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Peimisine and peiminine production by endophytic fungus *Fusarium* sp. isolated from *Fritillaria unibracteata* var. *wabensis*

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ABSTRACT

Steroidal alkaloids, as the major biologically active components in Bulbus *Fritillariae*, possess a variety of toxicological and pharmacological effects on humans. The objective of this work was to determine whether endophytic fungi isolated from fresh bulbs of *Fritillaria unibracteata* var. *wabensis* can produce one or more alkaloids like its host plant. Four classical reagents including Wagner's, iodine-potassium iodide, Mayer's and improved Dragendorff's were used for primary screening. Then thin-layer chromatography (TLC) and high performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD) were employed to identify the fermentation products of the selected strains. The results showed that extract from one stain (WBS007) has positive reactions in process of primary screening. A further TLC scan and HPLC-ELSD showed that strain WBS007 had two components with the same TLC relative front (Rf) value and HPLC retention time (RT) as authentic peimisine and peiminine. In addition, strain WBS007 was identified as *Fusarium* sp. based on phylogenetic analysis of ITS sequences. Thus, strain WBS007 produced the bioactive ingredient peimisine and peiminine, as does its host plant, and could be used for the production of peimisine and peiminine by fermentation.

Introduction

Bulbus Fritillariae Cirrhosae (BFC) (Chuan Beimu in Chinese) is the most commonly used antitussive and expectorant traditional Chinese medicinal (TCM) herb. It has been used as one of high-efficiency antitussive and expectorant drugs in China and other Asian countries for a long time (Wang et al. 2012). The bulbus of *Fritillaria unibracteata* Hsiao et K.C. Hsia var. *wabuensis* (S.Y. Tang et S.C.Yue) Z.D. Liu, S. Wang et S.C. Chen (BFW) (Wabu Beimu in Chinese) is one of the sources for BFC in China due to its positive therapeutic effects and few side effects. Abundant utilization experiences of such plants have been accumulated. It is now officially recorded in the National Pharmacopoeia of China (Editorial Board of the Pharmacopoeia of the PR China, 2010). Since the natural resource of this medicinal material was collected unduly, it is not easy to find its wild populations currently. And cultivated BFW has difficulty to satisfy the existing market demand because this plant grows slowly and mainly distributes at the altitude of 2500–3000 m in Qinghai-Tibet Plateau where is inconvenient to manage under adverse natural environment (Yan et al. 2012). In fact, BFW is in shortage and becomes vulnerable as cultivar (Liu et al. 2009).

It is well known that steroidal alkaloids are the main medicinal active ingredients of *Fritillaria* species (FS) (Tang and Eisenbrand 1992; Zhou et al. 2010), such as peimisine, peiminine, peimine etc. The pure alkaloid compounds from FS were done on pharmacological studies in the late 20th century. For example, verticine and verticinone showed antitussive effects, hypotensive effect, sedative effect *in vivo* and inhibition of PAF-induced thrombocyte aggregation and ADP-induced thrombocyte aggregation *in vitro*, and cevane alkaloid(s) showed inhibition of c-AMP phosphodiesterase and anticholinergic activity, and imperialine showed spasmolytic activity (Wagner et al. 2011). In addition, various *Fritillaria* extracts or pure compounds isolated from different FS have also been studied on their pharmacological aspects recently. The ethanol extracts from bulbs of *F. unibracteata* (BFU), BFW and bulbs of *F. mellea* investigated by Mo et al. and were similar in treating coughing in mice, expectoration in rats, asthma in guinea pigs, bronchodilation of isolated lungs in mice, and cyclic nucleotide (cAMP, cGMP) in the plasma and lungs of mice. Moreover, BFW was significantly superior to BFU in terms of asthma relieving and cAMP level increment in the lungs (Mo et al. 1998). Studies

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still showed that there were equal or similar activities among different pure *Fritillaria* alkaloids (FAs) not only among various FS. For instance, 3β-acetylimperialine, imperialine and sinpeinnine A might signally inhibit muscarinic receptor in spite of difference mechanism (Lin et al. 2006). Chan et al. investigated and compared the relaxant effect on five major FAs (imperialine, verticine, verticinone, ebeiedine and puqietinone) using rat isolated tracheal and bronchial preparations pre-contracted with carbachol, and they found that all five FAs caused a concentration-dependent relaxation with the relaxing potency (Chan et al. 2011). In addition, Wang et al. studied imperialine, chuanbeinone, verticinone and verticine from BFC and the results turned out that the alkaloids significantly inhibited cough frequency and increased latent period of cough in mice induced by ammonia, particularly imperialine, verticinone and verticine markedly increased mice tracheal phenol red output (Wang et al. 2011). The ability of inhibited cough frequency and increased latent period of cough of *F. wabuensis* had a close relationship to its alkaloids ingredient (Wang et al. 2012). Those alkaloids shows not only obvious biological activities as the single constituents but also the synergy effects with multi-target therapy described by H. Wagner and G. Ulrich-Merzenich (Wagner and Ulrich-Merzenich 2009).

The screening of plants for fungi is important as it opens the possibility to start fermentation processes and genetic Engineering methods to produce valuable drugs on an industrial scale, such as penicillin produced from multiple fungi (Cole 1966). It is, however, the pivotal and basal step to isolate and screen fungi synthesizing bioactive compounds. Since Stierle et al. found that an endophytic fungal strain isolated from *Taxomyces andreanae* produced the same bioactive taxol and taxane compounds as its host plant (Stierle et al. 1993), much renewed attention and time are paid to endophytic fungi which possess the potential to produce bioactive compounds which are equal or similar to their host (BCESH) (Kusari et al. 2013; You et al. 2013). It is a good example that producing paclitaxe or its similar compound(s) endophytic fungi were isolated from *Taxus wallichiana* Zucc., *T. yunnanensis*, *T. Cuspidata*, *T. baccata*, *T. chinensis* (Pilger) Rehd. (Wang et al. 2007). And taxol from the fermentation of endophytic fungi of plants has been demonstrated a high potential for efficient production of taxol (Kang et al. 2011). Therefore, BCESH from endophytic fungi isolated from other precious plants, especially to the precious medicinal plants, is a feasible way to develop and utilize microbial resources as well as protect precious plant resources. Endophytic fungi are considered as novel sources for bioactive compounds which have important application in the fields of medicine and agriculture. Now, a sipeimine-producing endophytic fungus has isolated from *F. ussuriensis* by Yin et al. (Yin and Chen 2008). In addition, the endophytic fungi from BFU (Chen et al. 2012a) and *F. przewalskii* (Chen et al. 2012b) were studied in our research group. But little published work has focused on testing endophytic fungi from BFW.

As previous details, *F. wabuensis* as one of the precious medicinal plant is far from meeting the market demands. Therefore, it is of great significance to find an alternative way to produce FAs of BFW. In this study, we isolated 10 endophytic fungal strains from fresh BFW collected from Sichuan province, southwest China. We screened out one strain which could produce alkaloids stably with improved Dragendorff's reagent and other three common-used reagents. Then, the extract solution of hyphae and fermentation liquor of this stain both were analyzed with TLC and HPLC-ELSD. This strain, for another, was identified with phylogenetic analysis of ITS sequences. In this way, we hope to develop a new method of producing these plant-derived pharmaceutical components, to resolve the conflict between natural resource protection and the requirement for BFW.

Materials and methods

Plant material

Fresh BFW were collected from the Mao County, Sichuan Province, China, in October 2012, which were identified by Prof. Wu Wei, Agronomy College, Sichuan Agricultural University, China. The altitude of the above site is 3040 m.

Authentic drug

Authentic sipeimine (Fig. 1D) and peimisine (Fig. 1A) (purity ≥ 98%) were purchased from Shanghai YuanYe Biotechnology Co., Ltd (Shanghai, China); Authentic peimine (Fig. 1C) (purity ≥ 98%) was purchased from Chengdu Sikehua Biological Technology Co., Ltd. (Chengdu, China); Authentic peiminine (Fig. 1B) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, Chain). The partial BFW collected above were dried at 60 °C in the oven as a positive control herb. The alkaloid of sample was extracted as described previously (Zhou et al. 2010).

Media

Potato dextrose agar (PDA) medium were used for the isolation and purification of endophytic fungi, and for the maintenance of pure strains. Potato-dextrose liquid (PDB) medium was prepared for fermentation. All media were sterilized by autoclaving.

Isolation of endophytic fungi

The collected samples were washed under running water thoroughly and then air-dried. The cleaned bulbs were surface-sterilized as follows: 75% ethanol (v/v) for all 30 s and rinsed in sterile water five times; followed by 0.1% $HgCl_2$ (g/v) 5 min; then, similarly, rinsed in sterile distilled water five times. The sterilized samples were cut into pieces of about 0.5 cm × 0.5 cm × 0.1 cm and placed on the PDA (containing more than 30 μg ml^{-1} streptomycin sulfate) to incubate at 28 °C for 3–15 d. The hypha of the endophytic fungus growing out from the bulbs pieces was transferred onto another PDA plate in time and incubated for 7 d. Testing was not repeated until a pure culture was obtained according to colony morphology. The purified strains were numbered and stored in a refrigerator at 4 °C for later use.

Culture conditions in fermentation

The mycelium of each isolated strain was inoculated in equal amounts (1%) into an Erlenmeyer flash (250 ml) containing 150 ml PDB medium and incubated at 28 °C for 7 d. The rotation rate of incubator was fixed to 130 rev min^{-1}.

Preparation of the isolated strains extract

Fungal mycelia were separated from the culture broth by vacuum filtration after the procedures of fermentation. The filtrate was all collected and concentrated up to 10% of the original (v/v) in a vacuum rotary evaporator at 40–45 °C. Having been pre-alkalized with strong ammonia (pH 9–10), the concentrated filtrate were extracted three times of the liquid-liquid partition with dichloromethane (DCM) 1:1 (v/v). The obtained crude extracts were collected and concentrated up to dryness, then dissolved into 2 ml methanol solvent per 1000 ml filtrate, followed by filtered with 0.45 μm Millipore filters to get the DCM extracting solution. Fungal mycelia were collected and rinsed in distilled water to remove residues of the filtrate, followed by dried inside of the

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