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Temperature-dependent production of cyclic lipopeptides by *Pseudomonas fuscovaginae* UPB0736 and its impact on *Rhizoctonia solani* suppression

Jasmine De Rop [a,b], Durga Prasad [c], Niels Geudens [c], Lu Zhou [b], Bishnu Marahatta [b], Pieter Spanoghe [a], José C. Martins [c], Monica Höfte [b,*]

[a] Lab of Crop Protection Chemistry, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium
[b] Lab of Phytopathology, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium
[c] NMR and Structure Analysis Unit, Department of Organic and Macromolecular Chemistry, Faculty of Sciences, Ghent University, Ghent, Belgium

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ABSTRACT

Aims: Pseudomonas fuscovaginae, the causal agent of rice sheath brown rot, produces the cyclic lipopeptides (CLiPs) syringotoxin and fuscopeptin, with a dual role in phytotoxicity and antagonism against competing microorganisms. Up to now, produced CLiP quantities are too low for further biological studies. We aimed to optimize CLiP production and extraction to further investigate their antifungal effects.
Methods and results: Bacteria were cultured at different temperatures in different liquid media. CLiPs were extracted using solid phase extraction and quantified and characterized by UPLC-MS/MS and NMR. Antifungal activity was assessed via IC_{50} determinations in bioassays against *Rhizoctonia solani*. The highest CLiP production was reached at 18°C in liquid King's B Medium in baffled flasks, reaching a 1000-fold increase for syringotoxin and a 5-fold increase for fuscopeptin, compared with incubation at 28°C in non-baffled flasks. Especially syringotoxin showed potent activity against *Rhizoctonia*, with IC_{50} values of 0.02–0.09 mg/L.
Conclusions: CLiP production in *P. fuscovaginae* is boosted at 18°C, promoting higher yields and stronger antifungal activity, especially for syringotoxin. These findings highlight the importance of environmental conditions in shaping antifungal efficacy and disease outcomes in rice-pathogen interactions.

1. Introduction

Pseudomonas species are renowned for their capacity to produce specialized metabolites such as cyclic lipopeptides (CLiPs), which play multifaceted roles in their ecological and physiological functions. These amphiphilic molecules exhibit antimicrobial properties, cause phytotoxicity or promote induced systemic resistance (ISR) in plants, and support developmental traits including motility, biofilm formation, and root colonization (D'aes et al., 2014; Ferrarini et al., 2022a; Köhl et al., 2019; Pieterse et al., 2014; Raaijmakers et al., 2010). Among the most bioactive of these compounds are the Mycin and Peptin families, which show distinct antimicrobial and phytotoxic activities by targeting cellular membranes, disrupting their integrity, and permeabilizing lipid bilayers (Balleza et al., 2019; Dexter and Middelberg, 2008; Ferrarini et al., 2022a; Girard et al., 2020).

P. fuscovaginae is a member of the *P. asplenii* subgroup within the *P. fluorescens* group and a rice pathogen causing bacterial sheath brown rot (Bigirimana et al., 2015; Quibod et al., 2015). *P. fuscovaginae* is typically causing disease at high altitudes, with temperatures ranging from 18 to 23°C or cooler (Cottyn et al., 1996; Duveiller et al., 1988; Jaunet et al., 1996; Musonerimana et al., 2020; Quibod et al., 2015). Strain UPB0736, isolated from diseased rice plants in the highlands of Madagascar, produces three CLiPs: syringotoxin, fuscopeptin, and asplenin. The structure and retention times of these metabolites synthesized by large nonribosomal peptide synthetases (NRPSs), are presented in Fig. 1. They exhibit varying degrees of phytotoxicity, antimicrobial activity, and ecological functions. Syringotoxin belongs to the Mycin family, characterized by a nine-amino-acid macrolactone ring and a 3-hydroxy fatty acid tail comprising 14 carbons. It displays potent antifungal activity against *R. solani*, a major plant pathogen, and plays a role in phytotoxicity in combination with fuscopeptin (Ferrarini et al., 2022b; Serra et al., 1999; Sorensen et al., 1996; Surico & DeVay, 1982). Fuscopeptins, classified within the Peptin family, comprise two variants, fuscopeptin A and B, differing in the length of their acyl chains, C8:0 and C10:0, respectively. These CLiPs contain 19 amino acids, with a distinctive macrolactone ring formed by five of them. Fuscopeptins

* Corresponding author.
E-mail address: monica.hofte@ugent.be (M. Höfte).

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exhibit phytotoxicity through inhibition of plant H^+-ATPases and membrane permeabilization, thereby disrupting host cellular processes (Coraiola et al., 2008, Zhou et al., 2024) and show some antifungal activity against *Botrytis cinerea* and *Rhodotorula mucilaginosa* (Ballio et al., 1996). The dual role of syringotoxin and fuscopeptins as phytotoxins and antimicrobial agents positions them as key contributors to the virulence of UPB0736. In addition to these, UPB0736 produces asplenin, a distinct CLiP predicted to have a 13-amino-acid sequence, including an eight-amino-acid macrolactone ring. Unlike syringotoxin and fuscopeptins, asplenin lacks significant antifungal or phytotoxic activity but plays a pivotal role in bacterial motility (Ferrarini et al., 2022b). It was shown that *P. fuscovaginae* UPB0736 is able to inhibit the growth of *R. solani*, due to the antifungal effect of syringotoxin. Mutants lacking syringotoxin production exhibited no significant in vitro antifungal

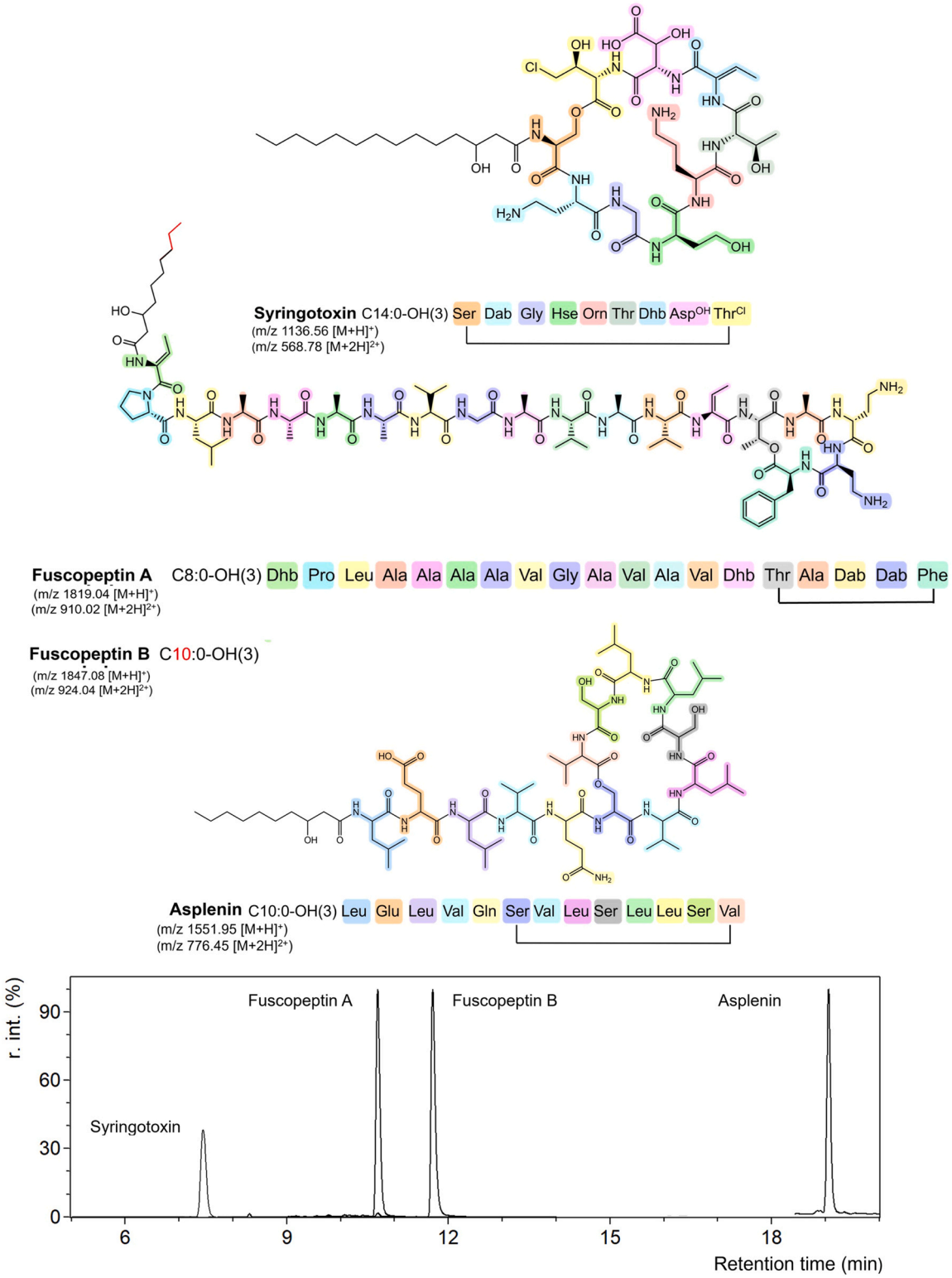


Fig. 1. Chemical structures and chromatographic full MS scan (cone 30 V) of syringotoxin, fuscopeptin A, fuscopeptin B and the predicted structure of asplenin.

activity against *Rhizoctonia* strains. However, fuscopeptin was identified as the primary contributor to sheath brown rot symptoms in rice (Ferrarini et al., 2022b).

Rhizoctonia solani consists of 14 multinucleate anastomosis groups (AG 1–13 and BI), which have been divided based on hyphal anastomosis behavior, cultural morphology, host range, pathogenicity and other characteristics (Carling et al., 1999; Ogoshi, 1976). These multinucleate *Rhizoctonia* isolates mainly cause damping-off and hypocotyl rot, but can also cause foliar symptoms. *R. solani* AG 1-IA is the causal agent of sheath blight, a major disease of rice cultivated in intensive production systems (Lee, 1983; Taheri et al., 2007). *R. solani* AG 2–2 can aggressively infect cauliflower, endive, maize and bean, resulting in a severely reduced yields (Fenille et al., 2002; Ghosh et al., 2014; Nerey et al., 2010; Pannecoucque et al., 2008; Tu et al., 1996; Verma, 1996).

In order to investigate the biological effects of the isolated CLiPs, there is a need for an increased CLiP production. Optimization of CLiP production requires a holistic approach which includes screening of producer strains, determination of optimal production parameters, and finally extraction and purification (Coutte et al., 2017; Henkel et al., 2017). Screening of producer strains as well as metabolic and genetic engineering strategies have been thus developed to increase the specific productivity (Coutte et al., 2015). Most efforts have focused on the optimization of lipopeptide production in *Bacillus* species (Ongena and Jacques, 2008), while little work has been done so far in *Pseudomonas* spp. Under optimized conditions in King's B (KB) medium, *Pseudomonas fluorescens* BD5 achieves production levels of pseudofactin, up to 1.2 g/L at 28°C, a substantial increase from the 0.01 g/L observed in unoptimized conditions. This enhancement is primarily attributed to a high glycerol concentration (up to 100 g/L), supplementation with leucine (9.2 g/L) or valine, tryptone (12.1 g/L), and increased aeration achieved with a 20 % filling volume. Comparative studies indicated that KB medium outperformed other media in supporting maximum pseudofactin production (Biniarz et al., 2018). In comparison, syringomycin production levels by *Pseudomonas syringae* in still cultures of potato dextrose broth (PDB) reached a more modest 26 mg/L, nevertheless representing a yield 20–136 times higher than that on potato dextrose agar (PDA) or in shaken PDB cultures. The most favorable condition for syringomycin production by *P. syringae* consisted of 1.5 % dextrose and 0.4 % casamino acids in still PDB cultures at 23°C to 25°C (Gross & DeVay, 1977). In contrast, thanamycin yields in *P. fluorescens* DSM11579 are very low with levels between 0.1 mg/L and 0.33 mg/L in Yeast Mannitol liquid medium (Johnston et al., 2015).

This study examined the influence of incubation temperature on CLiP production by *Pseudomonas fuscovaginae* UPB0736 and how this regulation influences its antifungal effect against *Rhizoctonia solani* AG 1-IA and AG 2–2. To this end, we investigated the production level of specialized metabolites by ultra-performance liquid chromatography (UPLC) coupled with tandem mass spectrometry. Syringotoxin and fuscopeptin were fully characterized by NMR spectroscopy to confirm their structure. Furthermore, the sensitivity of *R. solani* AG 1-IA and AG 2–2 to the CLiPs at both temperatures is also evaluated. This study further focuses on optimizing syringotoxin and fuscopeptin production by *P. fuscovaginae* UPB0736 under varying culture conditions. In particular, we examined the impact of growth medium composition (King's B liquid medium or potato dextrose broth), temperature (18°C vs. 28°C), and aeration (shaking or still cultures, baffled or non-baffled flasks) on CLiP yields. By leveraging these optimizations, sufficient quantities of CLiPs were produced to enable IC_{50} values and additional bioactivity assays.

2. Material and methods

2.1. Strains and culture conditions

The strains used in this study are listed in Table 1. All *Pseudomonas* strains were cultured in King's B liquid medium (10 g/L proteose peptone No.3 (Difco), 1.5 g/L K_2HPO_4, 2.96 g/L $MgSO_4$ x 7 H_2O, 10 mL/L glycerol) or potato dextrose broth (PDB, Becton Dickinson) at different temperatures. Solidified KB medium was prepared by supplementing with 15 g/L of bacteriological agar (Difco). Liquid cultures were incubated in baffled flasks with a 15 % filling volume and shaken at 150 rpm. *Rhizoctonia* strains (Table 1) were cultured on potato dextrose agar (PDA; Becton Dickinson) at 28°C, while liquid cultures were grown in potato dextrose broth (PDB) at either 18°C or 28°C under the same shaking conditions (150 rpm in baffled flasks with a 15 % filling volume).

Table 1
Strains used in this study and their main characteristics.

	Origin and characteristics	Reference
Bacteria		
Pseudomonas fuscovaginae UPB0736 WT	Wild type (*Oryza sativa*, Madagascar)	(Ferrarini et al., 2022b)
UPB0736 Δ*fst*[1]	Deletion in fstA gene	(Ferrarini et al., 2022b)
UPB0736 Δ*fus*[2]	Deletion in fusA gene	(Ferrarini et al., 2022b)
UPB0736 Δ*fst*Δ*fus*	Deletion in fstA and fusA genes	(Ferrarini et al., 2022b)
UPB0736 Δ*fst* Δ*asp*[3]	Deletion in fstA and aspA genes	(Ferrarini et al., 2022b)
UPB0736 Δ*fus* Δ*asp*	Deletion in fusA and aspA genes	(Ferrarini et al., 2022b)
Fungi		
Rhizoctonia solani AG 2–2 CuHav-Rs18	*Phaseolus vulgaris*, Cuba	(Nerey et al., 2010)
Rhizoctonia solani AG 1-IA STMX04–3	Cabbage, Vietnam, pathogenic on rice	(Hua et al., 2014)

[1] fst: syringotoxin
[2] fus: fuscopeptin
[3] asp: aspleni

2.2. Quadruple culture interaction

A plug with diameter 6 mm of a 48-hour old culture of *Rhizoctonia solani* AG 1-IA or AG 2–2 grown on PDA was added in the center of a PDA plate. *Pseudomonas* strains were cultured in KB broth at 28°C (150 rpm) for 24 h. Ten μL of the *Pseudomonas* broth was spotted on the four sides of the plate at 2.25 cm from the central plug. The plates were incubated at 18°C or 28°C and evaluation took place 24, 48 and 72 h after incubation. ImageJ software was used to determine the mycelium area. The *Pseudomonas* strains were scraped off the plate with 10 mL saline water (0.85 % NaCl), transferred to an Eppendorf tube and vortexed to homogenize the bacterial suspension. From this suspension, 20 μL was diluted with 180 μL saline water for OD measurements (Tecan Infinite 200 Pro, Switzerland).

2.3. Pseudomonas spp. metabolite extraction from liquid broth

For the determination of the CLiP concentration in liquid KB, 50 mL of the inoculated broth was centrifuged and the cell-free supernatant was collected. Extraction of the CLiPs was done by solid-liquid extraction using Waters Oasis HLB extraction columns. Columns were activated with 5 mL methanol and 5 mL MilliQ. Prior to elution, the column was washed with 5 mL 20 % acetonitrile (ACN). The CLiPs were eluted with 5 mL 50 % ACN, Samples were taken for UPLC-MS/MS analysis. The recovery of this method for syringotoxin is 96.0 ± 7.2 % and the recoveries of fuscopeptin A and B are, and 75.10 ± 6.1 % and 78.12 ± 4.1 % respectively (n = 6).

2.4. IC_{50} determination with purified compounds

For the extraction of syringotoxin and fuscopeptin, extraction columns were washed with 20 % methanol (MeOH) and subsequently eluted with 100 % MeOH. The collected extracts were evaporated to dryness. For IC_{50} determination of syringotoxin and fuscopeptin, the dried extracts were reconstituted in methanol, and serial dilutions were prepared by diluting 50 µL of the reconstituted extract in 450 µL of sterile water. Potato dextrose agar (PDA) was then added to the dilutions. The concentrations of the compounds were quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). In the initial suspension, fuscopeptin A and fuscopeptin B were present at concentrations of 640.3 mg/L and 947.1 mg/L, respectively.

2.5. Chemical analysis of CLiPs produced by Pseudomonas spp

Detailed NMR characterization and quantification of syringotoxin and fuscopeptin is provided in Supplementary Materials S1. CLiPs were identified based on a previously reported method (Andrić et al., 2021) using UPLC coupled with tandem MS in positive mode with the parameter set up as follows: capillary voltage of 2.5 kV, source temperature 130°C, desolvation temperature 450°C, desolvation gas flow 650 L/h and cone gas flow 50 L/h. The ionization was kept in positive mode (ES+). Mass spectra were recorded for *m/z* ratio's ranging from 100 to 1900. All component-specific MS/MS parameters are given in Supplementary Table S4. The UPLC-MS1 full scan mode was executed at capillary 2.5 kV and a cone voltage of 30 V.

An ACQUITY UPLC® HSS T3 (2.1 ×100 mm, 1.8 µm particle size) was used at a flow rate of 0.5 mL/min at 40°C. A gradient of acidified MilliQ-water (0.1 % formic acid) (solvent A) and acidified ACN (0.1 % formic acid) (solvent B) was used as a mobile phase starting at 10 % B and rising to 100 % B in 20 min. Solvent B was kept at 100 % for 4 min before going to the initial ratio in 1 min and kept at 10 % for 2 more minutes. Due to the unavailability of sufficient purified asplenin standard, absolute quantification in mg/L was not feasible. Consequently, asplenin levels were reported based on relative peak area measurements rather than concentration in mg/L.

2.6. Statistical analysis

Statistical analyses were performed using R software version 2023.09.1 (Posit team, 2023). The packages ggplot2, multcomp, multcompView, cld, drc, lsmeans, biostat, ggpubr, and tidyverse were used. For comparison between treatment groups, normality and homoscedasticity were checked with Shapiro-Wilk's and Levene's test ($p = 0.05$). The evaluation results of normal distributed data were analyzed using the Tukey's Range test (homoskedastic), or the Welch's ANOVA (heteroskedastic) ($p = 0.05$). Data that were not normally distributed was analyzed with the Kuskal-Wallis Rank Sum Test and the Pairwise Wilcoxon Rank Sum Test. In all cases, significances were only

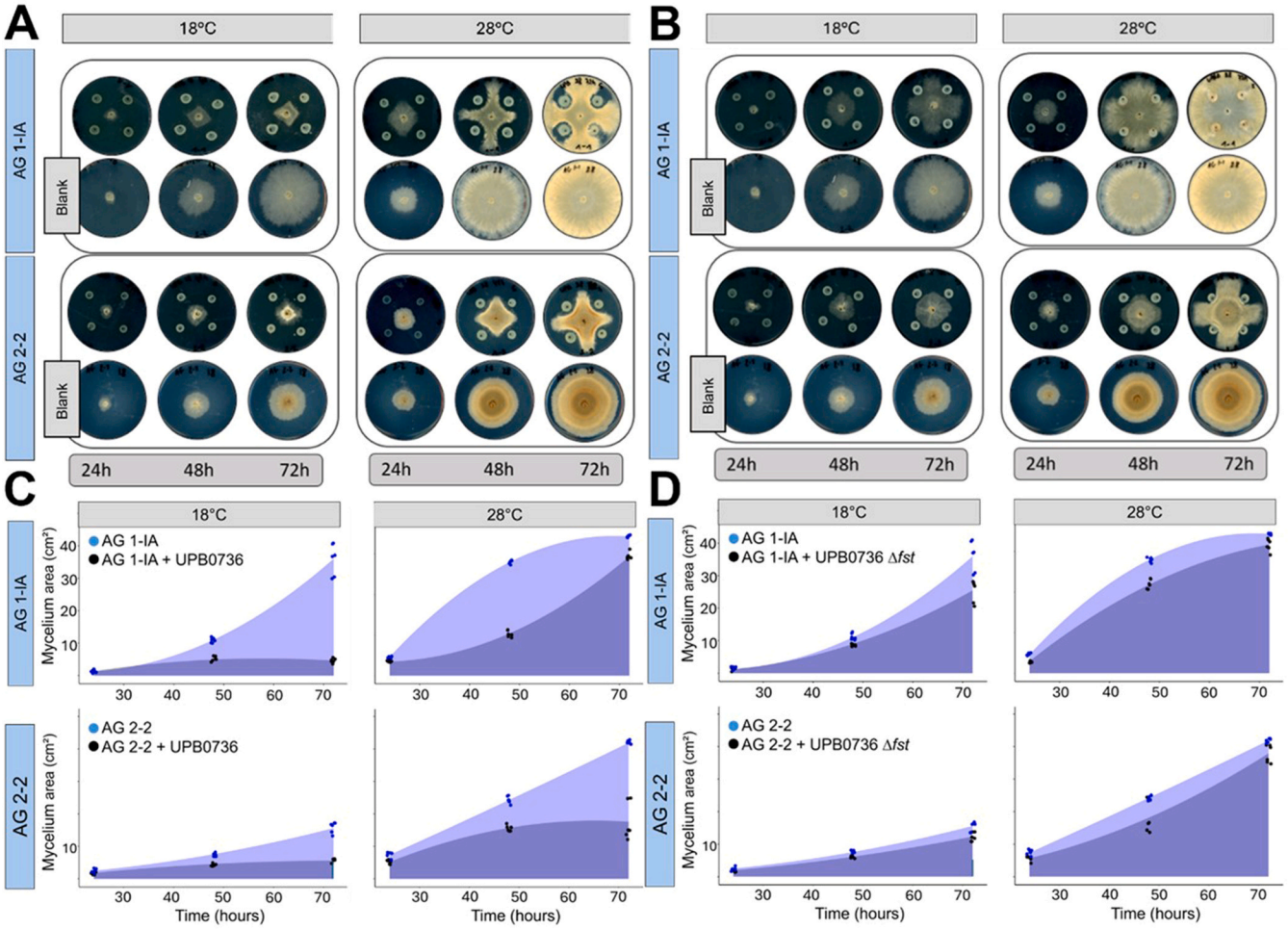


Fig. 2. Effect of *P. fuscovaginae* UPB0736 on the mycelium growth of *R. solani* AG 1-IA and AG 2–2 under different temperature conditions (18°C and 28°C) over time (24, 48, and 72 h). Representative images illustrate mycelial growth on PDA in the presence and absence of (A) the *P. fuscovaginae* UPB0736 wild-type (WT) strain and (B) the syringotoxin-deficient mutant (*UPB0736Δfst*). The lower row displays control plates without *P. fuscovaginae*. Quantification of mycelium area (cm^2) under the same conditions with (C) UPB0736 WT and (D) UPB0736*Δfst*, comparing growth dynamics.

accepted if $p < 0.05$.

3. Results

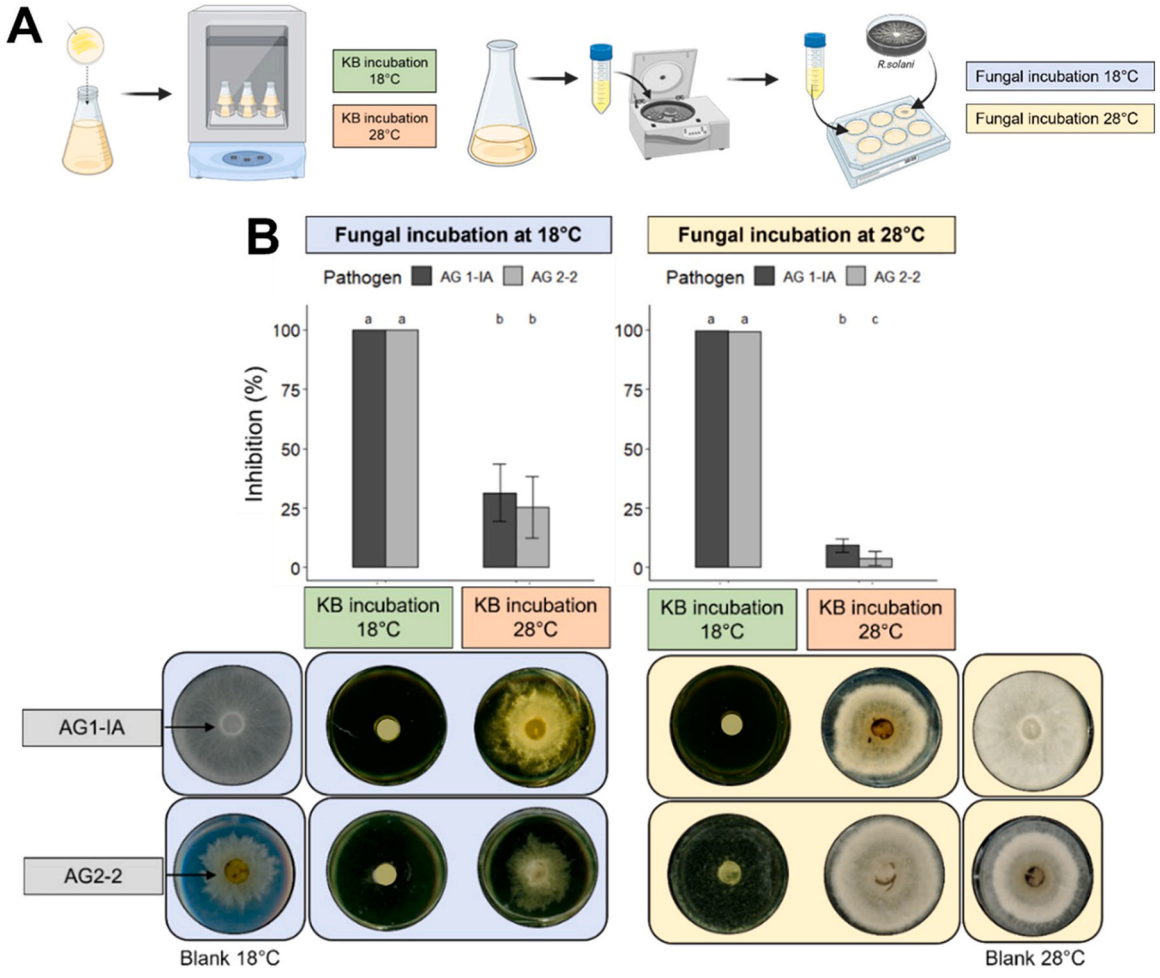
3.1. UPB0736 is more effective against R. solani at 18°C compared to 28°C

In studies assessing the antifungal activity of *P. fuscovaginae* UPB0736 over different incubation periods (24, 48, and 72 h) and temperatures (18°C and 28°C) on potato dextrose agar (PDA), the strain exhibited significant antagonistic activity against *R. solani* AG 1-IA and AG 2–2 mycelial growth at both temperatures, with the strongest inhibitory effect observed at 18°C. The evolution of the antagonistic activity of UPB0736 wild type (WT) and the syringotoxin mutant UPB0736 Δ*fst* is given in Fig. 2. There is more inhibition at 18°C compared to 28°C although the general growth of *R. solani* at 18°C is much slower. The relative inhibitory activity of the syringotoxin-deficient mutant *P. fuscovaginae* UPB0736 Δ*fst* against *R. solani* isolates was limited and exhibited similar effects on both AG 1-IA and AG 2–2 across both tested temperatures. Overall, the mutant strain producing only fuscopeptin and asplenin exhibited significantly reduced inhibitory activity compared to the wild-type strain, highlighting the crucial role of syringotoxin in mycelial inhibition. The mycelial surface area covered in each treatment was also monitored over time and is presented in Supplementary Table S5, while the logarithmic colony-forming unit (log CFU) values are provided in Supplementary Table S6.

To determine whether the enhanced antifungal effects at 18°C are attributable to increased CLiP production or an increased sensitivity of *R. solani* AG 1-IA and AG 2–2, two additional experiments were conducted. The first experiment evaluated CLiP production at 18°C and 28°C, while the second assessed the dose-response relationship of *R. solani* AG 1-IA and AG 2–2 to CLiPs under the same temperature conditions.

3.2. CLiP production is increased in King's B (KB) medium at 18°C

In order to get a better understanding in the temperature-mediated differences in CLiP production in liquid medium, an experiment in



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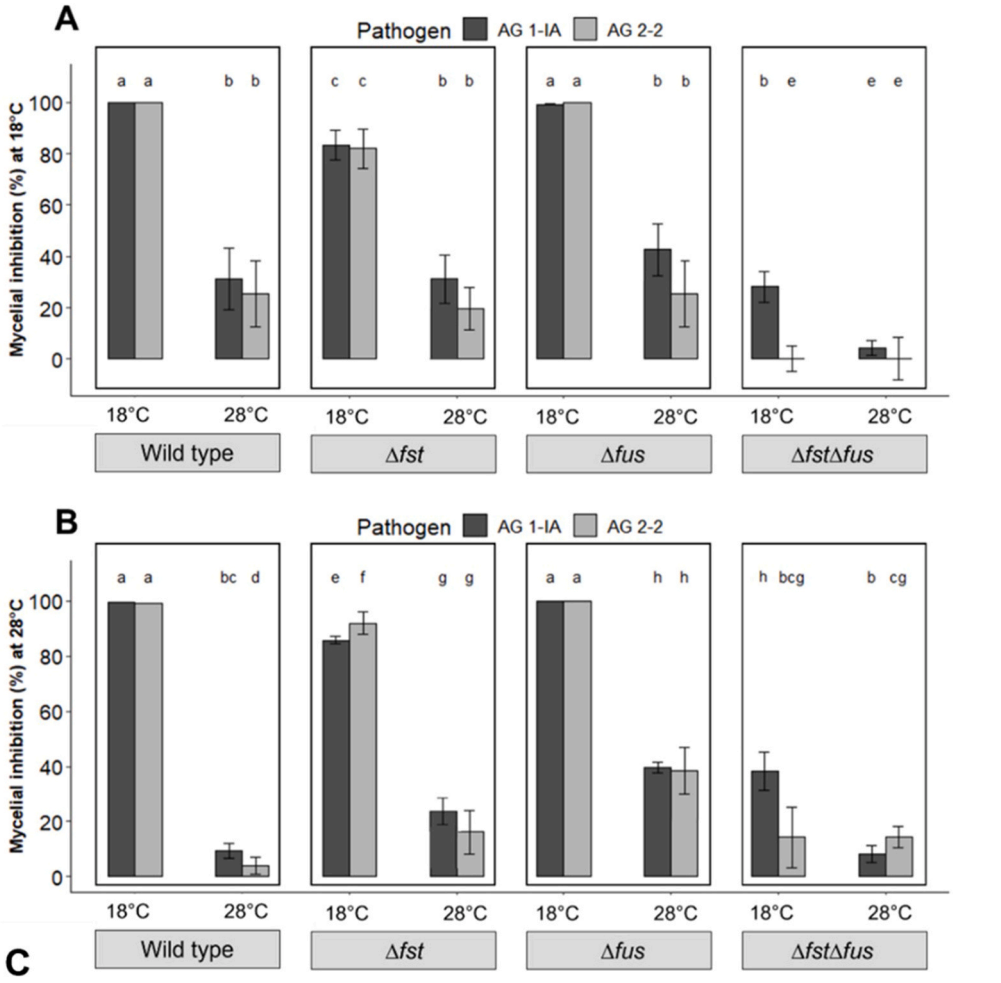
UPB0736	Syringotoxin		Asplenin		Fuscopeptin A		Fuscopeptin B	
Temperature	mg/L	SD	Log(area)	SD	mg/L	SD	mg/L	SD
18°C	6.58 a	0.01	6.74 a	0.01	8.35 a	0.01	5.67 a	0.01
28°C	0.04 b	0.02	5.23 b	0.53	1.98 b	0.26	1.32 b	0.05

Fig. 3. (A) Schematic representation of the methodology used for the mycelium inhibition assay. (B) Mycelium inhibition (mean % ± SD) of *R. solani* AG 1-IA and AG 2–2 at 18°C or 28°C. Significance: Wilcoxon rank sum test ($p < 0.05$, $n = 6$). (C) CLiP concentration (mg/L or mean log(area)). Significances were calculated with TukeyHSD ($p < 0.05$, $n = 3$).

liquid KB medium was set up. To assess the ability of the supernatant of UPB0736 WT to inhibit the mycelium growth of *R. solani* AG 1-IA and AG 2–2 at 18°C and 28°C, the cell-free supernatant of broth incubated at 18°C and 28°C was filter sterilized and mixed in a 1:1 ratio with double strength potato dextrose agar (PDA). A plug of *R.solani* was placed in the center and the plates were incubated for 48 h at 18°C or 28°C (Fig. 3). The highest significant enhancements in the inhibitory effect on mycelium growth were observed on the solid agar that contained the



UPB0736 WT	Syringotoxin		Asplenin		Fuscopeptin A		Fuscopeptin B	
Temperature	mg/L	SD	Log(area)	SD	mg/L	SD	mg/L	SD
18°C	6.58 a	0.05	6.74 a	0.01	9.62 a	0.01	5.65 a	0.01
28°C	0.03 b	0.02	5.23 b	0.53	2.67 b	0.32	1.21 b	0.06
UPB0736 Δ*fst*								
18°C			6.76 a	0.01	11.64 a	0.01	6.98 a	0.03
28°C			5.00 b	0.09	2.18 b	0.07	1.61 b	0.36
UPB0736 Δ*fus*								
18°C	8.13 a	0.03	6.76 a	0.01				
28°C	0.14 b	0.04	4.45 b	0.11				
UPB0736 Δ*fst*Δ*fus*								
18°C			6.78 a	0.01				
28°C			5.24 b	0.01				

Fig. 4. (A,B) Mycelium inhibition (mean (%) ± SD) of *Rhizoctonia solani* AG 1-IA and AG 2–2 at 18°C (A) and 28°C (B). Supernatant of King's B liquid medium containing *P. fuscovaginae* UPB0736 WT, the syringotoxin mutant (UPB0736Δfst), fuscopeptin mutant (UPB0736Δfus) and double syringotoxin and fuscopeptin mutant (UPB0736 ΔfstΔfus), incubated at 18°C and 28°C. Significance: Wilcoxon rank sum test ($p < 0.05$, $n = 3$). (C) CLiP concentration (mg/L or mean log(area)) and SD. Significances: TukeyHSD ($p < 0.05$, $n = 3$).

supernatant of UPB0736 that was subjected to incubation at 18°C. Simultaneously, the concentration of CLiPs in the supernatant was determined. The key distinguishing factor in CLiP concentration is the elevated concentration of syringotoxin, fuscopeptin A and B and asplenin in the broth exposed to 18°C incubation. The mycelium of *R. solani* AG 1-IA and AG 2–2 was also seemingly more effectively inhibited at 18°C compared to 28°C, indicating that the mycelium is possibly more sensitive to the secondary metabolites of UPB0736 at the lower temperatures. The experiment was also conducted using the single mutants UPB0736Δ*fst* (syringotoxin-deficient) and UPB0736Δ*fus* (fuscopeptin-deficient), as well as the double mutant UPB0736Δ*fst*Δ*fus* (Fig. 4). The results indicate that, while syringotoxin plays a dominant role in the antifungal effect, fuscopeptin also contributes to mycelial inhibition. In contrast, asplenin exhibits limited antifungal effects, reducing *R. solani* growth by no more than 30 %.

3.3. IC_{50} of UPB0736 supernatant against R. solani AG 1-IA and AG 2–2

Supernatant of UPB0736 and its mutants UPB0736Δ*fst,* UPB0736Δ*fus and* UPB0736Δ*fst*Δ*fus* was filter sterilized and subsequently combined with potato dextrose agar in different ratios. The extent of mycelium inhibition was evaluated for both anastomosis groups based on the dose-response relationships (Supplementary Fig. S3) and the IC_{50} values were determined (Table 2).

The sensitivity of *R. solani* to the secondary metabolites of the wild type of UPB0736 is mainly due to the presence of syringotoxin, since the combined effect of the syringotoxin and fuscopeptin does not seem to significantly influence the IC_{50}. The *R. solani* inhibition due to asplenin produced by UPB0736Δ*fst*Δ*fus* at a concentration of 50 % supernatant was at 18°C and 28°C respectively 12 % and 28 % for AG 1-IA and 3 % and 7 % for AG 2–2. The inhibition values of UPB0736Δ*fst*Δ*fus* are higher than 50 % supernatant, suggesting that asplenin and other metabolites present in the supernatant are not able to inhibit *R. solani* mycelium growth. *R. solani* AG 1-IA is slightly more sensitive to syringotoxin at 18°C while AG 2–2 is similarly sensitive for both in the tested temperature range (Table 2). For fuscopeptin, the IC_{50} value was determined based on the sum of the concentrations of fuscopeptin A and fuscopeptin B. The sensitivity to fuscopeptin was significantly lower compared to syringotoxin, resulting in higher IC_{50} values. Overall, *R. solani* AG 1-IA exhibited greater sensitivity to fuscopeptin than AG 2–2. Additionally, AG 2–2 demonstrated increased sensitivity to fuscopeptin at 28°C compared to 18°C. It was not possible to calculate an IC_{50} of UPB0736 Δ*fst*Δ*fus* due to its inability to inhibit the mycelium area for 50 %.

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3.4. Syringotoxin and fuscopeptin production is higher at 18°C in different liquid media

From the previous results, it can be concluded that the increased CLiP production by UPB0736 at 18°C is responsible for the increased antifungal effect towards *R. solani*. To further explore the growth conditions of UPB0736 that promote elevated CLiP production, we focused on optimizing microbial cultivation parameters in Potato Dextrose Broth (PDB) and King's B medium (KB). In the initial phase of experiment, both media were evaluated under static and shaking (150 rpm) conditions and at 18°C and 28°C as incubation temperatures, representing four distinct conditions. Standard 500 mL Erlenmeyer flasks were employed, each filled to 15 % of their total volume. Syringotoxin and fuscopeptin production was assessed after 2 and 5 days. Optical densities (OD) were measured at 620 nm using a spectrophotometer (Tecan Infinite 200 Pro, Switzerland) (Supplementary Table S7). Asplenin concentrations were not monitored.

The concentration of syringotoxin in the broth containing *P. fuscvaginae* UPB0736 reached its maximum level of ~12 mg/L after 5 days of incubation at 18°C in a shaken KB liquid medium (Fig. 5A). Importantly, shaking of the cultures was essential for achieving high syringotoxin concentrations at 18°C, regardless of whether incubation occurred in PDB or KB media. In PDB, syringotoxin concentration reached approximately 7 mg/L after 2 days, and remained stable after 5 days. At 28°C, syringotoxin production was very low and showed no significant differences across all tested culture conditions.

The concentration of fuscopeptin A and fuscopeptin B in the culture broth of *P. fuscovaginae* UPB0736 reached a maximum of approximately 5 mg/L for fuscopeptin A after 2–5 days of incubation at 18°C in shaken PDB and after 2 days in shaken KB medium (Fig. 5B,C). Fuscopeptin B reached its peak concentration of approximately 6 mg/L after 2 days of incubation at 18°C in shaken PDB or KB. Compared to syringotoxin, the temperature-dependent variation in fuscopeptin production between 18°C and 28°C was less pronounced. Also here, shaking of the cultures was essential for achieving high fuscopeptin concentrations at 18°C, regardless of whether incubation occurred in PDB or KB media. Syringotoxin and fuscopeptin concentrations were proven to stay stable in sterile, shaking conditions for at least 3 days (Supplementary Fig.S4).

3.5. Maximal syringotoxin and fuscopeptin production in shaken KB is situated around 18°C

Since lowering the incubation temperature from 28°C to 18°C significantly improves syringotoxin and fuscopeptin production, UPB was inoculated in shaking KB at different temperatures (16°C, 18°C, 21°C and 28°C) and the syringotoxin and fuscopeptin concentrations were monitored after 48 h (Fig. 5D,E,F). CFU measurements are given in Supplementary Table S7. Syringotoxin concentrations peaked sharply at 18°C, reaching approximately 6 mg/L. At 16°C and 21°C, syringotoxin concentrations were comparable to those observed at 28°C, remaining below 2 mg/L. In contrast, fuscopeptin A concentrations were highest within the 16–21°C temperature range, reaching approximately 7 mg/L, but decreased at higher temperatures, declining to ~4 mg/L at 28°C. A similar trend was observed for fuscopeptin B, with concentrations reaching ~6 mg/L between 16°C and 21°C and decreasing to ~2 mg/L at 28°C. Asplenin concentrations were not monitored.

Table 2
IC_{50} determinations and the 95 % confidence interval (CI) of syringotoxin and fuscopeptin (A+B) of the CLiPs in supernatant of *P. fuscovaginae* UPB0736 WT, the syringotoxin deficient mutant UPB0736Δ*fus*Δ*asp* and the fuscopeptin deficient mutant UPB0736Δ*fst*Δ*asp*) on mycelium of *R. solani* AG 1-IA and AG 2–2 at 18°C and 28°C.

IC_{50} determination based on the supernatant							
	Temperature	IC_{50} syringotoxin (mg/L)				IC_{50} fuscopeptin (mg/L)	
		UPB0736 WT		UPB0736Δ*fus*Δ*asp*		UPB0736Δ*fst*Δ*asp*	
		mg/L	95 % CI	mg/L	95 % CI	mg/L	95 % CI
R.solani AG 1-IA	18°C	0.06	0.03	0.05	0.02	3.07	0.74
R.solani AG 2–2	18°C	0.08	0.03	0.11	0.03	10.23	2.32
R.solani AG 1-IA	28°C	0.11	0.01	0.10	0.04	3.29	0.96
R.solani AG 2–2	28°C	0.08	0.02	0.11	0.13	5.24	1.19

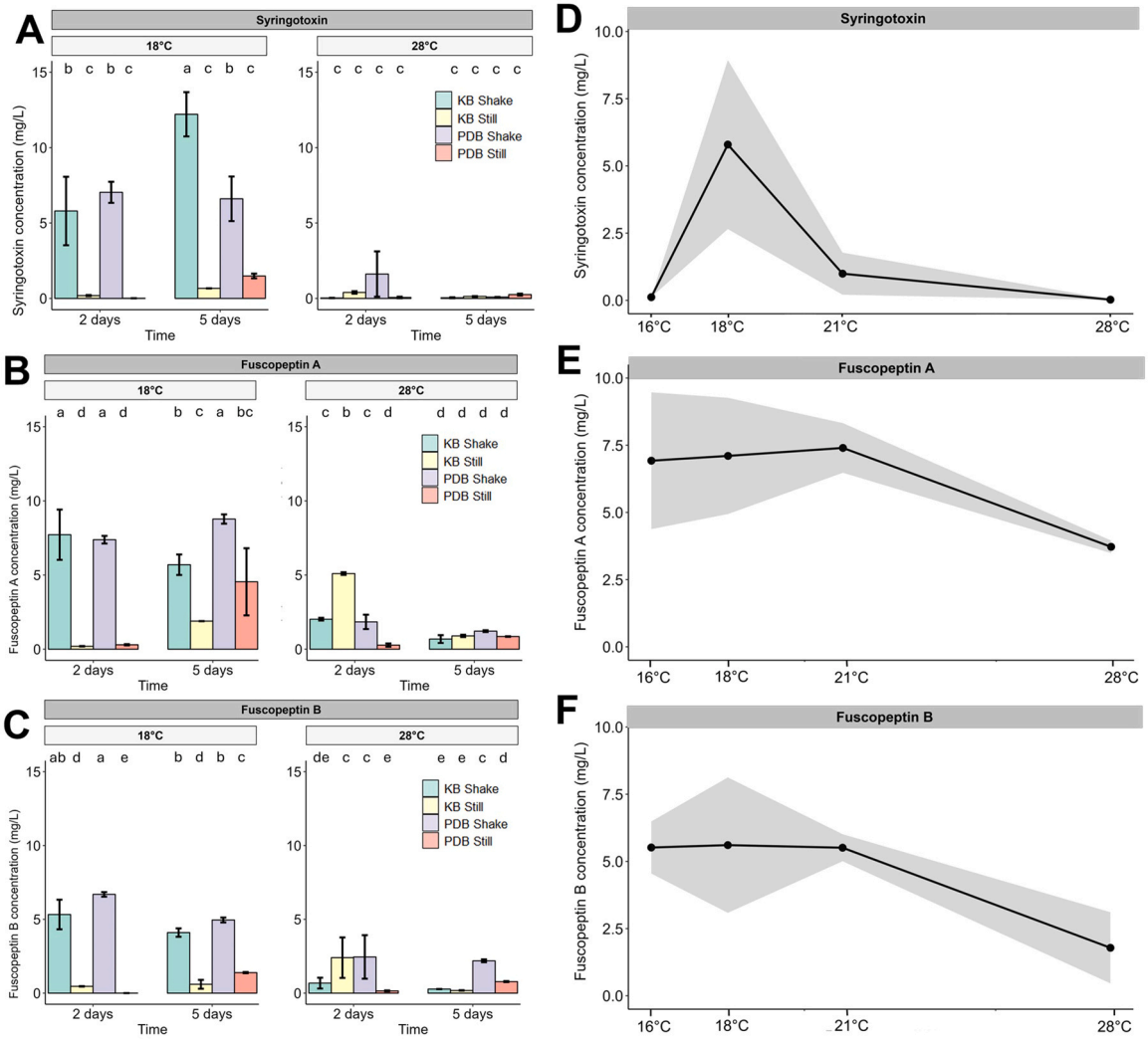

Fig. 5. (A) Syringotoxin, (B) fuscopeptin A and (C) fuscopeptin B concentration (mg/L) and SD in King's B liquid medium (KB) or potato dextrose broth (PDB) inoculated with *P. fuscovaginae* UPB0736 (at 18°C and 28°C) in still and shaken conditions. Significances were calculated using the Welch's ANOVA test. Bars with the same letters are not significantly different from each other ($p < 0.05$, $n = 3$). (D) Syringotoxin, (E) fuscopeptin A and (F) fuscopeptin B concentration (mg/L) at different incubation temperatures in KB. The shaded areas represent the 95 % confidence interval ($p > 0.05$, $n = 6$).

3.6. Syringotoxin and fuscopeptin production is further optimized by aerating KB broth in baffled flasks

In the next step, the CLiP production in baffled 1 L Erlenmeyer flasks with KB was tested at 18°C and 28°C and compared with production in 1 L standard Erlenmeyer flasks, to investigate if the cultures can perform better when supplied with extra oxygen. The results of this test are shown in Table 3. CFU and pH measurements of the supernatants can be found in Supplementary Table S8. Asplenin concentrations were not monitored.

At 18°C, syringotoxin production reached approximately 60 mg/L in baffled flasks, while non-baffled flasks produced only around 8 mg/L, indicating a significant reduction. At 28°C, syringotoxin production was considerably lower, with concentrations falling below 1 mg/L in non-baffled flasks and increased to 2 mg/L in baffled flasks, however, with a very high variability. Statistical analysis confirmed significant differences in syringotoxin concentrations between baffled and non-baffled flasks at both temperatures ($p < 0.05$). These results highlight the importance of shaking (baffling) and a lower incubation temperature (18°C) in optimizing syringotoxin production. The production of fuscopeptin A was significantly enhanced in baffled flasks at both 18°C and 28°C, though with increased variability. The production of fuscopeptin B was significantly enhanced in baffled flasks only at 28°C. Notably, under these conditions, the differences in fuscopeptin production between 18°C and 28°C were no longer significant. In baffled flasks, fuscopeptin A and fuscopeptin B concentrations reached approximately 21 mg/L and 12 mg/L, respectively.

Table 3
Syringotoxin, fuscopeptin A and B levels (mg/L) produced by *P. fuscovaginae* UPB0736 in King's B liquid medium after 48 h incubation at 18°C or 28°C under baffled and non-baffled conditions. Mean ± SD ($n = 6$) are shown. Significant differences ($p < 0.05$) were determined via pairwise t-tests, concentrations with the same letters are not significantly different from each other.

CLiP	Flask type	Incubation temperature			
		18°C		28°C	
		mg/L	SD	mg/L	SD
Syringotoxin	Non-baffled	7.8 b	2.27	0.03 d	0.01
	Baffled	58.6 a	32.9	1.81c	0.51
Fuscopeptin A	Non-baffled	7.72 b	1.70	4.02 c	0.09
	Baffled	21.23 a	6.83	11.13 ab	4.62
Fuscopeptin B	Non-baffled	7.33 a	1.00	3.68 b	0.36
	Baffled	11.61 a	4.51	8.30 a	3.26

3.7. IC_{50} of syringotoxin and fuscopeptin against R. solani AG 1-IA and AG 2–2

Growing the bacteria at 18°C in shaken liquid King's B in baffled flasks for 48 h allowed us to extract CLiPs in sufficient amounts to determine IC_{50} values. For fuscopeptin, the IC_{50} value was determined by taking the sum of the concentrations of fuscopeptin A and fuscopeptin B to obtain the total fuscopeptin concentration. The dried extracts of syringotoxin and fuscopeptins were suspended in methanol and subjected to a serial dilution, which was then mixed with PDA. The dose-response relationships are presented in Supplementary Fig. S5, with the calculated IC_{50} values provided in Table 4. The IC_{50} values obtained from the purified compounds were not significantly different from those derived using the KB supernatant, indicating that other metabolites did not interfere with the assay. Reference data obtained with the commercial fungicide fludioxonil demonstrated an EC_{50} of 0.04 mg/L (or 0.16 μM) against *R. solani* AG 2–2 at 28 °C. These results support the interpretation that syringotoxin exerts fungitoxic effects of similar magnitude.

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4. Discussion

In this study we demonstrate that *P. fuscovaginae* UPB0736 shows increased antifungal efficacy against *R. solani* AG 1-IA and AG 2–2 at 18°C compared to 28°C. This is mainly due to an enhanced production of both syringotoxin and fuscopeptin, rather than an increased sensitivity of *R. solani* to these CLiPs at lower temperatures. Asplenin, by contrast, appears to lack notable antifungal activity.

The molecular mechanisms by which temperature affects CLiP production are not fully understood. Microbial cultivation at 30°C seems to be optimal for most lipopeptide production, although some strains prefer lower temperatures: the production of putisolvin by *P. putida* strain PCL1445 increased at lower temperatures and higher salt concentration (Dubern & Bloemberg, 2006; Zhou et al., 2024). The positive regulator NunF that regulates the production of nunamycin and nunapeptin, two CLiPs that are produced by *P. nunensis*, is activated by low temperature. It was shown that the inhibition of fungi and oomycetes is actually more efficient at 10–15°C, rather than 20°C, and the synthesis of both CLiPs is regulated by the global regulator GacA by fungal- and oomycete-associated molecules (Christiansen et al., 2020). Other studies described that *P. putida* PCL1445 showed the highest putisolvin production at 11°C (Dubern & Bloemberg, 2006; Zhou et al., 2024) and that in the bean pathogen *P. syringae pv. syringae* B728a, syringofactin production is significantly higher at 20°C than at 30°C. The molecular mechanisms underlying temperature's influence on CLiP production remain unclear. It is hypothesized that temperature affects the formation of synthetases and alters iron uptake (Gross, 1985) In *P. putida* PCL1445, the DnaK stress response system has been shown to positively regulate putisolvin production at low temperatures, potentially due to the role of heat shock proteins like DnaK and DnaJ in ensuring proper folding of transcription factors or assembly of nonribosomal peptide synthetase (NRPS) complexes (Dubern & Bloemberg, 2006; Zhou et al., 2024). Understanding these temperature-mediated regulatory mechanisms is crucial for optimizing CLiP production for further applications. In general, lipopeptide production can also be influenced by other abiotic factors such as pH, oxygen levels and nutritional factors such as carbon, phosphorous, nitrogen and trace elements (Raaijmakers et al., 2006; Zhou et al., 2024).

Interestingly, although asplenin had no observable effect on mycelial inhibition in vitro, it was generally produced at a higher rate at 18°C compared to 28°C, aligning with overall trends observed in this study. However, the lack of an asplenin standard prevented quantification in mg/L, limiting the ability to draw definitive conclusions about its production levels. To date, low expression levels under laboratory conditions yielded marginal production of syringotoxin and fuscopeptin, limiting further investigations into their properties (Ferrarini et al., 2022b). This study shows that optimization of syringotoxin and fuscopeptin production is possible by growing the bacteria at 18°C in shaken liquid King's B or PDB medium in baffled flasks. This result does not align with findings in *P. syringae*, where the highest syringomycin production was achieved in static cultures of PDB (Gross & DeVay, 1977). Similarly, optimal production of lipopeptides by *P. cichorii* SF1–54 was attained at 25°C in non-shaken minimal medium with arbutin and fructose (SRMAF) cultures over 6 days (Pauwelyn et al., 2013). Additionally, viscosinamide production by *P. fluorescens* strain DR54 was reported to occur in still cultures or under conditions of carbon, nitrogen or phosphorus starvation (Nybroe & Sørensen, 2004). The importance of oxygenation however, has been well established for the production of other CLiPs. For instance, reducing the filling volume from 40 % to 20 % led to a higher microbial growth rate and a 50 % increase in pseudofactin yield in *P. fluorescens* BD5 (Biniarz et al., 2018). Similarly, increased aeration has been shown to boost surfactin production in *Bacillus mojavensis* (Hmidet et al., 2017). The aeration of the growth medium for *Bacillus subtilis* SPB1 significantly impacted the synthesis of lipopeptide biosurfactants (Ghribi and Ellouze-Chaabouni, 2011).These findings highlight the complex interplay between aeration and medium composition in CLiP production, emphasizing the need for tailored strategies to optimize metabolite yields under specific conditions.

Growing the bacteria at 18°C in shaken liquid King's B or PDB medium in baffled flasks for 48 h allowed us to extract CLiPs in sufficient amounts to determine IC_{50} values. The IC_{50} of syringotoxin against *R. solani* AG 1-IA and AG 2–2 ranged from 0.02 to 0.11 mg/L (0.02–0.10 μM), whereas fuscopeptin exhibited a significantly higher $IC_{50,}$ between 2.66 and 9.68 mg/L (1.45 and 5.28 μM), indicating that syringotoxin is at least ten times more effective than fuscopeptin. However, in this study, the IC_{50} of fuscopeptin A and B are assumed to be equal, although fuscopeptin B has a longer fatty acid chain than fuscopeptin A, which may influence its membrane interaction and bioactivity (Baré et al., 1999). Purified CLiPs and supernatant yielded similar IC_{50} values compared to those determined based on the supernatant, showing that there is no influence of other unknown compounds on the antifungal effect. Notably, the IC_{50} value of syringotoxin is comparable to that of the synthetic fungicide fludioxonil under the same experimental conditions (0.04 mg/L or 0.16 μM). The IC_{50} values revealed that at 18°C, *R. solani* AG 1-IA is more sensitive to both syringotoxin and fuscopeptin compared to AG 2–2, while at 28°C, both anastomosis groups appear similarly sensitive. However, in the direct confrontation assay, AG 1-IA seems to be less sensitive than AG 2–2, especially at 28°C. This suggests potential interaction effects between *R. solani* AG 1-IA and *P. fuscovaginae* UPB0736, which we are currently investigating. *R. solani* AG 1-IA, the causal agent of rice sheath blight, naturally thrives under warmer conditions, with optimal infection occurring at temperatures between 25°C and 30°C and in the presence of high humidity (Chen et al., 2022; Feng et al., 2017; Inagaki, 1998; Lee, 1983; Taheri et al., 2007). In contrast, *R. solani* AG 2–2 has been primarily detected in northern and central latitudes (Harikrishnan and Yang, 2004). Some AG 2–2 isolates can even cause disease at temperatures below 15°C, further demonstrating their ability to persist in temperate climates (Minier and Hanson, 2021).

Table 4
IC_{50} determinations and the 95 % confidence interval (CI) of syringotoxin and fuscopeptin on mycelium of *R.solani* AG 1-IA and AG 2–2 at 18°C and 28°C.

EC_{50} determination based on purified syringotoxin and fuscopeptin					
		EC_{50} syringotoxin (mg/L)		EC_{50} fuscopeptin (mg/L)	
		mg/L	95 % CI	mg/L	95 % CI
R.solani AG 1-IA	18°C	0.02	0.01	2.66	0.45
R.solani AG 2–2	18°C	0.09	0.02	9.68	0.63
R.solani AG 1-IA	28°C	0.11	0.02	4.13	0.69
R.solani AG 2–2	28°C	0.08	0.04	6.97	1.14

The ecological significance of temperature-dependent CLiP production is particularly relevant given that *P. fuscovaginae* is predominantly causing disease in high-altitude rice-growing regions where temperatures typically range between 18°C and 23°C or lower (Cottyn et al., 1996; Duveiller et al., 1988; Jaunet et al., 1996; Musonerimana et al., 2020; Quibod et al., 2015). This raises the question of whether cooler temperatures also favor the increased production of syringotoxin and fuscopeptin in the plant environment, thereby exacerbating symptoms of sheath brown rot. These environmental adaptations may also shape interactions between *P. fuscovaginae* and *R. solani* or other pathogens, potentially influencing the effectiveness of the produced CLiPs.

This study has several limitations that should be addressed in future work. The exclusive use of in vitro assays limits ecological relevance, as interactions with the plant host and microbial community are not accounted for. The absence of plant-based validation prevents firm conclusions about the role of CLiPs in disease severity under realistic conditions. It also remains unclear whether the observed temperature-driven increase in CLiP production is specific to *P. fuscovaginae* UPB0736 or conserved across related strains. Moreover, the underlying regulatory mechanisms remain unexplored. Integrating plant assays, comparative strain analysis, and transcriptomic or proteomic profiling would significantly enhance the mechanistic and translational value of the findings.

5. Conclusions

This study establishes that *P. fuscovaginae* UPB0736 produces CLiPs in a temperature-dependent manner, with syringotoxin and fuscopeptin biosynthesis significantly enhanced at 18°C. The increased production of these metabolites correlates with stronger inhibition of *R. solani* AG 1-IA and AG 2–2. Optimal production conditions were achieved by culturing the bacteria in shaken liquid King's B medium in baffled flasks at 18°C, facilitating CLiP extraction and IC_{50} determination. These findings raise the question of whether cooler temperatures similarly enhance the in planta production of virulence-associated metabolites, potentially influencing disease severity.

CRediT authorship contribution statement

Jasmine De Rop: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Durga Prasad:** Writing – review & editing, Validation, Resources, Investigation, Formal analysis. **Lu Zhou:** Writing – review & editing, Resources, Data curation, Conceptualization. **Bishnu Marahatta:** Writing – review & editing, Resources. **Niels Geudens:** Writing – review & editing, Validation, Resources, Investigation, Formal analysis. **Pieter Spanoghe:** Writing – review & editing, Supervision, Project administration. **Martins José:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Monica Höfte:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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