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Effects of Plant Stress Signal Molecules on the Production of Wilforgine in an Endophytic Actinomycete Isolated from *Tripterygium wilfordii* Hook.f.

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Abstract The endophytic actinomycete F4-20 was isolated from *Tripterygium wilfordii* Hook.f. and was confirmed to produce wilforgine, a secondary metabolite discovered in its host. F4-20 showed a close phylogenetic relationship to *Streptomyces* species. To seek elicitors that may enhance the production of wilforgine in F4-20, four plant stress molecules were applied to the in vitro liquid cultures. Results showed that methyl jasmonate (MeJA), salicylic acid (SA), and hydrogen peroxide (H_2O_2) inhibited bacterial growth, whereas glutathione (GSH) treatment significantly increased bacterial growth. The wilforgine contents in the mycelia of F4-20 were reduced by MeJA and GSH but were induced by SA and H_2O_2. When added in the end of the culture period (7 day), 1 mM SA and 5 mM H_2O_2 resulted in 69.35 ± 1.71 and 71.80 ± 3.35 μg/g DW of wilforgine production, 1.55 and 1.60 fold to that of control (44.83 ± 1.35 μg/g DW), respectively. Though this improved production was about 6.5 times lower than that of the natural root (454.00 μg/g dry root bark), it provided an alternative method for the production of valuable plant secondary metabolites.

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Introduction

Plant secondary metabolites are unique resources for pharmaceuticals, food additives, biopesticides, and fine chemicals and also used as raw materials in other fields. However, the natural contents of these active metabolites are always very low. The production of valuable secondary metabolites in plant cell, tissue, and organ cultures is an attractive alternative to extraction of the whole plant material and in chemical synthesis. Unfortunately, the issues that plagued plant-based production systems decades ago continue to exist today: low yields, biochemical or genetic instability, and scale-up challenges [34]. Manipulating biosynthetic pathways leading to secondary metabolites by plant metabolic engineering is a promising approach for enhancing metabolite production. Some studies have been conducted in this field in the past decades, however, due to the highly complicated biosynthesis pathway and manipulation network of plant, few successful examples could be listed [7].

Unlike plants, microorganisms grow rapidly, are easy to scale-up culture, and are convenient for genetic engineering because the genes involved in secondary metabolite biosynthesis are mostly clustered in fungal genomes and even present as polycistron in bacterial genomes. Some endophytic microorganisms produce the same secondary metabolites as their host plants through horizontal gene transfer or other unknown mechanisms [5, 21, 30, 32, 33, 40]. This provides great opportunities for them as substitutes of the host plants, of which the resources are often limited. However, the production of these valuable metabolites in the endophytes is often very low and may attenuate with subculture times [11].

Secondary metabolites protect endophytes and/or the host plant against aggressive microorganisms and/or

feeding insects [9, 13]. Endophytes may increase the biosynthesis of secondary metabolites after stress signal recognition. Many signaling molecules in the host plant are candidate messengers for this kind of recognition. The most well-known candidate messengers are jasmonic acid (JA) and salicylic acid (SA). The JA signal transduction pathway is involved in the formation of chemical and physical barriers against pathogens or herbivores [38]. SA serves different functions in growth and development. It responses to abiotic stresses and regulates both local disease resistance mechanisms, including host cell death and defense gene expression, and systemic acquired resistance [37]. JA and SA have highly antagonistic signaling pathways [36]. Pathogens with a biotrophic lifestyle are more sensitive to SA-dependent responses, whereas necrotrophic pathogens and herbivorous insects are commonly deterred by JA-dependent defenses [10].

Hydrogen peroxide (H_2O_2) exerts various effects on plant defense responses, including cell wall reinforcement, hypersensitive cell death, defensive gene activation, and defensive compound induction. Though the mechanism remains unclear, reactive oxygen species (ROS) induces the expression of many defense genes and secondary metabolite biosynthetic genes [17]. Oxidative damage in plant tissue is alleviated by a concerted action of both enzymatic and non-enzymatic antioxidant metabolisms, such as reduced glutathione (GSH) and glutathione reductase [22]. Similar to H_2O_2, the GSH content in plants is also an indicator of environmental stress. H_2O_2 and GSH are crucial to redox homeostasis and redox signaling, and also act as secondary messengers in JA and SA signaling pathways [20].

In this study, endophytic actinomycete F4-20 was isolated from *Tripterygium wilfordii* Hook.f. This strain produces host-contained wilforgine, a kind of sesquiterpene pyridine alkaloid, which elicits strong insecticidal activity [29, 41] and many pharmacological activities [15, 18]. Four plant stress molecules, namely, MeJA, SA, H_2O_2, and GSH, were separately applied to in vitro cultures of F4-20 to examine their effects on bacterial growth and wilforgine production.

Materials and Methods

Isolation of Endophytic Actinomycetes

Fresh root, stem, and leaf tissues of *T. wilfordii* Hook.f. were collected from the Training Mountainous Area in Fujian Province, China in July 2010. Postharvest moisture and endophytic activity were maintained and ensured, respectively. Fresh root, stem, and leaf samples were washed with tap water and cut into small pieces (i.e., 5 cm long for roots and stems, 3 cm × 3 cm for leaves). Surface disinfection was carried out through stepwise washing in 70 % ethanol for 5 min, sodium hypochlorite solution (2 % available Cl^-) for 5 min, and 70 % ethanol for 30 s, followed by four rinses in sterile distilled water. Then, the samples were further cut into small pieces (i.e., 0.5-cm long for roots and stems, 0.2 cm × 0.2 cm for leaves). The samples were placed under aseptic conditions on two solid culture plates, Gause's No.1 and humic acid–vitamin agar [2] both supplemented with 15 μg/mL of nalidixic acid and 50 μg/mL of nystatin. The samples were cultured upside down at 26 °C for 12 h and 20 °C for 12 h daily. The growth of the endophytes was observed daily. Newly grown mycelia were transferred onto new plates and further purified until pure strains were obtained. The last washings of sterile waters were spread on the plates and were cultured under the same conditions mentioned above as the controls to ensure thorough surface sterilization.

Alkaloid Identification via Chemical Chromogenic Reaction and LC–MS/MS Analysis

Ethyl acetate extracts of mycelia from the plates were spotted on silica gel thin-layer chromatography plate and detected by chemical chromogenic reaction with iodine-acid-modified Dragendorff's reagent to screen for alkaloid-producing strains [25]. Four alkaloids of *T. wilfordii* Hook.f. (wilforine, wilforjine, wilforgine, and wilfortrine) as external standards were subjected to LC–MS/MS analysis to isolate the strains that produce the same sesquiterpene pyridine alkaloid as the host plant. Mycelium (1 g) was filtered and ultrasonically extracted thrice using 20 mL of ethyl acetate. The supernatants were combined and evaporated. The solid extract was dissolved in 1 mL of 70 % acetonitrile aqueous solution and was filtered through a 0.22 μM filter before LC–MS/MS analysis. LC–MS/MS was performed on a Thermo ion trap LC–MS system (Thermo Scientific, USA) consisting of a surveyor autosampler, a surveyor MS pump, and an LTQ XL^{TM} linear ion trap mass spectrometer equipped with an ESI source that was operated in the positive mode. The data acquisition software used was Xcalibur 2.1. The LC separation was carried out using an Agilent ZORBAX Eclipse XDB C18 column (250 mm × 4.6 mm, 3.5 μm particle sizes). Solvent A was water, and solvent B was acetonitrile. The gradient program was as follows: a linear gradient was established from 5 % A to 75 % A for 20 min, followed by a holding period of 10 min, and then the concentration was returned to 5 % A for 5 min, with a holding period of 5 min (40 min total run time). The system was run at 25 °C, and the flow rate was 0.4 mL/min. Sample injections were 10 μL in volume. The LC–MS/MS conditions were as follows: ESI spray voltage, 4 kV; sheath gas flow

rate, 70 arb; auxiliary gas flow rate, 20 arb; capillary voltage, −38 V; capillary temperature, 350 °C; and tube lens, 95 V. Collision-induced dissociation-MS/MS experiments were performed on the precursor ions selected from MS_1 using the selected ion monitoring (SIM) mode: MS_1 was performed in the full scan mode (*m/z* 100–1,000); MS_2 was performed in the SIM mode. The molecular ions of the standard alkaloids were monitored at *m/z* 868, 764, 858, and 874 for wilforine, wilforjine, wilforgine, and wilfortrine, respectively. Finally, one root colonizing strain (F4-20) that produces wilforgine was isolated from the 23 isolated endophytic actinomycetes.

16S rRNA Gene Sequence Analysis of F4-20

The genomic DNA of F4-20 was extracted using the method of Wilson [39]. Highly conserved 16S rRNA gene primers, the sense (5′-GAAGAGTTTGATCATGGCT CAG-3′) and antisense (5′-AAGGAGGTGATCCAACC GCAGG-3′) primers were designed on the basis of the 16S rRNA gene sequence of *Escherichia coli* (J01859.1), with the product length of 1541 bp referred to work of Edwards et al. [8]. The PCR consisted of 15.8 μL of distilled water, 2.5 μL of 10× PCR buffer, 1.5 μL of 25 mM $MgCl_2$, 2 μL of 2.5 mM dNTPs, 1 μL of 10 μM sense primer, 1 μL of 10 μM antisense primer, 1 μL of template, and 0.2 μL if 1 U *Taq* Polymerase (Takara, Japan). The reaction mixture was incubated for 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 2 min at 72 °C. The PCR product was sequenced. The sequencing result was compared using the BLASTn method [1] and a phylogenetic tree was constructed using the neighbor-joining module of MEGA5.1 software to analyze the phylogenetic relationships between strains. The evolutionary distances were computed using the Maximum Composite Likelihood method. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.35). All positions containing gaps and missing data were eliminated from the dataset [27, 35].

MeJA, SA, H_2O_2, and GSH Preparation and Application

A 0.5 cm diameter agar disk of F4-20 was inoculated into 50 mL of ISP-2 liquid medium [31] in a 250 mL Erlenmeyer flask, and cultured at 28 °C with shaking at 180 rpm for 7 days to establish a stock culture. Experimental cultures of F4-20 were derived from one stock culture to eliminate random errors with 2 % inoculum size (1 mL of stock culture to 50 mL of fresh medium). Each of the stock solutions of MeJA, SA, H_2O_2, and GSH was added to the medium on day 3.5 of culture to yield two final concentrations of 10 and 100 μM, 100 μM and 1 mM, 5 and 25 mM, and 300 μM and 3 mM, respectively. The control received 100 μL of ethanol or 1 mL of sterile water to match the solvents used in the preparation of stock solutions. Three repeated samples for each treatment were collected separately (50 mL each) after 12, 24, 48, and 72 h of treatment. The mycelia were filtered, blotted to dryness on filter paper, weighed to determine the FW, and freeze-dried to determine the DW.

Wilforgine Quantification via HPLC

The freeze-dried mycelia were first ground to powder. Ethanol was successively added (900, 600, and 450 μL) to 50 mg of the powdered sample prior to ultrasonication for 30 min. The pooled supernatant was then dried. The extracts were redissolved in 100 μL of 60 % acetonitrile aqueous solution and then filtered through a 0.22 μm filter before HPLC analysis. The culture media were extracted thrice using an equal volume of ethyl acetate to investigate the secretion of wilforgine after the treatments. The combined extract was concentrated and redissolved in 500 μL of 60 % acetonitrile aqueous solution and then filtered through a 0.22 μm filter before HPLC analysis.

The HPLC system (Waters 600E, Milford, USA) was equipped with a Waters 2478 UV detector and an Agilent Zorbax Eclipse XDB C18 column (250 mm × 4.6 mm, 3.5 μm particle size). Solvent A was acetonitrile, and solvent B was water. The gradient program was as follows: a linear gradient was established from 5 % A to 75 % A for 20 min, followed by a holding period of 10 min, and then the concentration was returned to 5 % A for 10 min, with a holding period of 5 min (45 min total run time). The system was run at 25 °C at a flow rate of 1 mL/min. The sample injections were 10 μL in volume. Wilforgine was quantified using external standard method and the wavelength was set at 230 nm. Five diluted concentrations of wilforgine standard samples, 400, 100, 20, 4, and 0.8 μg/mL were used and the established linear regression equation was $Y = 2.61e + 004X + 1.37e + 004$, $R^2 = 0.999991$. The precisions for all the concentrations were below 2 %.

Statistical Analysis

All data presented are the mean values of three replicates statistically analyzed using Duncan's multiple range test at $p < 0.05$ in SPSS (IBM, USA). The experiments were repeated twice with similar results.

Results and Discussion

Identification of F4-20

Good growth of F4-20 was observed on Gause's No. 1 medium. The colors of the aerial and substrate mycelia

were white and yellow, respectively. A hump was observed in the center of the colony, and no diffusible pigments were detected (Fig. 1). Based on other morphological and physiological characteristics (data not shown), F4-20 was assigned to the genus *Streptomyces*. A partial 16S rRNA gene sequence (GenBank accession no. KJ551912) was determined for strain F4-20. BLASTn analysis confirmed that strain F4-20 was closely related to *Streptomyces* species. The highest 16S rRNA gene sequence similarity was found with that of *Streptomyces blastmyceticus* NRRL 12747 (GenBank accession no. NR_043357). The close phylogenetic relationships between strain F4-20 and *Streptomyces* species are shown in Fig. 2.

LC–MS/MS and Chromatographic Analysis

The presence of wilforgine in the bacterial samples was first confirmed using LC–MS/MS (Fig. 3). The peak of *m/z* 840, the most abundant daughter ion of wilforgine, was observed in the sample extracted from mycelia at the statistically identical time to that of the standard (Fig. 3a, b). The mass spectrum of the sample extracts showed almost identical fragment ion peaks to that of the standard (Fig. 3c, d). HPLC analysis results also confirmed the presence of wilforgine through the peak with a retention time of 21.5 min, which was similar to that of standard wilforgine (Fig. 4).

Many studies for screening plant endophytes that produce host-containing secondary metabolites have been reported [5, 21, 30, 32, 33, 40]. These endophytes could produce identical metabolites to that of the host plant, however, the mechanism remains unknown. In recent years, attempts have been made to address various possibilities, including the possibilities that (a) endophytes possess genes of the secondary metabolite biosynthetic pathway [14] and (b) plant secondary metabolite synthesis in endophytic fungi may be harbored in extrachromosomal elements or plasmids, which by themselves may be contained in endohyphal bacteria [26]. The gene clusters coding for bioactive molecules, especially secondary metabolites, can be rendered silent under culture conditions and contribute to steady attenuation. Attenuation of wilforgine production was also observed when F4-20 was cultured in some other culture media (data not shown), but wilforgine production was roused again after inoculation into ISP-2 liquid medium.

Effects of MeJA, SA, H_2O_2, and GSH on the Growth of F4-20

The growth of F4-20 was significantly inhibited immediately after the application of 100 μM MeJA (Fig. 5a). Ceased growth was observed after 12 h of treatment with 1 mM SA, and reduced growth was observed after treatment with 100 μM SA (Fig. 5b). H_2O_2 also exhibited growth inhibitory effects after 72 h of treatment (Fig. 5c). Notably, the growth of F4-20 was significantly induced by GSH. The maximum mycelial fresh weight (FW) of 4.72 ± 0.40 g, which was 1.39 times to that of the control (3.40 ± 0.07 g), was observed after 12 h of treatment with 3 mM GSH. However, the FW of mycelia sharply decreased to the minimum value of 3.27 ± 0.04 g on day 5.5. Three hundred micromole per liter GSH showed similar effects on mycelial growth (Fig. 5d).

The FW of F4-20 decreased after MeJA, SA, and H_2O_2 treatments, but increased after GSH treatment. Given its powerful oxidant reactivity, H_2O_2 causes oxidative damage to nucleotides, proteins, and lipids when not properly removed from cells [3]. GSH functions as an electron donor for glutathione peroxidase, which reduces H_2O_2 to water and contributes to ROS degradation [19]. These facts may explain the effects of H_2O_2 and GSH on F4-20 growth. The growth inhibitory effects of MeJA and SA may indicate the existence of the receptor and signal transduction pathways. And through the signal reception and

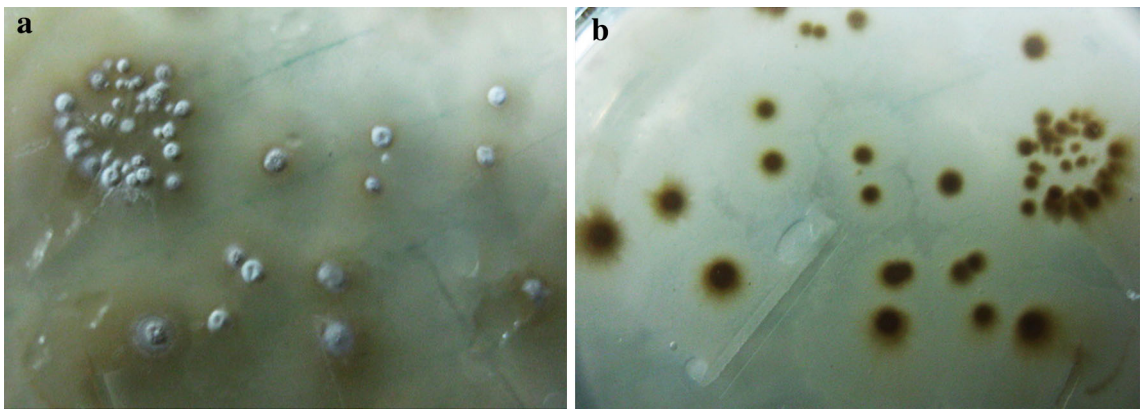


Fig. 1 Colony characteristics of strain F4-20 on Gause's No.1 medium plate observed from the *front* (**a**) and the *back* (**b**) of the plate

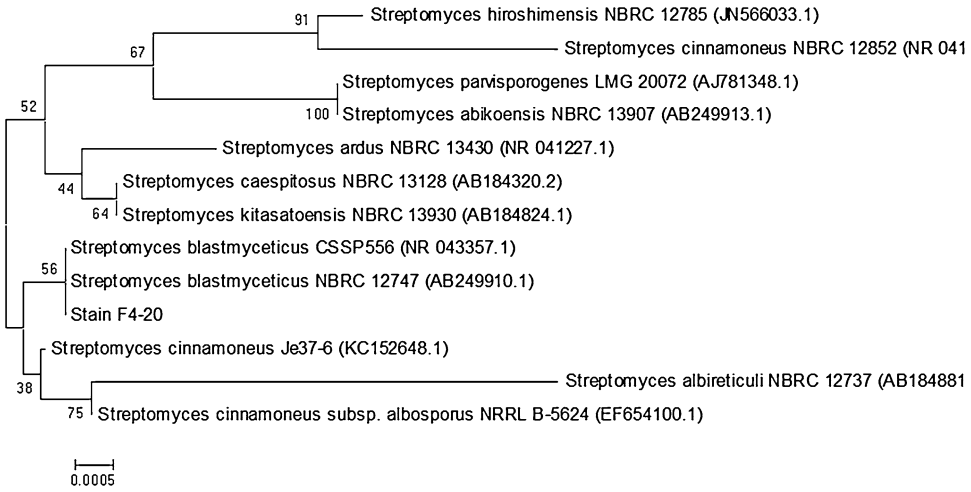


Fig. 2 Phylogenetic relationships of partial 16S rRNA sequences between the isolates and Streptomyces species. Numbers at the nodes indicate the levels of bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets. Accession numbers of the sequences are in parentheses. The *scale bar* represents the number of changes per base position

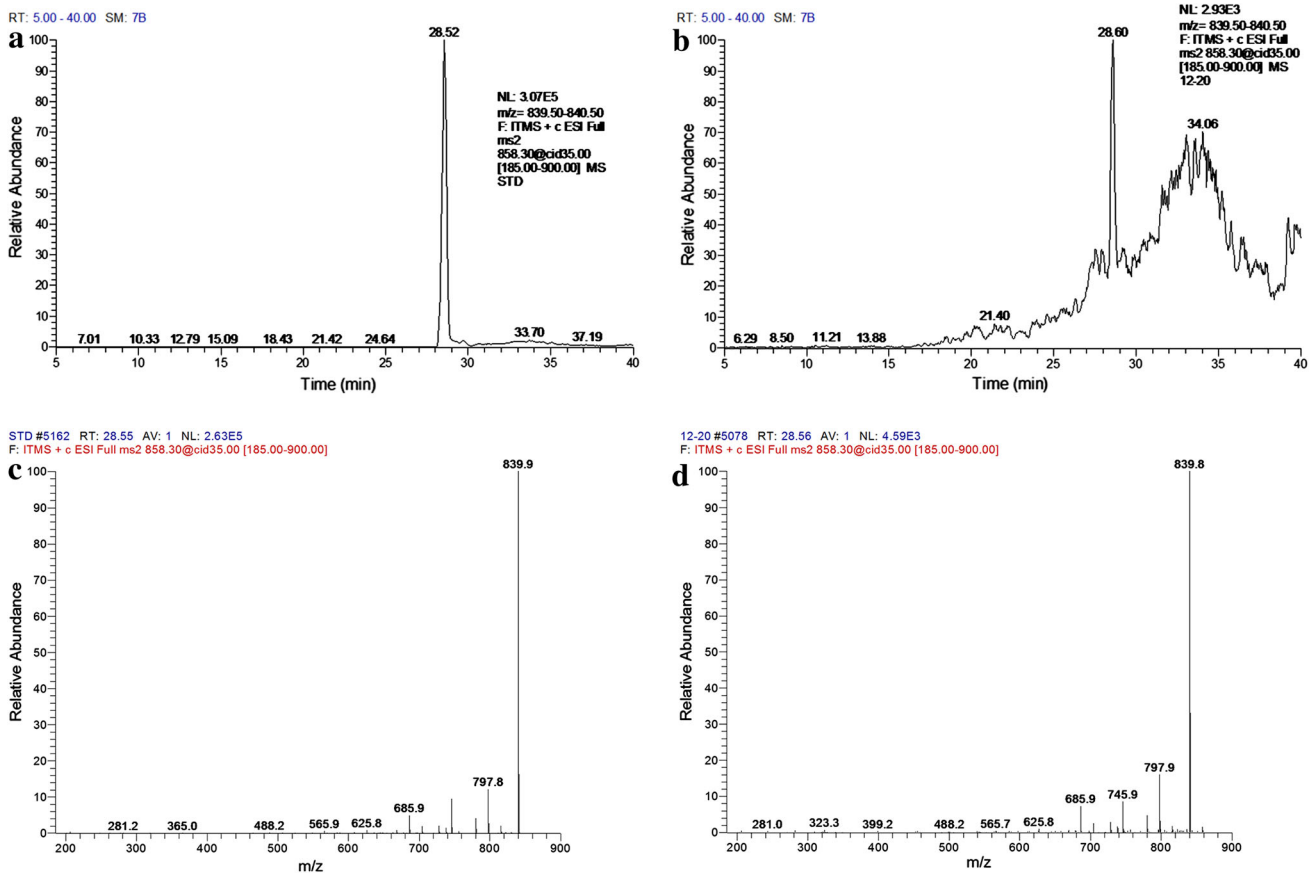


Fig. 3 LC-MS/MS analysis of standard wilforgine (**a**, **c**) and mycelial wilforgine extract (**b**, **d**). Collision-induced dissociation-MS/MS experiments (35 eV collision energy) were performed on the precursor ions selected from MS1 using the selected ion monitoring (SIM) mode: MS1 was performed in the full scan mode (*m/z* 100–1,000); MS2 was performed in the SIM mode. The molecular ions of *m/z* 858 were monitored. The chromatograms of the most abundant daughter ions (*m/z* 840) are presented for the standard wilforgine (**a**) and mycelial wilforgine extract (**b**). The mass spectra of the molecular ion (*m/z* 858) are presented for the standard wilforgine (**c**) and mycelial wilforgine extract (**d**). The concentration of standard wilforgine was 20 μg/mL. Mycelial ethyl acetate extract of 1 g dry mycelia was dissolved in 1 mL of 70 % acetonitrile aqueous solution. The sample injections were 10 μL in volume

transduction, MeJA and SA may reduce primary metabolism and/or enhance secondary metabolism of strain F4-20. SA is both an uncoupler and inhibitor of mitochondrial electron transport, which underlies the induction of some genes by SA in tobacco [23]. Therefore, SA may also act as a poison to inhibit the growth of F4-20.

Effects of MeJA, SA, H_2O_2, and GSH on Wilforgine Production of F4-20

Wilforgine production increased with the culture time for the control, which may be due to slow primary growth. After 24 h of MeJA treatment, the wilforgine contents significantly decreased to 11.68 ± 3.34 μg/g dry weight (DW), which was 2.77 times less than those of the control (32.39 ± 2.62 μg/g DW). Similar results were obtained for higher MeJA concentrations (Fig. 6a). Wilforgine contents increased significantly after 12 and 24 h of treatment with both 100 μM and 1 mM SA (Fig. 6b). The accumulation patterns of wilforgine after H_2O_2 and GSH treatments were highly similar to those of SA and MeJA treatments (Fig. 6c, d). No wilforgine release from the mycelia to the culture medium was observed, indicating that the accumulated wilforgine in mycelia represented all the wilforgine biosynthesized.

MeJA and SA may poison the strain, reduce primary metabolism and consequently increase wilforgine production. If this poisonous effect was dominant, wilforgine production would be higher than the control throughout the period after treatment. However, according to the experimental results, both MeJA and SA could only change wilforgine production rapidly (Fig. 6a, b). Thus, we speculated that the receptor and signal transduction pathways were involved in MeJA and SA treatments. *Streptomyces* species harbor many two-component systems (TCSs), however, the activating signals of most TCSs currently remain unknown [24]. The filamentous lifestyle provides endophytic and plant pathogenic *Streptomyces* species the ability to colonize nearby plant tissues, and subsequently directly penetrate plant cells to enter the host [28]. Intracellular hyphae of these species may recognize plant signals and modulate self-metabolism.

H_2O_2 can induce the expression of oxidative stress regulons in bacteria, such as OxyR and SoxRS [4]. The expression of the SoxR regulon is intimately linked with actinorhodin production in *Streptomyces coelicolor* [6]. Thus, H_2O_2 treatment may activate a similar signaling

Fig. 5 Effect of MeJA (**a**), SA (**b**), H_2O_2 (**c**), and GSH (**d**) on the ▶ growth of F4-20. The additives were added to the culture on the 3.5th day of culture. The mycelia were filtered, blotted to dryness on filter paper, weighed to determine their fresh weight (FW), and freeze-dried to weigh the dry weight (DW). The values are presented as means of triplicate ±SD. The *signs* on the *plots* indicate the significant difference of the FW or DW between the treatment and the control collected in parallel time day according to Duncan's multiple range test at $p < 0.05$ and "*filled star*" represents low concentration treatments and "*asterisk*" represents high concentration treatments

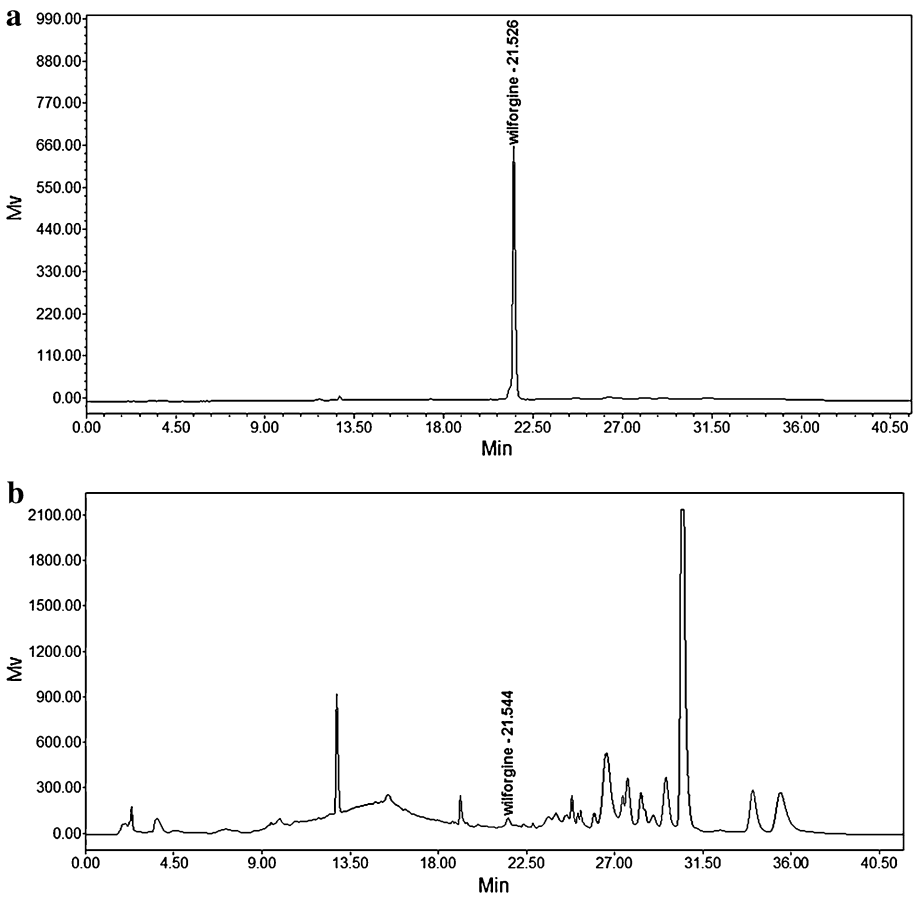
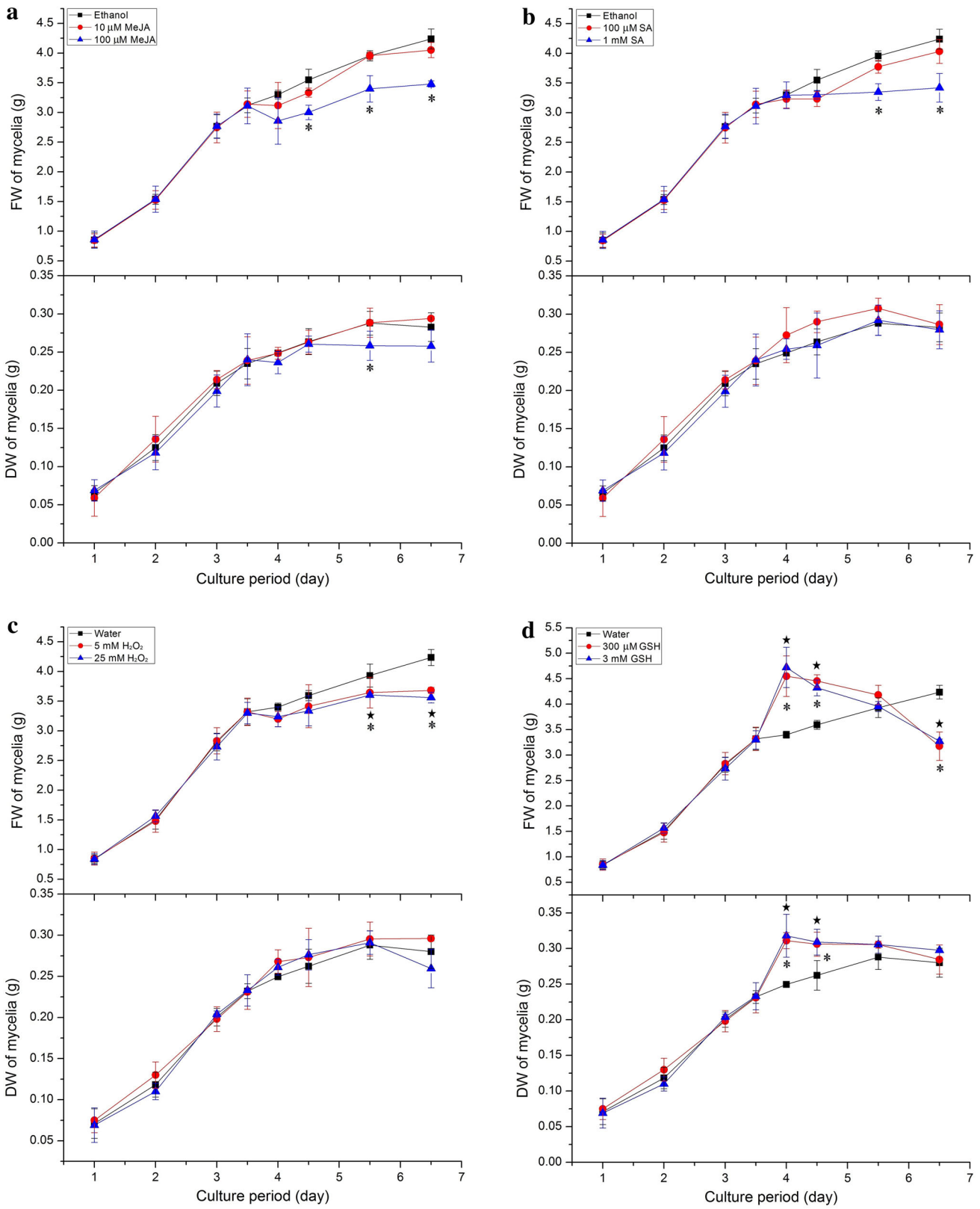


Fig. 4 High-performance liquid chromatogram of standard wilforgine (**a**) and mycelial wilforgine extract (**b**). Registrations of *peak* and retention times are recorded through UV detection at 230 nm. Bacterial sample showed a *peak*, with a retention time (21.5 min) identical to that of standard wilforgine. The concentration of standard wilforgine was 20 μg/mL. Mycelial ethanol extract of 50 mg dry mycelia was dissolved in 100 μL of 60 % acetonitrile aqueous solution. The sample injections were 10 μL in volume


a
Ethanol
10 μM MeJA
100 μM MeJA
FW of mycelia (g)
DW of mycelia (g)
Culture period (day)
b
Ethanol
100 μM SA
1 mM SA
FW of mycelia (g)
DW of mycelia (g)
Culture period (day)
c
Water
5 mM H2O2
25 mM H2O2
FW of mycelia (g)
DW of mycelia (g)
Culture period (day)
d
Water
300 μM GSH
3 mM GSH
FW of mycelia (g)
DW of mycelia (g)
Culture period (day)

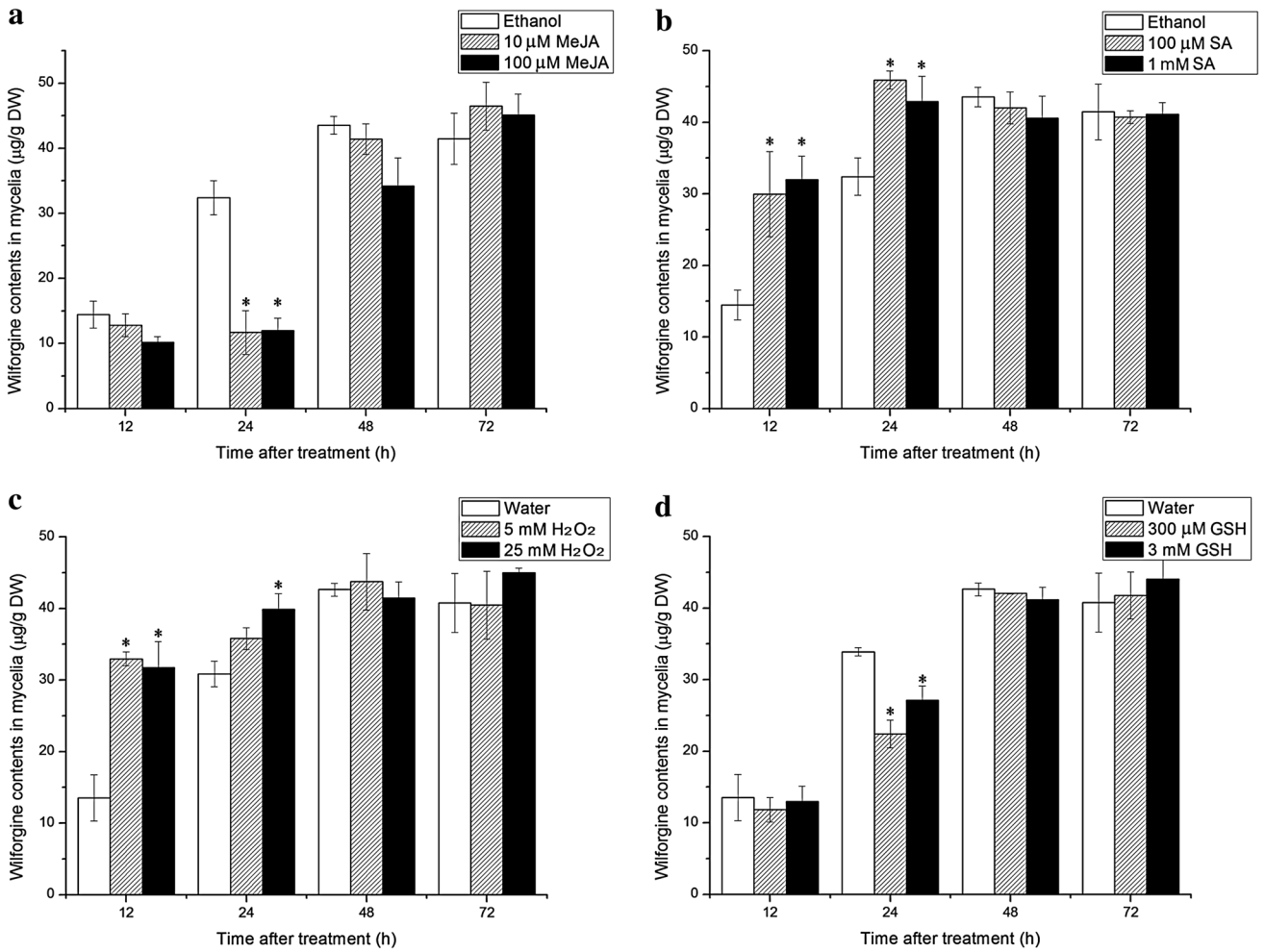


Fig. 6 Effects of MeJA (**a**), SA (**b**), H_2O_2 (**c**), and GSH (**d**) on the wilforgine contents of F4-20. The additives were added to the culture on the 3.5th day of culture. The samples were collected after 12, 24, 48, and 72 h of treatment. The values are represented as means of triplicate ±SD. The sign "*asterisk*" on the *top* of the plots indicates the significant difference of wilforgine contents between the treatment and the control collected at the same time according to Duncan's multiple range test at $p < 0.05$

pathway in F4-20, resulting in enhanced wilforgine production. GSH may have a function in wilforgine production of F4-20 directly by changing the status of oxidative stress regulators, or indirectly by eliminating ROS [4]. Endophytes may decide whether to synthesize defending compounds or not by recognizing these two redox-cycling chemicals, which mainly represent the two states of plants: threatened and secured.

The adding time point of SA and H_2O_2 was further investigated. When added in the end of the culture period (7 day), 1 mM SA and 5 mM H_2O_2 resulted in 69.35 ± 1.71 and 71.80 ± 3.35 μg/g DW of wilforgine production, 1.55 and 1.60 fold to that of control (44.83 ± 1.35 μg/g DW), respectively. The improved production was about 6.5 times lower than that of the natural root (454.00 μg/g dry root bark) [16] but much higher than that of a previously discovered endophytic fungi of *T. wilfordii* Hook.f. (8.03 μg/g DW) [12].

In conclusion, this study raises the hope of exploiting endophytes as alternative sources of valuable plant secondary metabolites like wilforgine. Other important parameters, such as pH and dissolved oxygen, should be optimized before industrial production. What's more, further studies should determine the signal recognition and transduction pathways in F4-20-like endophytes to understand the interactions between endophytes and their host plants and guide genetic engineering for increasing the efficiency of secondary metabolite production.

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