins. These two compounds are known to be produced by many members of the *Bacillus* genus (Foster and Woodruff, 1946; Landy et al., 1948; Deleu et al., 1999; Ben Slimene et al., 2012). Moreover, there were three other peptides (compound **1**-**3**) not present in the database Antibase. The *m/z* values of compounds **1**-**3** indicate that they differ by having a water molecule as compared to plipastatin A2, B1 and B2, respectively. This difference could be explained by compounds **1**-**3** being the linear analogues of the cyclic depsipeptides plipastatin A2, B1 and B2, respectively. Other studies reported that *Bacillus* spp. could secrete metabolites such as bacillin and bacillomycin, and lipopeptides such as surfactin, iturin, and fengycin, which could have antifungal activities against pathogens (Foster and Woodruff, 1946; Landy et al., 1948; Deleu et al., 1999; Carvalho et al., 2010; Ben Slimene et al., 2012).

We also investigated *B. subtilis* A18 in detail to evaluate the effect of different nutrients and culture age on the anti-fungal activity. Overall, the bacterial cultures had significantly higher and long-lasting anti-fungal activity compared to their corresponding cell-free culture filtrates. This result suggests that presence of bacteria might be essential for the continuous production of antifungal compounds, accomplishing higher concentrations. MALDI-TOF mass-spectrometry analysis of culture filtrates of *B. subtilis* A18 revealed the presence of compounds which could be classified into three mass ranges attributed to five biosurfactant families: 850–950 *m/z*, which includes kurstakins; 990–1100 *m/z*, which includes surfactins and iturins; 1100–1200 *m/z*,

Table 1
Lipopeptides produced from *B. subtilis* A18 detected by MALDI-TOF-MS. The peptide class was assigned based on comparison of mass spectra found in the literature. All filtrates are from day 4 unless differently noted.

Culture filtrate	Major Mass fragments (m/z)	Peak Intensity	Putative class of peptide	Reference
NB	1477.9, 1491.9, 1506.4	270	Fengycin	Vater et al., 2002
PDB	851.9, 867.8, 916.7, 967.9	2155	Kurstakin	Hathout et al., 2000
TSB	997	340	Iturin	Price et al., 2007
	1201.3	340	Polymixin	
SGB	1099.5, 1227.8, 1245.4	1165	Polymixin	Price et al., 2007
	1462.5, 1477.6, 1491.1, 1506.1	1165	Fengycin	Vater et al., 2002
LB	805.4, 854.7, 916.7, 996.8	498	Kurstakin	Hathout et al., 2000
TSB (7d)	996.9	912	Iturin	
	1449.4, 1463.7, 1478.2, 1492.2, 1495.3, 1506.2, 1523.7, 1531.3	1140	Fengycin	Vater et al., 2002
Positive control	993.7, 1007.6, 1021.6, 1029.7, 1035.7, 1073.8, 1089.6	1365	Surfactin	Current study

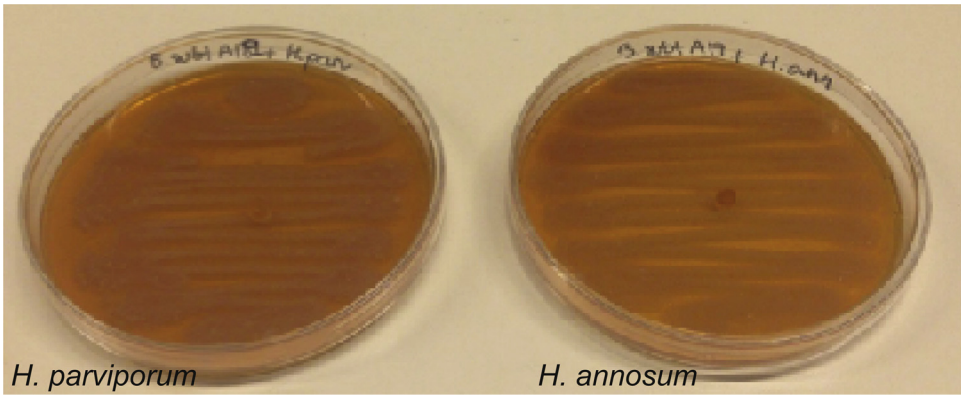

Fig. 8. Absence of *H. parviporum* and *H. annosum* growth in the presence of fully grown *B. subtilis* A18. The fungi were inoculated as mycelial plugs in the centre of the plates.

which includes polymixins, and 1450–1550 *m/z*, which includes fengycins. These biosurfactants are known as amphiphilic membrane active with antimicrobial properties. Attributions were made based on the mass numbers reported for lipopeptides from other *Bacillus* strains (Vater et al., 2002; Price et al., 2007; Kim et al., 2010; Ayed et al., 2014). Fengycins were identified in the culture filtrates of *B. subtilis* grown in NB, and SGB for 4 days; those culture filtrates also significantly inhibited *Heterobasidion* growth. On the other hand, kurstakins were found to be produced and secreted by *B. subtilis* A18 when cultured in PDB and LB which differs from the observations by Price et al. (2007), where kurstakins were predominantly found retained by bacterial colonies, as opposed to being secreted in liquid cultures.

The mass spectra of extract from *B. subtilis* A18 cultured in NB and SGB during four days revealed the presence of peaks clustered at 1462–1506 *m/z* that could be assigned as putative fengycin lipopeptides (Ayed et al., 2014; Kim et al., 2009; Vater et al., 2002; Price et al., 2007). The mass spectra of the crude extract from bacteria cultured in PDB for four days revealed the presence of well-resolved clusters of peaks at the *m/z* 851–967 regions. By comparing the mass numbers reported for lipopeptides from other *Bacillus* strains, this group of peaks could be attributed to kurstakins isomers (Price et al., 2007), which were identified in year 2000 and defined as the lowest molecular group of lipopeptides (Hathout et al., 2000). In fact, these lipopeptides are usually retained by cells and not secreted (Price et al., 2007), but *B. subtilis* A18 seems to be able to produce and secrete them when specific nutrients were used for its growth. *B. subtilis* A18 also produced kurstakin and iturin type peptides when cultured in LB for four days and showed higher inhibitory effect against *H. annosum*. Recently, Dimkić et al. (2017) reported that iturins were very effective lipopeptides.

Direct contact growth between *Heterobasidion* species and *B. subtilis* A18 revealed the complete growth inhibition of fungi for more than two months, whereas in co-culture studies, when fungi grew first followed by bacterial inoculation, the effect was not so strong and fungi started to compete with the bacterial inhibition. These results advocate the use of *B. subtilis* A18 as a bio-control agent to be applied on the fresh stump during the thinning process. However, further studies are needed to make way to establish bacteria on the stumps.

The properties which make using bacteria as a bio-control agent is advantageous due to their ability to multiply rapidly, secrete metabolites, and be effective in a short period of time. To our knowledge, this is the first report on antifungal activity of *B. subtilis* against *H. annosum* and *H. parviporum*. Further studies are needed to test this isolate in field trials in order to provide a suitable and alternative biocontrol agent against forest fungal pathogens.

5. Conclusions

The present study reports the results of the antifungal activity of *B. subtilis* A18 cultures and cell-free culture filtrates against the conifer pathogenic fungi *H. annosum* and *H. parviporum*. The growth inhibition of *Heterobasidion* species was found to depend on the composition of culture medium and the age of the culture, which affect the production of antifungal metabolites. The chemical analysis of *B. subtilis* A18 culture filtrates indicated the presence of kurstakins, surfactins, iturins, polymixins and fengycins and their production was found to be depended on the medium used for bacterial growth as well the age of culture.

Conflict of interest

All the authors of this manuscript declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.04.006.

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