MYCOLOGY
2025, VOL. 16, NO. 2, 929–940
https://doi.org/10.1080/21501203.2024.2426480

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

OPEN ACCESS Check for updates

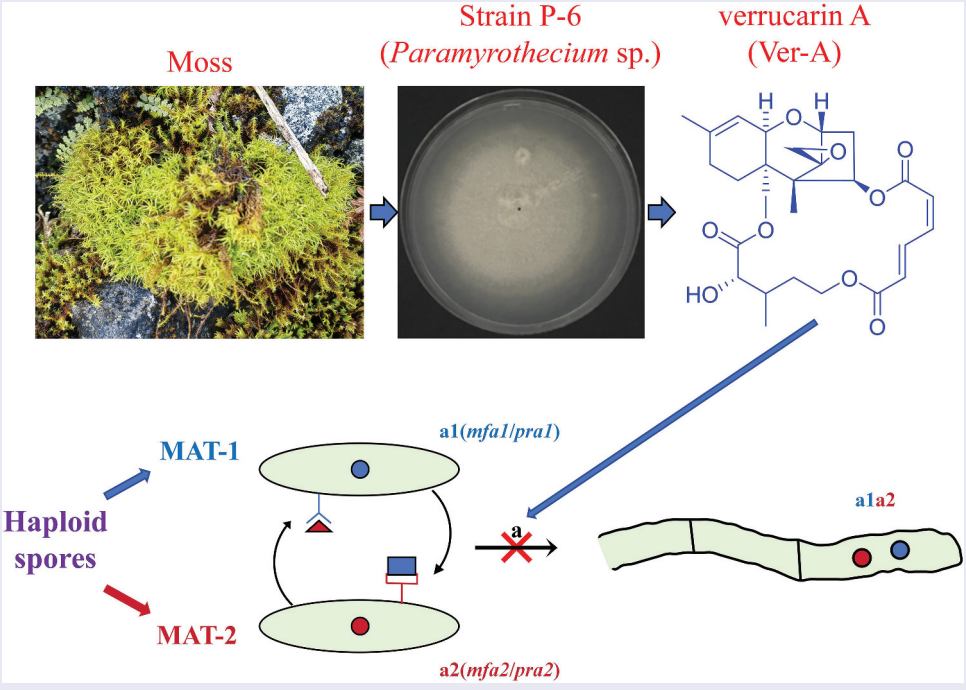
Interference with sexual mating of *Sporisorium scitamineum* by verrucarin A isolated from *Paramyrothecium* sp.

Ranying He[a,b*], Haifei Xie[c,d*], Jin Xie[a*], Bin Wang[a], Zhigang Li[c], Xihui Liu[d] and Wenxia Fang[a]

[a]Institute of Biological Sciences and Technology, Guangxi Academy of Sciences, Nanning, China; [b]College of Life Science and Technology, Guangxi University, Nanning, China; [c]College of Agriculture, Guangxi University, Nanning, China; [d]Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China

ABSTRACT

Sugarcane smut, caused by *Sporisorium scitamineum*, poses a significant global threat, leading to substantial economic losses. The pathogenic process involves haploid spores engaging in sexual mating to produce diploid mycelia, which then initiates the disease by penetrating sugarcane tissues. Targeting the mating process has thus emerged as the Achilles' heel in controlling sugarcane smut. In this study, we isolated a fungus designated as P-6 from a bryophyte, which impeded the mycelia formation of *S. scitamineum*. Phylogenetic and morphological analyses classified the strain P-6 within the genus *Paramyrothecum*. Through ethyl acetate extraction, subsequent separation, and nuclear magnetic resonance (NMR) analysis, we identified the active compound responsible for inhibiting the mating process as verrucarin A (Ver-A). Specifically, Ver-A inhibited the sexual mating of *S. scitamineum* by modulating the gene expression of loci *a* and *b*. Greenhouse pot experiments underscored the efficacy of strain P-6's fermentation products in reducing the incidence of sugarcane smut. These findings lay a robust groundwork for the development and application of P-6 as a novel biocontrol strain against sugarcane smut.



ARTICLE HISTORY
Received 29 June 2024
Accepted 1 November 2024

KEYWORDS
Sugarcane smut; *Sporisorium scitamineum*; sexual mating; verrucarin A; biocontrol agent

CONTACT Xihui Liu xihuiliu2006@126.com Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China; Wenxia Fang wfang@gxas.cn Institute of Biological Sciences and Technology, Guangxi Academy of Sciences, Nanning, Guangxi 530007, China
*These authors contributed equally to this work.

Supplemental data for this article can be accessed online at https://doi.org/10.1080/21501203.2024.2426480

1. Introduction

Sugarcane, an essential crop for sugar and energy production, thrives in diverse regions, spanning temperate, subtropical, and tropical climates. With approximately two-thirds of the world's sugar originating from sugarcane (Singh et al. 2004; Que et al. 2014; Marques et al. 2017), its prolonged growth cycle exposes it to various biotic and abiotic threats. Among these threats, sugarcane smut, caused by *S. scitamineum*, stands out as one of the most destructive diseases, leading to significant reductions in productivity for this perennial crop. The disease adversely affects the sucrose content of sugarcane, posing a substantial obstacle to the advancement of the sugarcane industry (Su et al. 2016). China ranks as the world's fourth-largest sugar producer, with Guangxi contributing to a staggering 92% of sugarcane production (Singh et al. 2019). However, besides causing considerable yield losses, smut also prompts the elimination of certain sugarcane varieties due to their susceptibility to the disease. In China, the average incidence of sugarcane smut ranges from 10% to 20%, with severely affected areas experiencing smut rates of up to 50% (Rajput et al. 2021). These statistics underscore the urgent need for effective strategies to mitigate the impact of smut on sugarcane cultivation.

The disease cycle of *S. scitamineum* sets it apart from most plant diseases. It hinges on the sexual mating between two haploid spores, resulting in a binucleate stage that subsequently evolves into dikaryotic hyphae, ultimately leading to sugarcane infection (Yan et al. 2016a). In this process, the haploids of these two mating types are named MAT-1 and MAT-2 (Que et al. 2014). Formed hyphae play a crucial role by facilitating nutrient acquisition and evading the host immune system (Zhong et al. 2018). Given that the dimorphic switch is essential for hyphae development, which in turn is pivotal for pathogenicity, targeting this switch emerges as a key point for sugarcane smut control. However, due to the initially inconspicuous symptoms and the prolonged disease course, managing the disease becomes increasingly complex. Presently, the primary strategies against sugarcane smut involve the development of resistant cultivars and chemical control methods. However, the physiological diversity within *S. scitamineum* renders resistant varieties susceptible to losing their resistance easily. Likewise, chemical control methods raise environmental concerns and pose risks to food safety. Therefore, there is a pressing need for safe and eco-friendly control strategies (Liu et al. 2017).

Biological control has emerged as a highly effective strategy for managing fungal diseases (He et al. 2021). In recent years, there has been a growing emphasis on leveraging microorganisms to combat sugarcane smut, resulting in notable experimental advancements. Various bacterial genera, including *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Enterobacter*, *Erwinia*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Stenotrophomonas*, *Streptomyces*, and *Xanthomonas*, have been identified for their potential to offer protection against both fungal and bacterial pathogens in plants (He et al. 2021). For example, secondary metabolites produced by *Bacillus amyloliquefaciens* have demonstrated significant inhibition of the growth and reproduction of *S. scitamineum* (Liu et al. 2020a). *In vitro* experiments have revealed the remarkable inhibitory effects of *T. harzianum* against *S. scitamineum* (Tegene et al. 2021). Similarly, *Streptomyces griseorubiginosus* BTU6 has been found to enhance sugarcane resistance to smut by regulating stress-related enzyme activities, including peroxidase (POD), phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and total phenol (TP) (Wang et al. 2021). Microorganism-derived antimicrobial peptides, furanones, and alcohols play a crucial role in preventing the onset of sugarcane smut (Shi et al. 2012; Yassin et al. 2022).

In this study, we successfully isolated and screened a fungus (P-6) from moss. The crude extract from the fermentation of the P-6 strain effectively inhibited spore coalescence and dikaryotic mycelium formation in *S. scitamineum*. Through the isolation and purification of its secondary metabolites, verrucarin A (Ver-A) was identified. Interestingly, Ver-A hindered the sexual mating of *S. scitamineum* without killing its haploid spores. Furthermore, pot experiment revealed that the application of P-6's fermentation products significantly reduced the incidence of smut disease and showed no toxicity to the host plant. These findings suggest that P-6 holds great potential as a biocontrol strain for future sugarcane smut management.

2. Materials and methods

2.1. Culture media and growth conditions

Haploid spores MAT-1 and MAT-2 of *S. scitamineum* were cultivated at 28 °C in yeast extract peptone sucrose (YEPS) medium supplemented with 50 µg/mL ampicillin, either in liquid form or solidified with 1.8% agar. Strain P-6 was cultivated on PDA plates at 28 °C for 14 d for crude extract preparation. *Fusarium oxysporum* and *Aspergillus fumigatus* were cultured at 28 °C or 37 °C as required, using PDA or YPD media.

2.2. Isolation of strains

Strains were isolated from lichen-moss samples collected from Jiuling Skyscraper, Du'an County (23.9°N 108.1°E), Guangxi Zhuang Autonomous Region. Strain isolation was performed using a concentration gradient dilution method as previously reported (Sun et al. 2023). Initially, 5 g of moss sample was introduced into a conical flask containing 45 mL of sterile water. The flask was agitated at 150 r/min for 30 min to eliminate surface debris and impurities. Following this, the sample was washed thoroughly with sterile water and then disinfected in 75% ethanol. An additional three sterile water rinses were performed, and any excess surface moisture was removed by blotting with sterile absorbent paper. The prepared moss samples were ground into a paste in a mortar with 10 mL of sterile water. One-millilitre aliquot of the paste was then extracted and subjected to a 10-fold gradient dilution, resulting in final dilutions of 10^{-3}, 10^{-4}, and 10^{-5}. Subsequently, 50 µL of these diluted solutions were spread onto PDA, LB, or Gauze's Synthetic Medium No.1 plates, with 15 plates for each type. After the incubation at 28 °C in a controlled culture environment for 7 d, morphologically distinct single colonies were selected for strain purification.

2.3. Extract preparation and antifungal activity assay against S. scitamineum

The isolated fungi were cultured on PDA plates at 25 °C for 14 d. Post-fermentation, they were extracted using an ethyl acetate (EtOAc)/methanol (MeOH)/acetic acid (HAc) solution (80:15:5, v/v/v). After the evaporation of organic solvents, the extracts were reconstituted in dimethyl sulphoxide (DMSO) and subjected to two-fold serial dilutions to prepare stock solutions at concentrations of 100, 50, and 25 mg/mL. Antifungal screening assays were conducted against *S. scitamineum*, *Fusarium oxysporum*, and *Aspergillus fumigatus* in YAG medium using a 96-well plate format.

2.4. Identification of the P-6 strain

Genomic DNA was extracted from the mycelium of the P-6 strain cultured on PDA. DNA amplifications were performed by polymerase chain reaction (PCR) with primer pairs listed in Table S1. Amplifications were conducted in a gradient thermocycler (Bio-Rad) in a 50 µL reaction mixture comprising 25 µL 2× *Taq* PCR Mix, 1 µL each of forward and reverse primers, 1 µL DNA template, and ddH_2O to adjust the final volume to 50 µL. The PCR conditions were set as follows: an initial denaturation at 96 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 10 s, annealing at 58 °C for 15 s, and extension at 72 °C for 1 min. PCR products were visualised by agarose gel electrophoresis, purified, and then sent to QingKe Biotech Company (Guangzhou, China) for sequencing. The sequencing results were compared to gene sequences in the NCBI BLAST database, and highly similar sequences were downloaded. A phylogenetic tree was constructed using MEGA-X software (Zheng et al. 2017; Tamura et al. 2021).

2.5. Isolation and purification of secondary metabolites from P-6 strain

The P-6 strain was cultured on 5 L PDA plates at 28 °C for 20 d. The solid fermentation product was fragmented and extracted three times using an ethyl acetate (EtOAc)/methanol (MeOH)/acetic acid (HAc) solution (80:15:5, v/v/v) to prepare a crude extract. The crude extract was dissolved in water and subjected to liquid-liquid extraction using equal volumes of ethyl acetate. The resulting solution was concentrated under reduced pressure and subsequently dried.

The ethyl acetate extract (2.1 g) was fractionated using the Sephadex LH-20 column (Amersham Pharmacia, MeOH, 2 × 100 cm, 1 mL/min), yielding four fractions (Fr.1–Fr.4). Following activity-guided fractionation, Fr.2 (779.1 mg) was further purified by semi-preparative HPLC under the following conditions: solvent A: water; solvent B: methanol; flow rate: 3 mL/min. The gradient involved isocratic elution

at 65% B for the first 17 min, followed by isocratic elution at 100% B from 17 to 30 min, and a return to 65% B from 30 to 37 min, resulting in four fractions (Fr.2.1–Fr.2.4). The dried Fr.2.4 (201.5 mg) was further separated using a silica gel column (Qingdao Marine Chemical, GF254, 3 cm × 50 cm, 70 g), first eluting with a gradient of petroleum ether/acetone (from 10:1 to 9:1, v/v) and then methanol, yielding three fractions (Fr.2.4.1–Fr.2.4.3). Fr.2.4.2 (9.6 mg) was further purified using the same HPLC conditions, resulting in the isolation of compound 1 (6.7 mg).

2.6. Structure identification of compound 1

The structural determination of compound 1 was carried out using an Agilent NMR system equipped with an 800 MHz NMR spectrometer (Agilent Technologies, Colorado Springs, CO, USA) to record NMR spectra. Additionally, a Waters Xevo G2-S QTOF MS mass spectrometer (Waters, Milford, MA, USA) was utilised for both electrospray ionisation mass spectrometry (ESI-MS) and high-resolution electrospray ionisation mass spectrometry (HR-ESI-MS) to confirm the molecular weight and obtain structural insights of compound 1 isolated from the strain P-6.

2.7. The effect of Ver-Aon the sexual mating and growth of S. scitamineum

To evaluate the impact of Ver-A on the sexual mating of haploid spores, it was dissolved in DMSO and subjected to two-fold serial dilutions to achieve varying concentrations. These dilutions were added to the YAG medium, resulting in final Ver-A concentrations of 128, 64, 32, 16, 8, 4, 2, and 1 μg/mL (Liu et al. 2017). An equivalent volume of DMSO was added to the YGA medium as a control. A 2 μL mixture of MAT-1 and MAT-2 spores (1×10^8/mL) was then introduced into the culture medium, followed by incubation at 28 °C for 48 h. The resulting colonies were selected and examined under a microscope. To further investigate the impact of Ver-A on growth rates, solid media containing 8 μg/mL Ver-A and control medium without Ver-A were used to plate varying concentrations of MAT-1 and MAT-2 spores. Additionally, to assess whether Ver-A has a direct effect on the sexual mating ability of either MAT-1 or MAT-2 spores, both types of spores were cultured separately in liquid media containing either 8 μg/mL Ver-A or Ver-A for 24 and 48 h, respectively. After centrifugation, the spores were collected, and the liquid culture media were removed. Subsequently, 10^8 spores of MAT-1 and MAT-2 were combined in pairs and spotted onto YAG plates.

2.8. Greenhouse pot experiment

The MAT-1 and MAT-2 spores of *S. scitamineum* were separately inoculated into 100 mL of YEPS liquid medium and cultivated at 28 °C with shaking at 200 r/min for 2 d. Cells were harvested by centrifugation at 3,000 r/min for 10 min, washed twice with sterile water, and re-suspended in PBS buffer solution ($OD_{600} \approx 1.0$). The ethyl acetate extract of P-6 was reconstituted in DMSO and mixed with MAT-1 and MAT-2 suspensions to prepare a 500 μg/mL working solution. Using a sterile needle, 1 mL of the stock solution was inoculated into the stem base (near the four-leaf stage) of young sugarcane seedlings of the smut-sensitive sugarcane variety 05136. A mixture of MAT-1 and MAT-2 spores of *S. scitamineum* was used as a positive control, while an equal volume of PBS buffer solution served as the negative control. Each treatment involved 30 seedlings (Yan et al. 2016b), and the experiment was conducted twice. After 21 d, both control and treated sugarcane plants were dissected for analysis. Stem tissues were sectioned into approximately 1 cm slices and observed under a microscope (Singh et al. 2021a, 2021b).

2.9. Quantitative analysis of gene expression

Sporisorium scitamineum was cultured on solid YAG plates at 28 °C for 2 d for RNA extraction. RNA was isolated using the TransZol Plus RNA Kit, followed by cDNA synthesis using the HiScript III RT Kit. Gene expression analysis was conducted on a LightCycler 96 system using SPAPKscript II, targeting genes involved in the pheromone and nuclear fusion pathways, including *mfa1*, *pra1*, *mfa2*, *pra2*, *bE1*, *bW1*, *bE2*, *bW2*, and *prf*. Primer sequences for qRT-PCR are listed in Table S1. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with the *actin* gene serving as the reference (Livak and Schmittgen 2001).

3. Results

3.1. Isolation, characterization, and phylogenetic analysis of the antagonistic P-6 strain

In this study, we isolated a total of 61 microorganisms from moss samples, comprising 50 bacteria and 11 fungi, using a gradient dilution method as described previously (Chen et al. 2014). We evaluated the antagonistic activity of all strains against *S. scitamineum*, and found that the P-6 isolate notably hindered mycelial formation (Figure 1a). The P-6 isolate displayed rapid growth, producing fluffy mycelia after 14 days of cultivation on PDA medium (Figure 1b). Moreover, it generated soluble yellow pigments, and its oval conidia ranged in colour from black or greyish-green (Figure 1c–e). Phylogenetic analysis based on the ITS region, Bt2, and RBP2 genes revealed that the P-6 strain belongs to the *Paramyrothecium* genus and clustered closely with *P. foliicola* and *P. amorphophalli* (Figure 1f). Due to incomplete species identification data, the P-6 strain has been provisionally assigned to the genus *Paramyrothecium* sp. (accession number MZ373255.1).

3.2. Broad-spectrum antifungal activity of secondary metabolites from the P-6 strain

To further validate the antagonistic activity of the P-6 strain against *S. scitamineum*, we evaluated the crude extract of its secondary metabolites. These extracts exhibited potent inhibitory effects on *S. scitamineum*, with significant inhibition observed at concentrations as low as 0.625 mg/mL (Figure 2a). No mycelial formation was detected, indicating a strong suppression of sexual mating (Figure 2b). Furthermore, the crude extract demonstrated inhibitory activity against the human pathogenic fungus *Aspergillus fumigatus* and the phytopathogen responsible for causing banana wilt, *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (Figure 2c). Collectively, these findings highlight the broad-spectrum inhibitory effects of the P-6 strain.

3.3. Identification of verrucarin A as the active antifungal compound in strain P-6

Extensive fermentation, extraction, and isolation procedures were conducted on strain P-6 to isolate

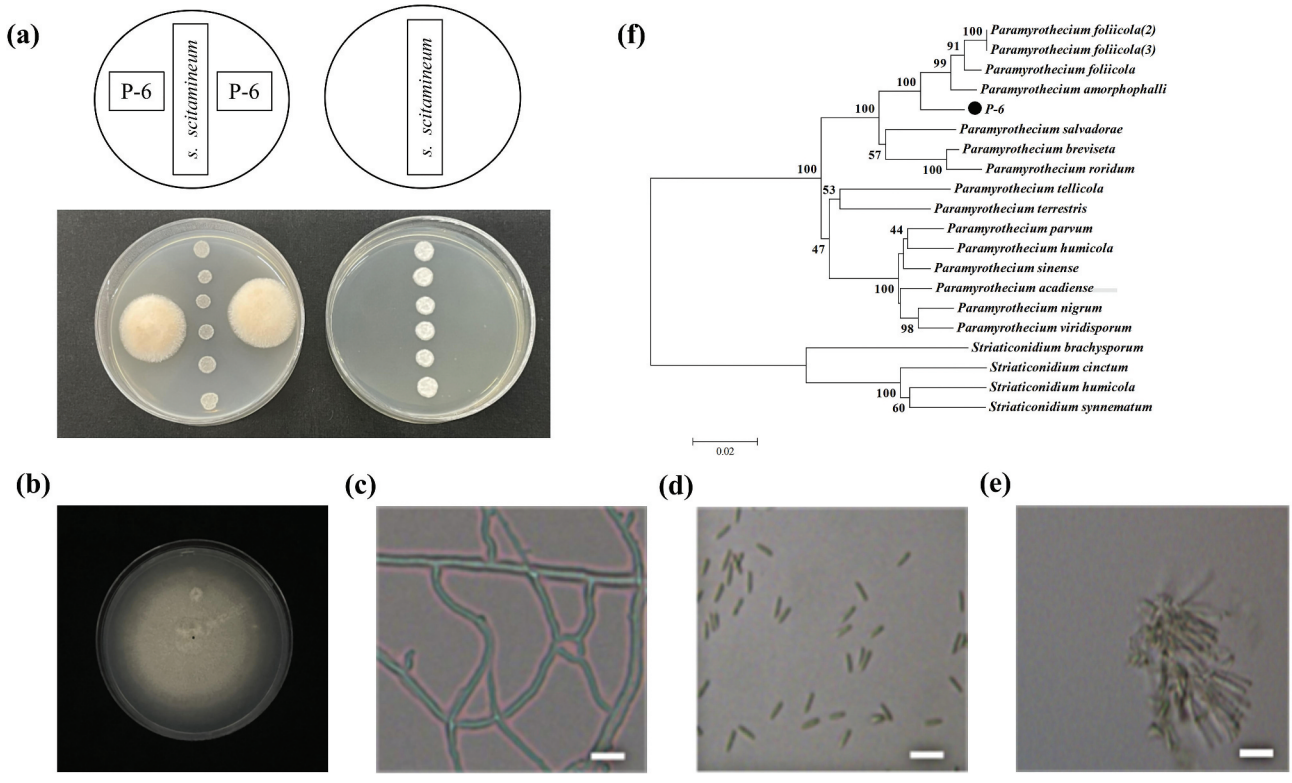


Figure 1. Isolation and morphological characteristics of the P-6 strain. (a) Inhibitory effect of strain P-6 against *Sporisorium scitamineum*. (b) Colony morphology of the P-6 strain on PDA plate. (c) Mycelia morphology of the P-6 strain in liquid PDA. Scale bar is 10 μm. (d) Conidial morphology of the P-6 strain. Scale bar is 10 μm. (e) Conidiophore morphology of the P-6 strain. Scale bar is 10 μm. (f) Phylogenetic tree of the P-6 strain. Bootstrap values are presented as percentages. The neighbor-joining method with 1,000 replicates was employed to construct the phylogenetic tree. The scale bar denotes sequence divergence.

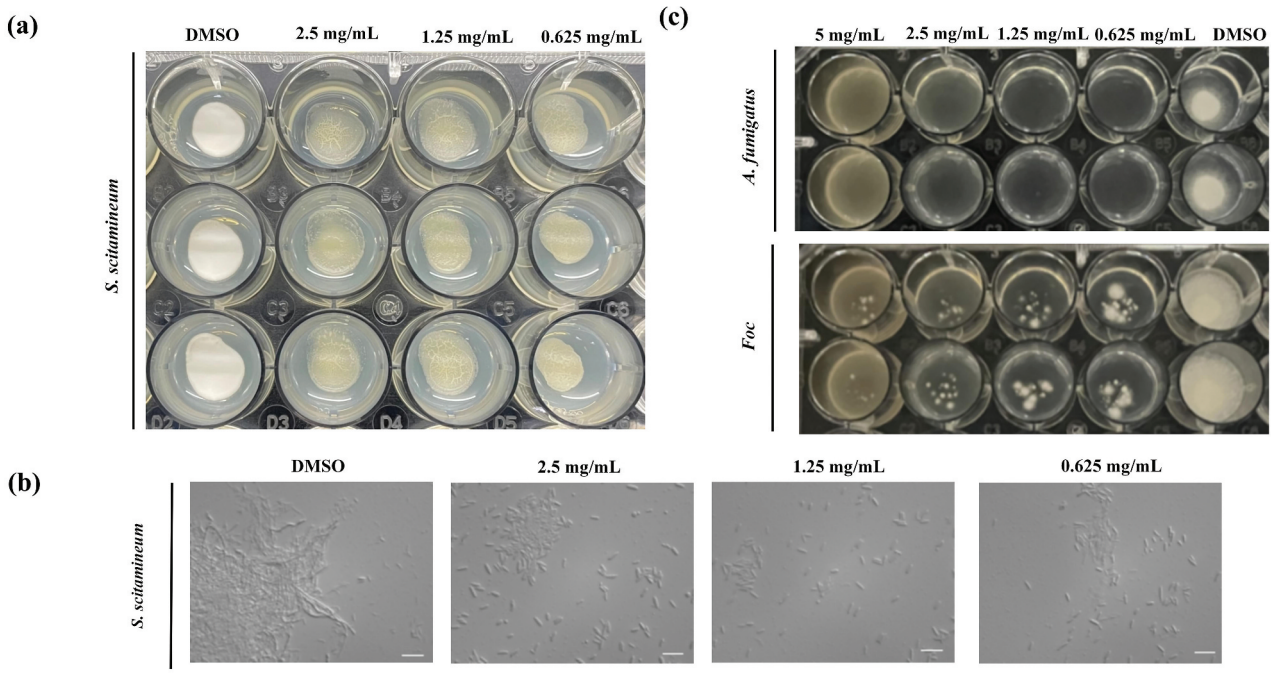


Figure 2. Inhibitory effect of P-6 fungal crude extracts on *Sporisorium scitamineum*, *Aspergillus fumigatus*, and *Foc*. (a) Growth inhibition of *S. scitamineum* on YAG plates supplemented with varying concentrations of P-6 crude extracts. DMSO was used as a control. (b) Microscopic observation of *S. scitamineum* cultures grown in YAG containing different concentrations of P-6 crude extracts after 24 h of incubation at 28 °C. Scale bar is 25 μm. (c) Growth of *A. fumigatus*, and *Foc* on YAG plates supplemented with varying concentrations of P-6 crude extracts. DMSO served as a control.

bioactive compounds and determine their chemical structures. A series of chromatographic techniques, including methanol gel column chromatography, high-performance liquid chromatography (HPLC), and silica gel column chromatography, were employed for separation and activity monitoring (Figure 3a). Results showed that only yeast-like haploid colonies grew in fractions Fr.2 and Fr.2.4, while hyphae growth occurred in the other groups, indicating that the active compound prevented sexual mating (Figure 3b). Ultimately, compound 1 from P-6 presented as a white powder and was characterised using high-resolution electrospray ionisation mass spectrometry (HR-ESI-MS), which showed an m/z $[M + Na]^+$ of 525.2107 (Figure S1b). Based on these data, the molecular formula of this compound was determined to be $C_{27}H_{34}O_9$ (Cacl. for $C_{27}H_{34}O_9Na^+$: 525.2101). ^{1}H NMR (MeOD, 800 MHz, Figure S2): δ 8.08 (1 h, ddd, *J* = 1.2, 11.6, 15.76 hz, H-8′), 6.80 (1 h, t, *J* = 11.36 hz, H-9′), 6.21 (1 h, d, *J* = 11.12 hz, H-10′), 6.09 (1 h, d, *J* = 11.12 hz, H-7′), 5.88 (1 h, dd, *J* = 4.0, 8.24 hz, H-4), 5.41 (1 h, d, *J* = 5.04 hz, H-10), 4.70 (1 h, d, *J* = 12.24 hz, H-15a), 4.46 (1 h, ddd, *J* = 2.48, 5.12, 11.36 hz, H-5′a), 4.21 (1 h, d, *J* = 12.16 hz, H-15b), 4.16 (1 h, d, *J* = 12.16 hz, H-2′), 3.99 (1 h, td, *J* = 3.12, 12.0 hz, H-5′b), 3.75 (1 h, d, *J* = 5.2 hz, H-2), 3.71 (1 h, d, *J* = 5.36 hz, H-11), 3.04 (1 h, d, *J* = 4.0 hz, H-13a), 2.85 (1 h, d, *J* = 4.0 hz, H-13b), 2.51 (1 h, dd, *J* = 8.24, 15.44 hz, H-3a), 2.39 (1 h, m, H-3′), 2.15 (1 h, ddd, *J* = 4.16, 5.12, 15.44 hz, H-3b), 1.95 (2 h, m, H-8), 1.89 (1 h, m, H-7a), 1.76 (1 h, m, H-7b), 1.72 (3 h, q, H-16), 1.31 (2 h, m, H-4′), 0.89 (3 h, d, *J* = 6.8 hz, H-12′), 0.85 (3 h, s, H-14); ^{13}C NMR (MeOD, 200 MHz, Figure S3): δ 174.0 (C-1′), 166.1 (C-6′), 165.7 (C-11′), 140.3 (C-9), 139.1 (C-8′), 138.3 (C-9′), 126.6 (C-7′), 125.8 (C-10′), 118.1 (C-10), 78.9 (C-2), 75.8 (C-4), 73.8 (C-2′), 66.7 (C-11), 65.0 (C-12), 62.2 (C-15), 61.0 (C-5′), 49.3 (C-5), 47.0 (C-13), 44.0 (C-6), 34.3 (C-3), 32.8 (C-3′), 32.1 (C-7), 27.1 (C-8), 21.9 (C-16), 19.7 (C-4′), 9.3 (C-12′), 6.5 (C-14). All the obtained data were consistent with the published NMR data for verrucarin A (Ver-A) (Shimada et al. 2004), confirming Ver-A as the primary active antifungal compound produced by strain P-6 (Figure 3c).

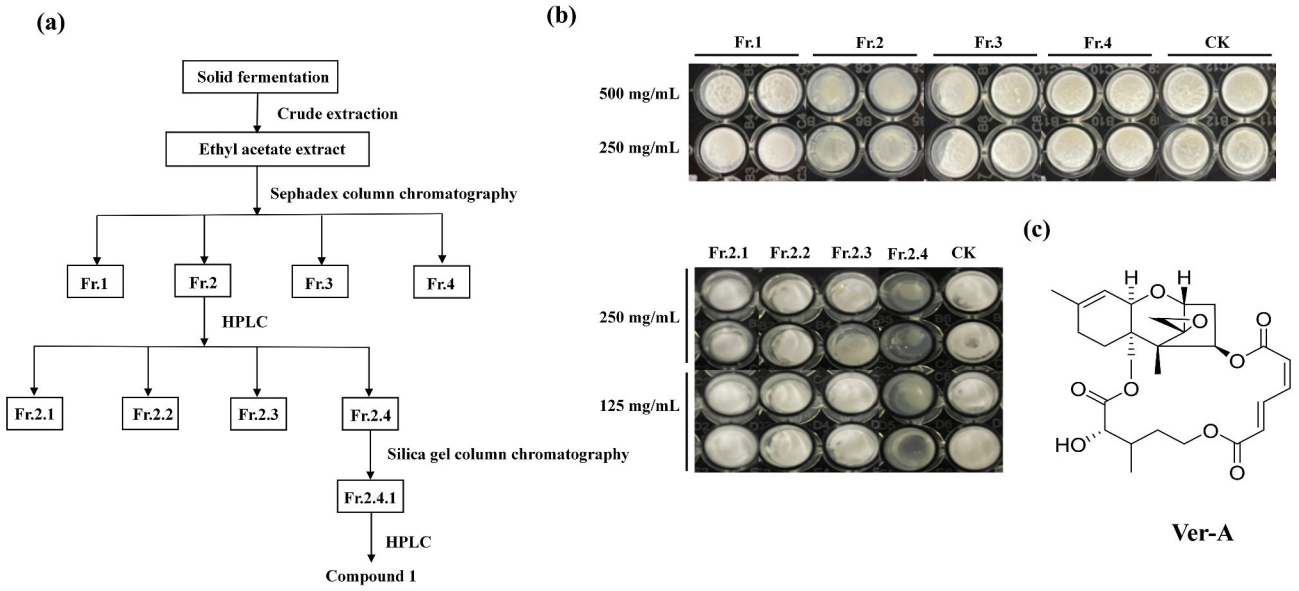


Figure 3. Isolation and characterization of the main active compound from strain P-6. (a) Flowchart depicting the extraction and separation procedure of compound 1 from strain P-6. (b) Antifungal activity of Fr.1–4 and Fr.2.1–2.4 against *Sporisorium scitamineum* after 48 h at concentrations of 500 µg/mL, 250 µg/mL, and 125 µg/mL. (c) Chemical structure of compound 1 (Ver-a).

3.4. Evaluation of the minimum inhibitory concentration (MIC) of Ver-A and its effects on S. scitamineum sexual mating

To determine the MIC of Ver-A against *S. scitamineum*, conidia were exposed to concentrations ranging from 1 to 128 µg/mL. A concentration-dependent inhibition of *S. scitamineum* mycelial growth was observed, with no hyphal formation detected at concentrations above 8 µg/mL (Figure 4a,b). To investigate whether Ver-A affected growth or sexual mating, MAT-1 and MAT-2 spores were cultured on solid media containing 8 µg/mL Ver-A. While the growth was initially inhibited within the first 48 h, it returned to normal by 72 h (Figure 4c), suggesting that the effect of Ver-A was not dependent on growth inhibition. Further investigation was conducted to determine whether Ver-A had a direct effect on the sexual mating ability of either MAT-1 or MAT-2 spores. Both types of spores were cultured separately in liquid media with or without 8 µg/mL Ver-A for 24 and 48 h, respectively. After centrifugation and thorough removal of the compound, the treated spores were paired and spotted onto plates, resulting in resumed normal mating behaviour and mycelial growth (Figure 4d). This demonstrates that Ver-A only affects sexual mating when both MAT-1 and MAT-2 spores are present, indicating that Ver-A may disturb mating signals between spores. Additionally, antifungal assays revealed MIC values of 16 µg/mL for *A. fumigatus* and 4 µg/mL for *Candida albicans* (Figure S3), showcasing its broad-spectrum antifungal activity.

3.5. Evaluation of Ver-A toxicity and P-6 fermentation extract in controlling sugarcane smut

To assess the potential toxicity of Ver-A on sugarcane, we punctured the leaves with a needle to facilitate its penetration and monitored them regularly. Remarkably, Ver-A did not cause any lesions on the leaves (Figure S4). Due to the limited availability of Ver-A, we substituted the ethyl acetate extract from strain *p*-6 for the smut disease infection test. On the 20th day post-inoculation, microscopic examination of sugarcane stem samples revealed mycelium in tissues inoculated with MAT-1 and MAT-2 spores. However, samples from the treatment group displayed no smut symptoms and contained only haploid spores. This result is consistent with the plate experiments, confirming that the fermentation extract from *p*-6 effectively inhibited the sexual mating of MAT-1 and MAT-2, thus preventing disease onset (Figure 5). At day 120, the disease incidence was recorded, 80% of the plants inoculated with MAT-1 and MAT-2 spore suspension developed black spike symptoms. In contrast, plants treated with the *p*-6 ethyl acetate extract (500 µg/mL) showed a significantly reduced incidence of 37%, demonstrating the efficacy

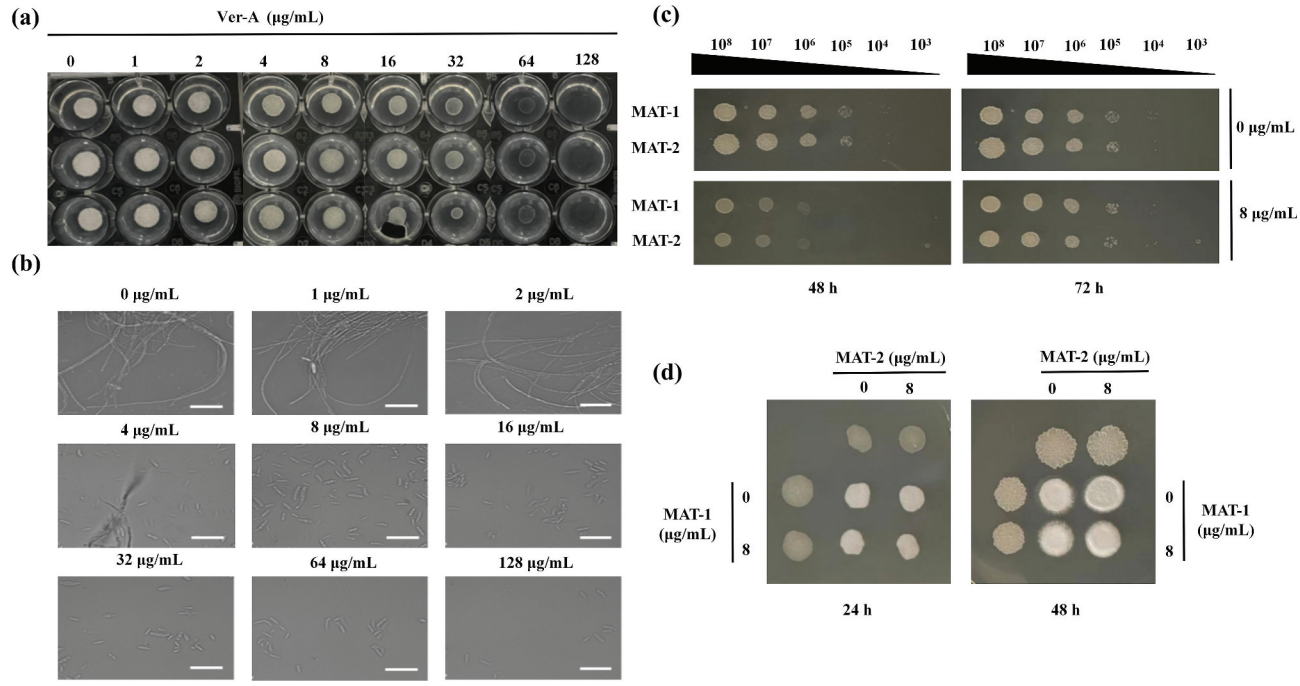


Figure 4. Inhibitory effect of Ver-A on *Sporisorium scitamineum* growth and sexual mating. (a) Growth inhibition of *S. scitamineum* by varying concentrations of Ver-A over 48 h. (b) Colonies from (a) were selected, and images were captured using a Leica DMi8 inverted microscope; scale bar is 25 μm. (c) Impact of Ver-A on the growth of MAT-1 and MAT-2 spores. (d) Influence of Ver-A-treated MAT-1 and MAT-2 on sexual mating.

of fermentation extract in controlling sugarcane smut (Table 1).

3.6. Ver-A disrupts mating dynamics in S. scitamineum by modulating gene expression at mating loci a and b

Building on previous findings, Ver-A influences the mating behaviour of the sugarcane smut fungus, *S. scitamineum*, whose genome shares high homology with *Ustilago maydis* (Fedler et al. 2009). Sexual mating in *U. maydis* is regulated by loci *a* and *b*. Locus *a* encodes pheromones and receptors, facilitating spore recognition and fusion, while locus *b* contains transcription factors essential for nuclear fusion and plant invasion (Zhu et al. 2019). The pheromone response transcription factor, *prf*, regulates the expression of loci *a* and *b* (Urban et al. 1996; Hartmann et al. 1999). *Ustilago maydis* has been extensively studied as a model pathogenic fungus due to its complete genome sequence and symptomatic assessment capabilities (Fedler et al. 2009; Wahl et al. 2010; Vollmeister et al. 2012). In *U. maydis*, *mfa1* and *pra1* for *a1* are expressed in the positive mating type (+), and *mfa2* and *pra2* for *a2* in the negative mating type (-), with hyphal fusion occurring when *mfa1* interacts with *pra2* and vice versa (Yan et al. 2016a).

To investigate Ver-A's impact on loci *a* and *b* in *S. scitamineum*, quantitative real-time PCR (qRT-PCR) was conducted to assess the expression of genes involved in pheromone signalling and nuclear fusion pathways (Figure 6). Treatment with 8 μg/mL of Ver-A induced substantial alterations in gene expression profiles compared to untreated samples. Genes at locus *b*, including *bE2*, *bW1*, and *bW2*, were up-regulated under Ver-A treatment, while *mfa1* and *pra1* at locus *a* showed minimal expression. Moreover, there was a notable increase in *mfa2* and *pra2* expression, indicating that Ver-A disrupts the interactions between positive and negative spores by modulating gene expression at both loci. This disruption hampers successful mating and highlights Ver-A's influence on mating dynamics in *S. scitamineum*.

4. Discussion

To promote the sustainable development of the sugarcane industry, various strategies have been employed to combat sugarcane smut disease. These

Figure 5. Effects of Ver-A on *Sporisorium scitamineum* infection in sugarcane. Sugarcane variety 05136 was applied for infection, scale bar is 20 μm. (a) Inoculation with PBS as a negative control. (b) Inoculation with a mixture of MAT-1 and MAT-2 spores. (c) Inoculation with a mixture of MAT-1 and MAT-2 supplemented with 500 μg/mL *p*-6 ethyl acetate extract.

Table 1. Disease incidence of sugarcane plantlets inoculated with *Sporisorium scitamineum* spores and *p*-6 ethyl acetate extract.

Inoculum	No. plantlets inoculated	No. whips	Total infection rate
PBS	30	0	0
MAT-1 and MAT-2 spores	30	24	80%
MAT-1 and MAT-2 supplemented with 500 μg/mL *p*-6 ethyl acetate extract	30	11	37%

methods encompass the utilisation of disease-resistant varieties, chemical pesticides, and microbial biocontrol. The practice of microbial biocontrol has been globally recognised for over 70 years (Howell 2003), with microorganisms emerging as promising agents for biological disease management and enhancing plant growth (Raupach and Kloepper 1998; Montesinos et al. 2002; Islam et al. 2016). These beneficial agents employ a range of mechanisms, including antibiotic secretion, resource competition, direct parasitism, induction of plant resistance, and facilitation of plant growth under stress conditions (Van et al. 2008; Kannan and Sureendar 2009; Sousa and Olivares 2016; Viaene et al. 2016).

Sugarcane smut, caused by the pathogenic fungus *S. scitamineum*, poses a significant threat to the

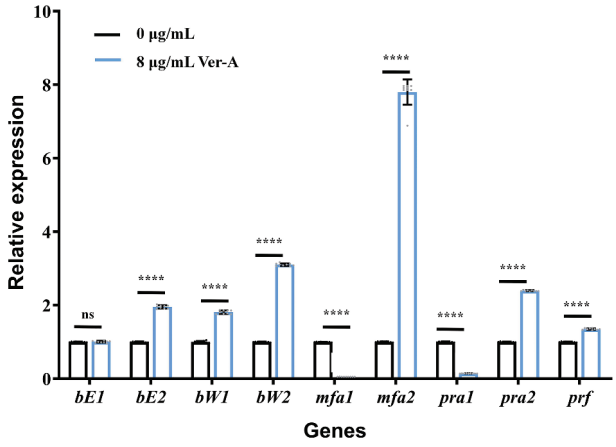


Figure 6. qRT-pcr gene expression analysis for mating loci *a* and *b*. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method, with the actin gene of *Sporisorium scitamineum* as the internal control.

sugarcane industry. The fungus produces two types of haploid spores, MAT-1 and MAT-2 (Zhong et al. 2018), which undergo sexual mating to form diploid cells encapsulated within a resilient outer wall, known as zygospores. These zygospores enable the fungus to penetrate and infect sugarcane, underscoring the significance of the morphological transition from haploid spores to filamentous hyphae in the pathogenicity of *S. scitamineum* (Que et al. 2014; Taniguti et al. 2015; Yan et al. 2016b). Research has identified natural products, such as mycophenolic acid (MPA), capable of inhibiting this dimorphic transition, effectively suppressing sugarcane smut disease in greenhouse trials (Zhong et al. 2018). Furthermore, the bacterium *Pseudomonas* sp. ST4 produces indole-3-carboxaldehyde, which has been shown to inhibit the sexual mating of both *S. scitamineum* and *U. maydis* (Liu et al. 2020b).

The unique natural conditions of Guangxi provide an exceptional environment for the survival and proliferation of diverse microorganisms, making it a valuable reservoir of microbial resources. These microorganisms exhibit distinct biological activities with promising applications in pharmaceuticals, environmental protection, and agriculture. One such fungus, strain *p*-6, was isolated from bryophytes in Guangxi's distinct ecosystem. Following separation, purification, and natural product identification processes, Ver-A was isolated, which significantly inhibited the mycelial formation of *S. scitamineum* (Figure 4). MIC assays revealed that Ver-A effectively inhibits *S. scitamineum* at a concentration of 8 μg/mL. Furthermore, it demonstrated broad-spectrum antifungal activity, with MIC values of 16 μg/mL against *A. fumigatus* and 4 μg/mL against *C. albicans*. Experimental trials on potted plants and leaves confirmed Ver-A's efficacy in controlling the sugarcane smut with minimal toxicity (Figure 5). These findings highlight Ver-A's potential as an effective solution for managing sugarcane smut and combating other pathogenic fungi.

Sporisorium scitamineum, a heterothallic fungus, requires two distinct haploid types to recognise and fuse, resulting in the formation of invasive binucleate mycelium. Its life cycle and sexual coordination closely resemble those of *U. maydis*, both employing a quadripolar sexual mating system (Kämper et al. 2006; Schirawski et al. 2010). This sexual mating is regulated by two unlinked mating type loci, *a* and *b* (Wahl et al. 2010; Vollmeister et al. 2012). In *U. maydis*, the *a* locus contains two bialleles, with the positive mating type (+) encoding *mfa1* pheromone and *pra1* receptor and the negative mating type (-) encoding *mfa2* and *pra2*. Mating occurs when *mfa1* from one mating type interacts with *pra2* from the opposite type, or *mfa2* interacts with *pra1*, leading to the fusion of compatible strains and the formation of bud tubes (Froeliger and Leong 1991). The *a* locus governs pheromone synthesis and haploid recognition, while the *b* locus regulates binucleate filament maintenance and host plant penetration. Studies have reported that deletion of the cytochrome c-peroxidase coding gene *SsCcp1* can cause defects in mating and filamentation, and significantly reduces the expression of *mfa1* and *pra1* in the mating type (Cai et al. 2023). In addition, deletion of the MAP kinase *SsKpp2* in the MPKA signalling pathway of *S. scitamineum* also inhibits the sexual mating process, with the *a* locus genes *mfa1* and *pra1* showing downregulation, while the *b* locus shows an upregulation (Bölker et al. 1995). Our research revealed that Ver-A effectively inhibits sexual mating in *S. scitamineum*. qRT-PCR analysis revealed Ver-A's regulatory effect on the *a* and *b* locus-associated genes. Specifically, *mfa1* and *pra1* were markedly downregulated, while *mfa2* and *pra2* were significantly upregulated (Figure 6). This suggests that Ver-A disrupts the mutual recognition between the MAT-1 and MAT-2 mating types, thus preventing the formation of invasive hyphae.

In conclusion, we identified Ver-A as a potent inhibitor of the dimorphic transition in sugarcane smut

disease. It disrupts sexual mating in *S. scitamineum* without affecting haploid spores. Virulence assays on leaves showed no development of reddish-brown spots, and sugarcane plants treated with Ver-A-containing mixtures showed no mycelium growth or black whip symptoms. These findings highlight Ver-A's potential as a biocontrol agent against sugarcane smut, offering valuable insights for the development of effective biological strategies to combat this damaging disease.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Guangxi Natural Science Foundation [2023GXNSFFA026011] to W.F., Guangxi Science and Technology Base and Talent Special Project [AD23026030] to B.W., National Natural Science Foundation of China [32160486] to X.L., Guangxi Natural Science Foundation [2023GXNSFBA026235] and Research Start-up Funding of Guangxi Academy of Sciences [2021YBI704] to J.X.

References

Bölker M, Genin S, Lehmler C, Kahmann R. 1995. Genetic regulation of mating and dimorphism in *Ustilago maydis*. Can J Bot. 73(S1):320–325. doi: 10.1139/b95-262.

Cai E, Jia H, Feng R, Zheng W, Li L, Zhang L, Jiang Z, Chang C. 2023. Cytochrome c-peroxidase modulates ROS homeostasis to regulate the sexual mating of *S. scitamineum*. Microbiology Spectr. 11(6):0205723. doi: 10.1128/spectrum.02057-23.

Chen Y, Huang H, Liu M, Bao S. 2014. Isolation and identification of an actinomycetes strain against root-knot nematode from mangrove soil. Biotechnol Bull. 0(10):174–178. https://biotech.aiijournal.com/EN/Y2014/V30/I10/174.

Fedler M, Luh K, Stelter K, Nieto-Jacobo F, Basse CW. 2009. The *a2* mating-type locus genes *lga2* and *rga2* direct uniparental mitochondrial DNA (mtDNA) inheritance and constrain mtDNA recombination during sexual development of *Ustilago maydis*. Genetics. 181(3):847–860. doi: 10.1534/genetics.108.096859.

Froeliger E, Leong S. 1991. The a mating-type alleles of *Ustilago maydis* are idiomorphs. Gene. 100:113–122. doi: 10.1016/0378-1119(91)90356-g.

Hartmann H, Krüger J, Lottspeich F, Kahmann R. 1999. Environmental signals controlling sexual development of the corn smut fungus *Ustilago maydis* through the transcriptional regulator *Prf1*. Plant Cell. 11(7):1293–1305. doi: 10.1105/tpc.11.7.1293.

He D, He M, Amalin D, Liu W, Alvindia D, Zhan J. 2021. Biological control of plant diseases: an evolutionary and eco-economic consideration. Pathogens. 10(10):1311. doi: 10.3390/pathogens10101311.

Howell C. 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. Plant Dis. 87(1):4–10. doi: 10.1094/PDIS.2003.87.1.4.

Islam S, Akanda A, Prova A, Islam M, Hossain M. 2016. Isolation and identification of plant growth promoting rhizobacteria from cucumber rhizosphere and their effect on plant growth promotion and disease suppression. Front Microbiol. 6:165532. doi: 10.3389/fmicb.2015.01360.

Kämper J, Kahmann R, Bölker M, Ma L, Brefort T, Saville BJ, Banuett F, Kronstad J, Gold S, Müller O. 2006. Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. Nature. 444(7115):97–101. doi: 10.1038/nature05248.

Kannan V, Sureendar R. 2009. Synergistic effect of beneficial rhizosphere microflora in biocontrol and plant growth promotion. J Basic Microbiol. 49(2):158–164. doi: 10.1002/jobm.200800011.

Liu H, Wang Y, Yang Q, Zhao W, Cui L, Wang B, Zhang L, Cheng H, Song S. 2020a. Genomics and LC-MS reveal diverse active secondary metabolites in *Bacillus amyloliquefaciens* WS-8. J Microbiology Biotechnol. 30(3):417. doi: 10.4014/jmb.1906.06055.

Liu S, He F, Lin N, Chen Y, Liang Z, Liao L, Lv M, Chen Y, Chen S, Zhou J. 2020b. *Pseudomonas* sp. ST4 produces variety of active compounds to interfere fungal sexual mating and hyphal growth. Microb Biotechnol. 13(1):107–117. doi: 10.1111/1751-7915.13289.

Liu S, Lin N, Chen Y, Liang Z, Liao L, Lv M, Chen Y, Tang Y, He F, Chen S. 2017. Biocontrol of sugarcane smut disease by interference of fungal sexual mating and hyphal growth using a bacterial isolate. Front Microbiol. 8:778. doi: 10.3389/fmicb.2017.00778.

Livak K, Schmittgen T. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 25(4):402–408. doi: 10.1006/meth.2001.1262.

Marques J, Appezzato-da-Glória B, Piepenbring M, Massola J, Monteiro-Vitorello C, Vieira M. 2017. Sugarcane smut: shedding light on the development of the whip-shaped sorus. Ann Bot. 119(5):815–827. doi: 10.1093/aob/mcw169.

Montesinos E, Bonaterra A, Badosa E, Frances J, Alemany J, Llorente I, Moragrega C. 2002. Plant-microbe interactions and the new biotechnological methods of plant disease control. Int Microbiol. 5(4):169–175. doi: 10.1007/s10123-002-0085-9.

Que Y, Xu L, Wu Q, Liu Y, Ling H, Liu Y, Zhang Y, Guo J, Su Y, Chen J. 2014. Genome sequencing of *Sporisorium scitamineum* provides insights into the pathogenic mechanisms of sugarcane smut. BMC Genomics. 15(1):1–20. doi: 10.1186/1471-2164-15-996.

Rajput M, Rajput N, Syed R, Lodhi A, Que Y. 2021. Sugarcane smut: current knowledge and the way forward for management. J Fungi. 7(12):1095. doi: 10.3390/jof7121095.

Raupach G, Kloepper J. 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology. 88 (11):1158–1164. doi: 10.1094/PHYTO.1998.88.11.1158.

Schirawski J, Mannhaupt G, Münch K, Brefort T, Schipper K, Doehlemann G, Di Stasio M, Rössel N, Mendoza-Mendoza A, Pester D. 2010. Pathogenicity determinants in smut fungi revealed by genome comparison. Science. 330 (6010):1546–1548. doi: 10.1126/science.1195330.

Shi M, Chen L, Wang X, Zhang T, Zhao P, Song X, Sun C, Chen X, Zhou B, Zhang Y. 2012. Antimicrobial peptaibols from *Trichoderma pseudokoningii* induce programmed cell death in plant fungal pathogens. Microbiology. 158(1):166–175. doi: 10.1099/mic.0.052670-0.

Shimada A, Takeuchi S, Kusano M, Fujioka S, Kimura Y. 2004. Roridin a and verrucarin A, inhibitors of pollen development in *Arabidopsis thaliana*, produced by *Cylindrocarpon* sp. Plant Sci. 166(5):1307–1312. doi: 10.1016/j.plantsci.2004.01.009.

Singh N, Somai B, Pillay D. 2004. Smut disease assessment by PCR and microscopy in inoculated tissue cultured sugarcane cultivars. Plant Sci. 167(5):987–994. doi: 10.1016/j.plantsci.2004.05.006.

Singh P, Singh R, Li H, Guo D, Sharma A, Lakshmanan P, Malviya M, Song X, Solanki M, Verma K. 2021a. Diazotrophic bacteria *Pantoea dispersa* and *Enterobacter asburiae* promote sugarcane growth by inducing nitrogen uptake and defense-related gene expression. Front Microbiol. 11:600417. doi: 10.3389/fmicb.2020.600417.

Singh P, Song Q, Singh R, Li H, Solanki M, Malviya M, Verma K, Yang L, Li Y. 2019. Proteomic analysis of the resistance mechanisms in sugarcane during *S. scitamineum* infection. Int J Mol Sci. 20(3):569. doi: 10.3390/ijms20030569.

Singh P, Xie J, Qi Y, Qin Q, Jin C, Wang B, Fang W. 2021b. A thermotolerant marine *Bacillus amyloliquefaciens* S185 producing iturin A5 for antifungal activity against *Fusarium oxysporum* f. sp. *cubense*. Mar Drugs. 19(9):516. doi: 10.3390/md19090516.

Sousa J, Olivares F. 2016. Plant growth promotion by *Streptomycetes*: ecophysiology, mechanisms and applications. Chem Biol Technol Agric. 3(1):1–12. doi: 10.1186/s40538-016-0073-5.

Su Y, Wang Z, Xu L, Peng Q, Liu F, Li Z, Que Y. 2016. Early selection for smut resistance in sugarcane using pathogen proliferation and changes in physiological and biochemical indices. Front Plant Sci. 7:1133. doi: 10.3389/fpls.2016.01133.

Sun Y, Xie J, Tang L, Odiba AS, Chen Y, Fang W, Wu X, Wang B. 2023. Isolation, identification and molecular mechanism analysis of the nematicidal compound spectinabilin from newly isolated *Streptomyces* sp. DT10. Molecules. 28 (11):4365. doi: 10.3390/molecules28114365.

Tamura K, Stecher G, Kumar S. 2021. Mega11: molecular evolutionary genetics analysis version 11. Mol Biol Evol. 38 (7):3022–3027. doi: 10.1093/molbev/msab120.

Taniguti L, Schaker P, Benevenuto J, Peters L, Carvalho G, Palhares A, Quecine M, Nunes F, Kmit M, Wai A, et al. 2015. Complete genome sequence of *S. scitamineum* and biotrophic interaction transcriptome with sugarcane. PLoS One. 10(6): e0129318. doi: 10.1371/journal.pone.0129318.

Tegene S, Dejene M, Terefe H, Tegegn G, Tena E, Ayalew A. 2021. Evaluation of native *Trichoderma* isolates for the management of sugarcane smut (*Ustilago scitaminea*) in sugar plantations of Ethiopia. Cogent Food Agr. 7(1):1872853. doi: 10.1080/23311932.2021.1872853.

Urban M, Kahmann R, Bölker M. 1996. Identification of the pheromone response element in *Ustilago maydis*. Molec Gen Genet. 251(1):31–37. doi: 10.1007/BF02174341.

Van S, Van S, Pieterse C. 2008. Plant immune responses triggered by beneficial microbes. Curr Opin Plant Biol. 11 (4):443–448. doi: 10.1016/j.pbi.2008.05.005.

Viaene T, Langendries S, Beirinckx S, Maes M, Goormachtig S. 2016. *Streptomyces* as a plant's best friend? FEMS Microbiol Ecol. 92(8):fiw119. doi: 10.1093/femsec/fiw119.

Vollmeister E, Schipper K, Baumann S, Haag C, Pohlmann T, Stock J, Feldbrügge M. 2012. Fungal development of the plant pathogen *Ustilago maydis*. FEMS Microbiol Rev. 36 (1):59–77. doi: 10.1111/j.1574-6976.2011.00296.x.

Wahl R, Zahiri A, Kämper J. 2010. The *Ustilago maydis b* mating type locus controls hyphal proliferation and expression of secreted virulence factors in planta. Mol Microbiol. 75 (1):208–220. doi: 10.1111/j.1365-2958.2009.06984.x.

Wang Z, Solanki M, Yu Z, Anas M, Dong D, Xing Y, Malviya M, Pang F, Li Y. 2021. Genome characteristics reveal the biocontrol potential of actinobacteria isolated from sugarcane rhizosphere. Front Microbiol. 12:797889. doi: 10.3389/fmicb.2021.797889.

Yan M, Cai E, Zhou J, Chang C, Xi P, Shen W, Li L, Jiang Z, Deng Y, Zhang L. 2016a. A dual-color imaging system for sugarcane smut fungus *S. scitamineum*. Plant Dis. 100 (12):2357–2362. doi: 10.1094/PDIS-02-16-0257-SR.

Yan M, Zhu G, Lin S, Xian X, Chang C, Xi P, Shen W, Huang W, Cai E, Jiang Z. 2016b. The mating-type locus *b* of the sugarcane smut *S. scitamineum* is essential for mating, filamentous growth and pathogenicity. Fungal Genet Biol. 86:1–8. doi: 10.1016/j.fgb.2015.11.005.

Yassin M, Mostafa A, Al-Askar A. 2022. *In vitro* antagonistic activity of *Trichoderma* spp. against fungal pathogens causing black point disease of wheat. J Taibah Univ Sci. 16 (1):57–65. doi: 10.1080/16583655.2022.2029327.

Zheng W, Li D, Zhao J, Liu C, Zhao Y, Xiang W, Wang X. 2017. *Promicromonospora soli* sp. nov. a novel actinomycete isolated from soil. Int J Syst Evol Microbiol. 67(10):3829–3833. doi: 10.1099/ijsem.0.002207.

Zhong Y, Yan M, Jiang J, Zhang Z, Huang J, Zhang L, Deng Y, Zhou X, He F. 2018. Mycophenolic acid as a promising fungal dimorphism inhibitor to control sugar cane disease caused by *S. scitamineum*. J Agric Food Chem. 67(1):112–119. doi: 10.1021/acs.jafc.8b04893.

Zhu G, Deng Y, Cai E, Yan M, Cui G, Wang Z, Zou C, Zhang B, Xi P, Chang C. 2019. Identification and functional analysis of the pheromone response factor gene of *S. scitamineum*. Front Microbiol. 10:481526. doi: 10.3389/fmicb.2019.02115.