Study of the anti-sapstain fungus activity of *Bacillus amyloliquefaciens* CGMCC 5569 associated with *Ginkgo biloba* and identification of its active components

Bo Yuan, Zhe Wang, Sheng Qin, Gui-Hua Zhao, You-Jian Feng, Li-Hui Wei, Ji-Hong Jiang

Abstract

An endophytic bacterium, designated strain *Bacillus amyloliquefaciens* CGMCC 5569 was isolated from Chinese medicinal *Ginkgo biloba* collected from Xuzhou, China. Both the filtrate and the ethyl acetate extract of strain CGMCC 5569 showed growth inhibition activity against the sapstain fungi *Lasiodiplodia rubropurpurea*, *L. crassispora*, and *L. theobromae* obviously (>65%) based on the comparison of the length of zones on the petri dish. From the ethyl acetate extract of the filtrate, the antifungal compounds were obtained as a series of lipopeptides, which including series of fengycin, surfactin and bacillomycin. It showed strong growth inhibition activity *in vitro* against the *L. rubropurpurea*, *L. crassispora* and *L. theobromae* by about 70.22%, 69.53% and 78.76%, respectively. The strong anti-sapstain fungus activity indicated that the endophytic *B. amyloliquefaciens* CGMCC 5569 and its bioactive components might provide an alternative bio-resource for the bio-control of sapstain.

1. Introduction

The concept of biological control as a method for protecting crops and other perishable commodities has received increased attention from the scientific research community in recent years. This is a consequence, in part, of the increasing awareness of both industry and the general public of the environmental impact of chemicals used for crop protection and preservation purposes. Wood discoloration is a complex biological process that can involve a wide variety of microorganisms, often interacting with one another, and influenced by the changing environmental conditions under which the wood is placed. Sapstain is a major problem for timber producers as well as pulp and paper manufacturers. Over the years, the economic losses to the China lumber industry have been commercialized and used in controlling crop diseases, such as *Bacillus subtilis*, *B. polymyxa*, *B. pumilus*, *B. amyloliquefaciens*, *B. cereus*, and *B. licheniformis*. Recently, *Bacillus* species have been used widely as bio-control agents (Chen et al., 2009; Zhao et al., 2010). *Bacillus* spp. can produce structurally diverse secondary metabolites with a wide spectrum of antifungal activity. Several strains of *B. subtilis* and *B. amyloliquefaciens* have been found to produce lipopeptides, and these bioactive lipopeptides showed a great potential for biotechnological, biopharmaceutical and agricultural applications (Schallmey et al., 2004).

Many of these antifungal substances have been identified, including mycobacillin, iturin, bacillomycin, surfactin, mycosubtilin, fungi-stain, subsporins and rhizoctins (Fiddaman and Rossall, 1993; Kunst et al., 1997; Touré et al., 2004; Stein, 2005; Liu et al., 2010). These compounds, made of amino acids and a fatty acid, are easily biodegradable in the soils (Cho et al., 2003). Considerable interest lies in using *Bacillus* producing lipopeptide antibiotics, such as iturin A and surfactin as biocontrol agents due to their antagonistic and repressive activities against plant pathogens. These amphiphilic cyclic biosurfactants have many advantages over other fungicides: low toxicity, high biodegradability and environmentally friendly characteristics (Kim et al., 2004). The endophytic the wood discoloration (Yang, 2005); however, it may pose significant risks to environment and public health.

Significant advances in control the disease and stain fungi have been achieved both in research and application by the use of bio-control microorganisms, like bacteria and actinomycetes. Bacteria have been commercialized and used in controlling crop diseases, such as *Bacillus subtilis*, *B. polymyxa*, *B. pumilus*, *B. amyloliquefaciens*, *B. cereus*, and *B. licheniformis*. Recently, *Bacillus* species have been used widely as bio-control agents (Chen et al., 2009; Zhao et al., 2010). *Bacillus* spp. can produce structurally diverse secondary metabolites with a wide spectrum of antifungal activity. Several strains of *B. subtilis* and *B. amyloliquefaciens* have been found to produce lipopeptides, and these bioactive lipopeptides showed a great potential for biotechnological, biopharmaceutical and agricultural applications (Schallmey et al., 2004).

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**B. amyloliquefaciens** CGMCC 5569 used in this study was isolated in our laboratory from *Gingko biloba* and showed high levels of anti-sapstain fungus properties. We showed that CGMCC 5569 inhibited anti-sapstain fungus suggesting that the strain and antibiotic production were involved in sapstain-suppression. Identification of the antibiotics produced may improve our understanding of the mechanism involved in this and other biocontrol systems. The goal of this study was to purify and identify certain antibiotics produced by CGMCC 5569, which is responsible for the inhibition of sapstain fungi. Using chromatography, HSCCC and mass spectrometer, series of lipopeptides that inhibit sapstain fungi were isolated and identified in this study. The filtrate, ethyl acetate extract, and components showed a strong growth inhibition activity against the sapstain. And the results were significantly better than chemical fungicide nystafungi. The strong anti-sapstain fungus activity indicated that the endophytic *B. amyloliquefaciens* CGMCC 5569 and its bioactive components might provide an alternative bio-resource for the bio-control of sapstain.

2. Methods

2.1. Microorganism

Strain *B. amyloliquefaciens* CGMCC 5569 was isolated from ginkgo (*G. biloba*) collected from Xuzhou, China. The strain was deposited in the China General Microbiological Culture Collection Center (CGMCC) and maintained on LB medium (Lysogeny Broth Medium).

The sapstain strain of *Lasiodiplodia theobromae* was provided by the Forest Microbial Resources of China (CFCC). The stain of *L. rubropurpurea* and *L. crassispora* were kindly provided by Professor Gui-hua Zhao and maintained on PDA (Medium, Potato Dextrose Agar Medium).

2.2. Identification of the strain CGMCC 5569

Morphological observations were made with scanning electron microscope (SEM) using the method of Vendan et al. (2010). Utilization of carbon and nitrogen sources was carried out according to standard methods. Strain CGMCC 5569 was identified by biochemical and physiological methods by a kit (French, bio-Merieux, sa). The ability to produce enzymes was also studied and the morphological description was characterized by SEM.

Strain CGMCC 5569 was grown on LB medium. Genomic DNA extraction, amplification and 16S rRNA genes sequencing was analyzed according to described procedures (Liu et al., 2007). The 16S rRNA gene sequence data of the strain in this study were deposited in GenBank (JQ756988) and also compared with those of some type strains within the genus bacterium (retrieved from the GenBank/EMBL/DDBJ database). Phylogenetic analysis was performed using mega version 4.0 after multiple alignment of data by Clustal-X.

2.3. Fermentation and extraction

The fermentation was performed in LB medium at 30°C, 100 rpm for 72 h. The obtained culture broth (12 L) was centrifuged at 4000 rpm at room temperature for 5 min. The cell-free supernatant was extracted exhaustively five times with ethyl acetate (filtrate:ethyl acetate = 1:2 vol/vol). The solvent was removed by using a rotary vacuum evaporator R 206 D (SENCO, Shanghai, China) under reduced pressure to yield a brown viscous tarry residue (5.2 g) and stored at 4°C.

2.4. Anti-sapstain fungus assays

Petri dishes containing PDA were used for anti-sapstain fungus activity assay procedure by Duru et al. (2003). After removal of the cells by centrifugation of the culture broth at 4000 rpm for 5 min and filtration through 0.22 μm membrane filter. Culture filtrate (1 mL) mixed with 10 mL of the PDA medium was poured into a Petri dish. Discs of the target fungi, from the fresh margin of the mycelia, were spaced equally on the Petri dish. The dish was incubated at 28 ± 0.5°C for 72 h. The inhibitory activity of the filtrate against fungal growth was recorded as the percentage reduction of mycelia growth in comparison with that of the control plates (Liu et al., 2007). Make up different concentrations from 0.1 to 1.0 mL/mL and used similar procedure above to determine the anti-sapstain fungus activity of culture filtrate. The tests were conducted in triplicate for each treatment.

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**Fig. 1.** A neighbor-joining phylogenetic dendrogram based on 16S rRNA gene sequences showing the position of strain CGMCC 5569 among members of the genus *Bacillus* species. Numbers on branch nodes are percentage bootstrap values (1000 resamplings). The “T” in the figure means “type strain.”
The efficiency of the culture filtrate, ethyl acetate extract and active fraction of the strain CGMCC 5569 in control of three sapstain fungi.

2.5. Correlation between cell growth and anti-sapstain fungus activity

One hundred milliliter of LB medium in a 250 mL flask was inoculated with 1% of the overnight culture of strain CGMCC 5569 and incubated at 37°C, 180 rpm for 10–72 h. During incubation, 20 mL of the culture broth was sampled at time intervals about 6 h to measure its OD600 value and test its anti-sapstain fungus activity against L. theobromae.

2.6. Isolation and characterization of the bioactive metabolites

The antifungal compounds were isolated from the ethyl acetate extract using in vitro anti-sapstain fungus activity-guided chromatography on silica gel, Sephadex LH-20, High-Speed Counter-Current Chromatography (HSCCC) and identified by reverse phase HPLC (Agilent 1200 system) analysis coupled to a UV (UV Detector: Agilent 1200 UV Detector) and MS (Mass Spectrometer: Bruker microTOF-Q) detection. The results were obtained from comparison of the results to the data of standard compounds in the Syngenta natural product dereplication database (provided by Nanjing University) and references data from our laboratories in the long-term accumulation. The database is constructed based on standardized set of chromatographic conditions, and contains data of bioactive compounds isolated from bacteria, fungi or plants.

3. Results and discussion

3.1. Identification of the strain CGMCC 5569 from the G. biloba

The strain CGMCC 5569 was a Gram-positive, motile, endogenous spore bacterium by using the methods of morphological, biochemical and physiological characteristics. It is a rod-like or oval-shaped bacterium (2 × 0.6 μm) from the SEM analysis. The optimal temperature for its growth is 28–37°C. The β-galactoside and asusulin were only used in the test for origin carbon and other sources were not used (e.g. sorbitol, adonitol, xylose and raffinose). The phenylalanine and lysine test being positive means that the strain could use them as nitrogen sources. Urea was not hydrolyzed and acid was produced from glucose and malonate. Nitrate was reduced to nitrite and pyruvate was converted to acetoin. The aforementioned results support the identification of this strain as a member of genus *Bacillus*.

Furthermore, the partial 16S rRNA gene sequence analysis (1489 bp) also demonstrated that the strain CGMCC 5569 was most likely *B. amyloliquefaciens* NBRC 15535 (Fig. 1) and the similarity of the sequences between CGMCC 5569 and NBRC 15534 was 99.313%. Thus, we designated this strain to be *B. amyloliquefaciens* CGMCC 5569.

To the best of our knowledge, this is the first report of isolation of endophytic *B. amyloliquefaciens* from healthy stems of *G. biloba*. For over 5000 years, the fruits and seeds of the *G. biloba* have been used in traditional Chinese medicine for the treatment of asthma, cough, and enuresis (Smith and Luo, 2004). The compounds of *G. biloba* showed activities of cytotoxicity, antitumor and immunosuppressive. As part of our ongoing research program on the development of endophytic resources, several endophytic bacteria and fungi have been isolated from the stems and leaves of *G. biloba*. Strain CGMCC 5569 is one of the most active endophytic bacteria with respect to production of active substances against sapstain fungi, such as *L. rubropurpurea*, *L. crassispora*, and *L. theobromae*. Based on the physiological, biochemical characteristics, and 16S rRNA sequence analysis, strain CGMCC 5569 was identified as *B. amyloliquefaciens* which is closely related to *B. subtilis* and several strains were reported effective for control of plant pathogens (Yu et al., 2002; Chen et al., 2009; Calderia et al., 2011). However, there are no studies on the bio-activity about endophytic *B. amyloliquefaciens* isolated from medicinal plants. The antibiotic production by some bacteria plays a major role in plant disease suppression, including *Pseudomonas* and *Bacillus* spp. (Yu et al., 2002). *Bacillus*

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<tr>
<th>Item</th>
<th>Concentration a</th>
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<tr>
<td>Nystatin (μg/mL)</td>
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<tr>
<td>20</td>
<td>24.96 ± 1.04</td>
<td>51.23 ± 1.02</td>
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<tr>
<td>40</td>
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<td>60</td>
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<td>100</td>
<td>69.67 ± 1.02</td>
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<td>Culture filtrate</td>
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<tr>
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<td>46.10 ± 1.12</td>
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<tr>
<td>1.0</td>
<td>76.88 ± 0.44</td>
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a: Represent relative concentration of fermentation fluid in the PDA medium.
b: Represent relative concentration of ethyl acetate extract in the medium.
species are well known for their ability to control plant diseases through various mechanisms, including the production of secondary metabolites. Several strains belonging to the Bacillus displaying bio-control activities against fungal pathogens have already been isolated from various herbaceous plants and members of the genus Bacillus produce a variety of antibacterial and antifungal peptide antibiotics (Bargabus et al., 2003; Van den Broek et al., 2003; Calderia et al., 2011). Strains of B. subtilis also have been studied as bio-control agents of plant pathogens, but only a few antibacterials produced by some strains were isolated and identified and their role in bio-control have been studied (Schallmey et al., 2004; Chen et al., 2009; Zhao et al., 2010).

3.2. Growth curve of the strain CGMCC 5569 and its production of anti-sapstain metabolites

As shown in Fig. 2, the strain CGMCC 5569 grew relatively quickly. It reached the stationary phase at 26 h after inoculation, and there was almost no lag phase. The highest anti-sapstain fungus activity occurred at 48 h, and then declined over the following 24 h. It may be concluded that, the optimal time for the anti-sapstain fungus metabolites of the strain CGMCC 5569 was 48 h after inoculation.

The culture filtrate of strain CGMCC 5569 was evaluated for its anti-sapstain activity. The results in Table 1 showed that the culture filtrate significantly inhibited the growth of the sapstain fungi. The potency value of inhibited the growth of the for L. rubropurpurea, L. crassispora, and L. theobromae are 76.88%, 68.21%, and 73.40%, respectively at the concentration of 1.0 mL/mL. It was higher than the value of commonly used fungicidal nystafungin at the concentration of 100 μg/mL. The anti-sapstain activity of the culture filtrate was dose-dependent.

Sapstain fungi are one group of the wood-inhabiting fungi and most of these fungal species can result in economic losses to wood users. Sapstain fungi affect the appearance of wood due to colonization by pigmented hyphae but without producing significant strength losses (Yang, 2005). Study on the sapstain fungi is necessary for controlling their growth on wood and wood products. Any biological control strategy targeted against this type of deterioration will therefore be considered successful if it inhibits either fungal growth or pigment production. The use of biological agents to protect wood presents an alternative to the use of the current synthetic fungicides (Bruce et al., 2003; Yang, 2005). A few reports have been on the bio-control of sapstain fungi. Potential biocontrol agents have included bacteria and their culture filtrates, even their ingredients (Florencio and Sharma, 1990; Jin and Morrell, 1996). Other studies, however, have focused on the use of a variety of fungil or fungal filtrates to act as bio-protectants. For example, yeast has been considered as potential biocontrol agents (Walker et al., 1995), but it sometimes produces pigment. No studies have shown the anti-sapstain activity of Bacillus spp. against wood surface contaminants or sapstain fungi. It is well known that Bacillus spp. having the potential of synthesizing a wide variety of metabolites with antibacterial and/or antifungal activity. And most strains of Bacillus spp. and metabolites have been intensively exploited in medicine and industry (Leifert et al., 1995; Zhao et al., 2010), but no research has reported the activity against the sapstain fungi. In this study, endophytic B. amycoliquifaciens CGMCC 5569 belongs to the Bacillus and its culture filtrates have strong activities against the sapstain fungi. It probably a new resource for controlling the sapstain fungi and play an important role in the industry of bio-control.

3.3. Isolation and elucidation of the active compounds

To characterize the anti-sapstain compounds in the culture filtrate of strain CGMSS 5569, about 5.2 g of the ethyl acetate extract was obtained from 12 L of filtrate. The activity test showed that the ethyl acetate extract revealed significant growth inhibition of the test fungi. The inhibition percentage of the extract on the growth of test fungi at the concentration above 20 μg/mL was significantly higher than that of the commonly used fungicides nystafungin at the concentration of 60 μg/mL (Table 1). Of the phytopathogens tested, L. theobromae, L. rubropurpurea, and L. crassispora appeared to be the most sensitive species. The results also demonstrated that the anti-sapstain activity of the extract has dose-dependent responses.

By following the anti-sapstain activity tested, the active fraction was isolated from the ethyl acetate. The anti-sapstain chemicals were determined by reverse phase HPLC analysis of the isolated active fraction coupled to UV (270 nm) eluting with CH3OH/H2O (80:20, v/v), and MS detection and comparison of the results to the data base.

A LC-MS spectral analysis of the active fraction showed a cluster containing molecules that were obtained at m/z 1017.2−1500.6 and comparison of the results to the references and Syngenta natural product dereplication data base (Price et al., 2007). These peaks differ by 14, suggesting a series of homologous molecules with different length of fatty acid chain. The peak mass exhibited on those experimental conditions was compared. The results showed that these lipopeptides belonged to the family of surfactin, fengycin and bacillomycin. The described data were in agreement with results in literature and all MS spectra were all run with NaCl and the structure as described in the supplementary Fig. 1. The [M + Na]+ m/z 1017.2: 1031.5: 1054.2 and 10597. ions are probably components of a homologue series. According to molecular mass analysis, these five mass peaks secondary metabolites produced with molecular masses from m/z 1017.2 to 10597. corresponded to surfactin C12 to surfactin C15, respectively. Different number of −CH2− means homologue of surfactin. Surfactin is a general term for a class of cyclic lipopeptides, is considered as one of the most powerful biosurfactants for its prominent interfacial activity. Meanwhile, surfactin is an efficient biosurfactant produced by some B. subtilis strains. Surfactin also had been demonstrated to exhibit antiviral, antifungal, and hemolytic properties hold promise for the potential application of surfactin in microbial enhanced oil recovery, bioremediation of the environmental pollutant (Gang et al., 2011). It is a cyclic lipopeptides, consists of a heptapeptide (L-Glu, L-Leu, L-Val, L-Asp, L-Leu and D-Leu) linked to a β-hydroxyl fatty acid and closed via a lactone bond, and the length of the fatty acid chain varies from C12 to C17.

Two mass spectra of them displayed their major [M + Na]+ peaks at m/z 1053.4, 1067.5, corresponding to the molecular weight 1030.4 and 1034.5 respectively in accordance with bacillomycin D (C14) and bacillomycin D (C15). The difference of 14 mass units was due to the presence of the homologous C14 and C15 8-amino acids. The ion peak at m/z 1081.6 might be a methylene group in 8-amino acids. Results indicated that these were bacillomycin D (C16) (Zhao et al., 2010). Similarly, mass spectra of other ingredients showed that the [M + Na]+ peaks at m/z 1043.7 and 1057.5, respectively were defined bacillomycin L (C14) and bacillomycin L (C15) (Peypoux et al., 1984). Bacillomycin, which is a member of the iturin family along with mycosubtilin and iturin A, is made of one β-amino fatty acid and 7α-amino acid. Bacillomycin D consists of the sequence L-Asn, L-Tyr, L-Asn, L-Pro, L-Glu, L-Ser and L-Thr, but L-Asp, L-Ser at position 3, 4 are replaced by L-Asn, L-Ser in the heptapeptide of bacillomycin. The members of the bacillomycin family (e.g. bacillomycin D, L and F) exhibit strong antifungal and hemolytic activities and limited antibacterial activity (Koumoutsi et al., 2004).

The molecular masses from 1457.3 and 1528.2 were consistent with fengycin A (C14−C17) and fengycin B (C15). Homologues of m/z 1457.3; 1472.5; 1483.1 and 1500.6 belonged to fengycin A.
ever, synthesis of the antibiotics began with bacterial growth as approximately 26 h cultivation at the given fermentation; how-

In this study, the strain CGMCC 5569 and its products can be tested (Table 1). Meanwhile, the lipopeptides were obtained have strong activities against the sapstain fungi. The strong anti-sapstain fungus activity indicated that the endophytic B. amyloliquefaciens CGMCC 5569 and its bioactive components might provide an alternative bioresource for the bio-control on sapstain fungi.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.03.062.

References


Bhaskar, N., Sudeepa, E.S., Rashmi, H.N., Selvi, A.T., 2007. Partial purification and bioremediation of heavy metals (Guo et al., 2010). (Bhaskar et al., 2007), plant growth promoting (Pavlo et al., 2011), and bioremediation of 1528.2 was deduced fengycin B (C17) comparison of the results to the references (Wang et al., 2004). In fengycin A isofoms the peptide moiety consists of the sequence L-Glu, D-Ala, L-Pro, L-Gln, D-Tyr and L-Ile, while in fengycin B iso-

4. Conclusion

An endophytic bacterium B. amyloliquefaciens CGMCC 5569 was isolated from the healthy leaf of the plant G. biloba. The culture filtrate and ethyl acetate extract showed strong an inhibition activity against sapstain fungi L. theobromae, L. rubropurpurea, and L. crassissinor. A highest fraction was obtained following activity guide and identified by LC-MS. It was a mixture of series of seven residues of D- and L-amino acid and one residue of hydroxy fatty acid. It can be produced by industry technology and play a major role in sapstain suppression as a fungicide in the future.

Acknowledgements

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References


Bhaskar, N., Sudeepa, E.S., Rashmi, H.N., Selvi, A.T., 2007. Partial purification and bioremediation of heavy metals (Guo et al., 2010). (Bhaskar et al., 2007), plant growth promoting (Pavlo et al., 2011), and bioremediation of heavy metals (Guo et al., 2010). B. amyloliquefaciens CGMCC 5569 reached stationary phase after approximately 26 h cultivation at the given fermentation; however, synthesis of the antibiotics began with bacterial growth as showed by inhibition. The highest level of antifungal activity was observed during the stationary phase 48 h after inoculation. The synthesis of lipopeptides usually starts at the end of the exponen-

In this study, the strain CGMCC 5569 and its bioactive components might provide an alternative bioresource for the bio-control on sapstain fungi.


