JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Endophytic Fungi from Pigeon Pea [*Cajanus cajan* (L.) Millsp.] Produce Antioxidant Cajaninstilbene Acid

JinTong Zhao,[†,‡] YuJie Fu,*[,†,‡] Meng Luo,[†,‡] YuanGang Zu,[†,‡] Wei Wang,[†,‡] ChunJian Zhao,[†,‡] and ChengBo Gu[†,‡]

[†]Engineering Research Center of Forest Bio-Preparation and [‡]Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, Harbin 150040, China

ABSTRACT: In this study, novel endophytic fungi producing cajaninstilbene acid (CSA) from pigeon pea [*Cajanus cajan* (L.) Millsp.] were investigated and screened. CSA has prominent pharmacological activities. A total of 110 endophytic fungi isolates were grouped into 8 genera on the basis of morphological characteristics, and CSA-producing fungi were screened by liquid chromatography−tandem mass spectrometry (LC-MS/MS). According to ITS-rDNA sequences analysis, the CSA-producing fungi were identified as *Fusarium solani* (ERP-07), *Fusarium oxysporum* (ERP-10), and *Fusarium proliferatum* (ERP-13), respectively. The amount of CSA produced by the ERP-13 reached 504.8 ± 20.1 μg/L or 100.5 ± 9.4 μg/g dry weight of mycelium. In a DPPH radical-scavenging assay, when the concentration of fungal CSA was 500 μg/mL, inhibition percentage could reach 80%, which was almost the same as that of standard CSA. This study first reported the natural antioxidant CSA from endophytic fungi *F. solani* and *F. proliferatum* isolated from pigeon pea.

KEYWORDS: *Cajanus cajan, endophytic fungi, LC-MS/MS, ITS sequence, cajaninstilbene acid (CSA)*

INTRODUCTION

Pigeon pea [*Cajanus cajan* (L.) Millsp.] is a famous grain legume crop in semitropical and tropical areas of the world.[1] Its traditional medicinal value has been confirmed. The extracts of pigeon pea leaves exhibit therapeutic effects on sickle cell anemia, plasmodiosis, and hepatic disorders.[2] Moreover, pigeon pea roots are used as a sedative, a vulnerary preparation, and so on.[3] In our previous research, we have proved that the extract of pigeon pea exhibited good antimicrobial and antioxidant activities.[4,5] The active constituent investigations of pigeon pea have revealed that there are more types of flavonoids and stilbenes.[6,7]

Cajaninstilbene acid (CSA), 3-hydroxy-4-prenyl-5-methoxystilbene-2-carboxylic acid, is one of the major stilbenes found in pigeon pea. The chemical structure of CSA is shown in Figure 1. Some pharmacological studies have revealed that CSA has hypotriglycerimic and hypoglycemic activities[8] and also showed anti-inflammatory and analgesic effects.[9] In addition, CSA has an antioxidant activity similar to that of the natural antioxidant resveratrol.[10−12] Therefore, CSA may be used as a potential natural antioxidant for the food industry and other fields. Natural antioxidants have recently gained increasing interest, because natural food ingredients are better and safer than synthetic ones.[13] However, it is difficult to obtain a large amount of CSA, because the extraction CSA from pigeon pea is limited by time and space constraints, leading to a conflict of supply and demand. Furthermore, to protect plant resources, it is necessary to find an alternative way to produce CSA.

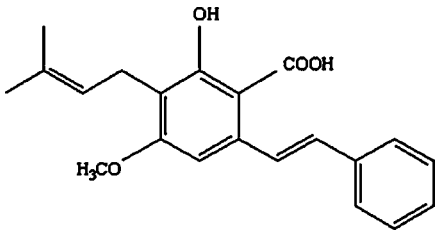

Figure 1. Chemical structure of cajaninstilbene acid.

Endophytic fungi have been found colonizing in all test plant species and have usually grown asymptomatically within the tissues of their host plants.[14] Endophytic fungi are also known as potential resources for producing secondary metabolites including antimicrobial, antifungal, antioxidant, and anti-inflammatory compounds.[15−18] Sometimes, endophytic fungi can produce the same active compounds as their host plants.[19] Thus, if large-scale fermentation of endophytic fungi isolated from pigeon pea can produce the same secondary metabolites, such as CSA, this will be an easier and more economically feasible source than plants.[20]

On the basis of this idea, this study aimed to isolate endophytic fungi from pigeon pea and screen special endophytic fungi that can produce CSA. The liquid chromatography−tandem mass spectrometry (LC-MS/MS) method was used to analyze endophytic fungi production and screen out CSA-producing endophytic fungi. The identification of endophytic fungi was carried out by phylogenetic analysis of their ITS-rDNA sequences. Furthermore, a DPPH radical-scavenging assay was conducted to evaluate the antioxidant activity of endophytic fungi CSA production.

Received: December 12, 2011
Revised: April 7, 2012
Accepted: April 12, 2012
Published: April 12, 2012

MATERIALS AND METHODS

Collection of Plant Material. Fresh roots, stems, and leaves of pigeon pea [*C. cajan* (L.) Millsp.] were collected from the botanical garden of the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, Harbin, China, and authenticated by Professor Shao-Quan Nie from the same laboratory. Voucher specimens (no. 052056001001001) were deposited in the herbarium of this Key Laboratory.

Isolation and Culture of Endophytic Fungi. Fresh roots, stems, and leaves of pigeon pea were surface sterilized with 75% ethanol for 3 min, 1 min, and 30 s, respectively, followed by treatment in 5% sodium hypochlorite solution for 7, 5, and 3 min, respectively. Then they were rinsed with sterile distilled water three times and dried with sterile filter paper. Small pieces of tissues were spaced onto Petri dishes (9 cm diameter) containing potato dextrose agar (PDA) medium. Surface sterilization of uncut plant tissues served as effectiveness control. Petri dishes were incubated at 28 ± 2 °C and monitored daily to check the growth of endophytic fungi colonies from the segments. After 7−14 days, the growth of endophytic fungi was observed from the fragments. Hyphal tips were isolated and brought into pure culture. Strains were stored presently on PDA slants at 4 °C.

Fermentation and Preparation of Crude Extract. All endophytic fungi isolated from pigeon pea were inoculated into 500 mL Erlenmeyer flasks containing 200 mL of potato dextrose liquid medium, respectively. The flasks were placed on an incubatory shaker at 120 rpm and 28 °C for 7 days. The mycelia and broth were treated separately. The mycelia were dried in an oven to obtain the dry weight and were resuspended in deionized water. This suspension was sonicated in an ultrasonicator and then was extracted three times with ethyl acetate (EtOAc). Evaporation of the organic solvent obtained the dry organic extract. The culture filtrate was extracted in a similar way. The final dry organic extract was dissolved in HPLC grade methanol for further analysis.

Preparation of Standard and Sample Solutions. A stock solution of reference sample CSA was prepared in methanol at a final concentration of 100 μg/mL. Solutions were all stored at 4 °C. Calibration standards were prepared by spiking working standard solutions and the isoliquiritigenin (ISL) (50 μL, 400 ng/mL in methanol) into 100 μL of pure methanol. The final concentrations of the standard curve samples were 10, 20, 50, 100, 200, 500, 1000, 3000, and 6000 ng/mL. Standard calibration samples were stored at −20 °C until analysis.

The ethyl acetate extract solutions (100 μL, 1 mg/mL in methanol) were added into 60 μL of pure methanol and the internal standard (ISL, 40 μL, 2 μg/mL in methanol). These solutions were filtered through a 0.22 μm membrane before injection into LC-MS/MS for analysis.

Quantitative Analysis of CSA. The chromatographic analysis used an Agilent 1100 series HPLC system (Agilent Technologies, San Jose, CA, USA), consisting of a G1312A binary pump, a 7725i manual injector, and a G1379A degasser. Chromatographic separations were conducted on a HIQ Sil C18 column (250 mm × 4.6 mm, KYA TEACH, made in Japan). The column effluent was monitored by an API3000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Concord, Canada) equipped with an electrospray ionization (ESI) source.

The determination of CSA performed using LC-MS/MS method was described by Hua et al.[21] The mobile phase consisted of water and methanol (9:91, v/v) containing 0.1% formic acid. The flow rate was 1.0 mL/min, and the sample injection volume was 10 μL.

Calibration curves were calculated on the basis of the relationship between the ratio of the peak area of CSA to that of the ISL and the concentration of analyte. The calibration curves showed good linearity in the range of 10−6000 ng/mL for CSA. The quantification of the CSA (m_{CSA}) was calculated according to the formula $m_{CSA} = (A_{CSA} \times m_{ISL})/A_{ISL}$, where A_{CSA} and A_{ISL} are the peak areas of the CSA and ISL, respectively, and m_{CSA} and m_{ISL} are the amounts of CSA and ISL, respectively. Then according to the dilution factor and sampling volume, the amount of CSA was calculated per liter of culture filtrate or per dry weight of mycelium.

DNA Extraction, PCR Amplification, and Sequencing. CSA-producing endophytic fungi were evaluated by the phylogenetic analysis of their ITS-rDNA sequences. Total DNA was extracted using a Genomic DNA Extraction Kit (Tiangen Corp., Beijing, China) according to the manufacturer's instruction. The resulting total DNA was used as a template to amplify fungal ITS-rDNA fragments using the primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′).[22] Amplifications were performed in a total reaction volume of 50 μL containing 25 μL of 2 × Taq Plus PCR Master Mix (Tiangen Corp.), 4 μL each of 10 μM primers, 2 μL of DNA template, and 15 μL of ddH_2O. PCR was carried out with an initial denaturation step of 3 min at 94 °C, 32 cycles of 30 s at 94 °C, 30 s at 53 °C, and 1 min at 72 °C, and a final extension at 72 °C for 5 min. PCR products were purified using a Gel Product Purification Kit (Tiangen Corp.). Sequencing of the PCR products was performed by the service of Sangon Engineering Technology and Service Co. Ltd. (Shanghai, China).

Molecular Phylogenetic Analysis. The ITS sequences of these endophytic fungi were compared with the data available in NCBI using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) to estimate the phylogenetic relationships of the endophytic fungi. The neighbor-joining (NJ) phylogenetic tree was constructed from evolutionary distance data by MEGA software ver. 4.0. The bootstrap was 1000 replications to assess the reliable level to the nods of the tree. The NJ tree was estimated using pairwise genetic distances based on all substitutions with the Jukes−Cantor distance parameter.[23]

Antioxidant Activity Assay. We selected the CSA strain (ERP-13) with the highest yield to evaluate the antioxidant activity. The scavenging of DPPH free radicals was determined according to the method of Wu et al.[24] with little modification. The ethyl acetate extracts from liquid culture and mycelia were combined. Before the antioxidant studies were begun, the final pooled sample was measured by LC-MS/MS. The final concentrations of fungal CSA that could be achieved were 100, 200, and 300 μg/mL and prepared at the same concentration of standard CSA. Then, 100 μL of the sample was mixed with ethanol (1.4 mL) and then added to 0.004% DPPH (1 mL) in ethanol. The mixture was vigorously shaken and then immediately placed in a UV−vis spectrophotometer (UNICO) to monitor the decrease of the absorbance at 517 nm. Monitoring was continued for 70 min until the reaction reached a plateau. Pure ethanol was used as a control sample. The percentage inhibition (Ip) of DPPH was calculated according to the formula $Ip = [(A_0 - A_1)/A_0] \times 100$, where A_0 and A_1 are the absorbance values of the blank sample and of the tested samples checked after 70 min, respectively. Samples were analyzed in triplicate.

Statistical Analyses. Results were expressed as the mean ± standard deviation (SD) ($n = 3$). The significant difference was calculated by one-way ANOVA, and values of $p < 0.05$ were considered to be significant.

RESULTS

Isolation and Culture of the Endophytic Fungi. The 110 endophytic fungi stains were isolated from roots, stems, and leaves (42, 19, and 39%, respectively). Macroscopic characteristics were described in detail according to the method of Wipornpan et al.[25] These endophytic fungi were closely related to *Hypocrea* sp., *Chaetomium* sp., *Neonectria* sp., *Colletotrichum* sp., *Alternaria* sp., *Fusarium* sp., *Aspergillus* sp., and *Exserohilum* sp., respectively. Among the endophytic fungi, four isolates were able to produce CSA (ELP-23, ERP-07, ERP-10, and ERP-13). However, the amount of CSA produced by ELP-23 was very low, so the other three strains were chosen for further study (Table 1). The morphologic and culture characteristics of these three endophytic fungi are shown in Figure 2. These three endophytic fungi were radially flat, lanose, and white in color, but after several days of culturing, the color

Table 1. Taxa of Endophytic Fungi of Pigeon Pea and Their Amounts of CSA Produced

host site	strain	taxa	CSA amount	
			mycelia (μg/g)	culture liquid (μg/L)
leaves	ELP-01	*Colletotrichum* sp.		
	ELP-03	*Exserohilum* sp.		
	ELP-06	*Neonectria* sp.		
	ELP-07	*Aspergillus* sp.		
	ELP-14	*Fusarium* sp.		
	ELP-23	*Alternaria* sp.	49.0 ± 20.6	109.5 ± 16.4
	ELP-30	*Chaetomium* sp.		
	ELP-33	*Hypocrea* sp.		
stems	ESP-01	*Fusarium* sp.		
	ESP-02	*Alternaria* sp.		
	ESP-03	*Colletotrichum* sp.		
	ESP-04	*Exserohilum* sp.		
	ESP-16	*Hypocrea* sp.		
roots	ERP-01	*Aspergillus* sp.		
	ERP-02	*Chaetomium* sp.		
	ERP-04	*Exserohilum* sp.		
	ERP-06	*Alternaria* sp.		
	ERP-07	*Fusarium* sp.	65.8 ± 10.2	325.6 ± 11.8
	ERP-08	*Colletotrichum* sp.		
	ERP-10	*Fusarium* sp.	82.6 ± 12.6	440.3 ± 15.6
	ERP-13	*Fusarium* sp.	100.5 ± 9.4	504.8 ± 20.1

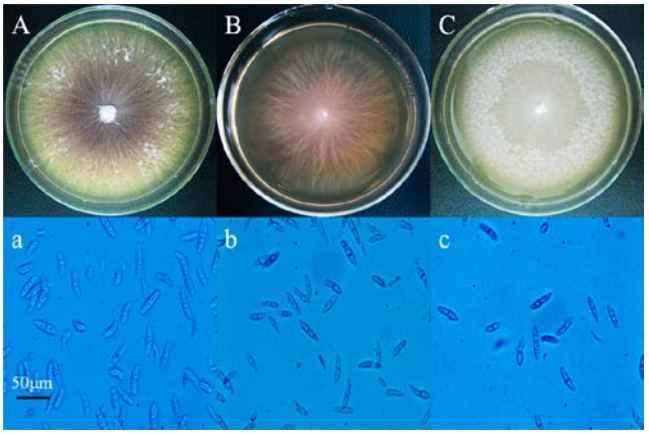

Figure 2. Colonial morphology (A–C) and micrographic characteristics (×400) (a–c) of endophytic fungi: (A) ERP-07; (B) ERP-10; (C) ERP-13.

of ERP-07 was variable from white to modena, spore shape was fusiform, and conidia were 30.0–51.2 μm × 5.0–7.5 μm. The color of ERP-10 was variable from white to deep red, and the spore shape was falcate; conidia were 19.0–50.0 μm × 2.5–5.0 μm. The color of ERP-13 was variable from white to yellow, spore shape was falcate or elliptical, and conidia were 24.2–56.1 μm × 4.8–5.0 μm. According to the morphological analysis, these three endophytic fungi were revealed to be closest to *Fusarium* sp. To establish the de novo production of CSA by the isolated fungi ERP-07, ERP-10, and ERP-13, the growing mycelium was serially transferred several times to fresh PDA to eliminate any contaminant possibility to the fungal hyphae by the host plant material.

Molecular Phylogenetics. The endophytic fungi were identified by sequencing the internal transcribed spacers (ITS) of the rDNA region. The results of the BLAST searches and their original sampling site are listed in Table 2. The length of the amplified rDNA fragment ranged from 500 to 600 bp. The ITS-rDNA sequences of ERP-07, ERP-10, and ERP-13 were most closely related to *Fusarium solani*, *Fusarium oxysporum*, and *Fusarium proliferatum*, respectively (Figure 3). Sequence

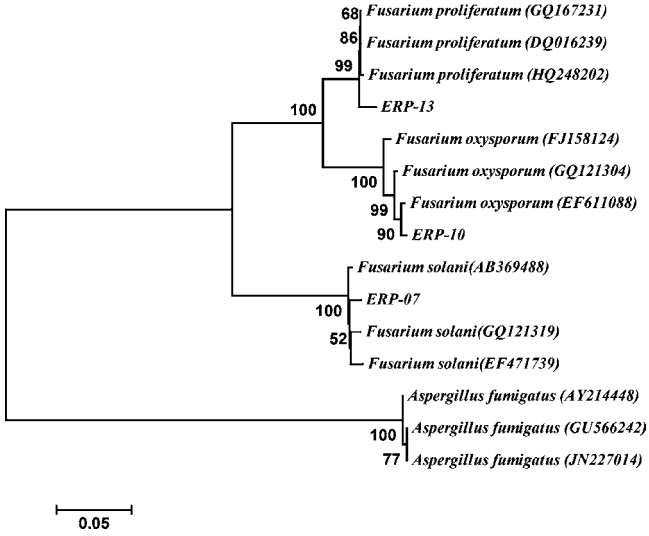

Figure 3. Phylogenetic tree constructed by the program neighbor-joining (NJ) based on ITS1-5.8S-ITS2 sequences of endophytic fungi. Bootstrap values (1000 tree interactions) are indicated at the nodes.

data have been submitted to Genbank under accession numbers JN222393 (ERP-07), JN222394 (ERP-10), and JN634960 (ERP-13).

Quantitative Analysis of CSA. Quantitative determination of CSA was done by accurate LC-MS/MS on the basis of fragmentation pathways, and the detection of CSA used mass spectrometry (MS). The extracts and standard CSA showed the same LC-MS/MS retention time for CSA (Figure 4A); also the isomers of CSA were detected. The MS spectra of the fungal extracts and standard CSA are shown in Figure 4B. The standard CSA yielded M − H^- at m/z 337.0, and the fungal extracts yielded the same peak. MS/MS of M − H^- at m/z 337.0 from fungal extracts was the same as that of standard CSA (Figure 4C), confirming that endophytic fungi can

Table 2. ERP Sequences and Their Closest Matches to Genbank Sequences

isolate	accession no. (present no.)	Genbank closest match	origin of closest match	accession no. (Genbank database)	sequence coverage (%)	max identity (%)	*E* value
ERP-07	JN222393	*Fusarium solani*	India	GQ121319.1	100	100	0
ERP-10	JN222394	*Fusarium oxysporum*	China	EF611088.1	100	100	0
ERP-13	JN634960	*Fusarium proliferatum*	Colombia	HQ248202.1	97	100	0

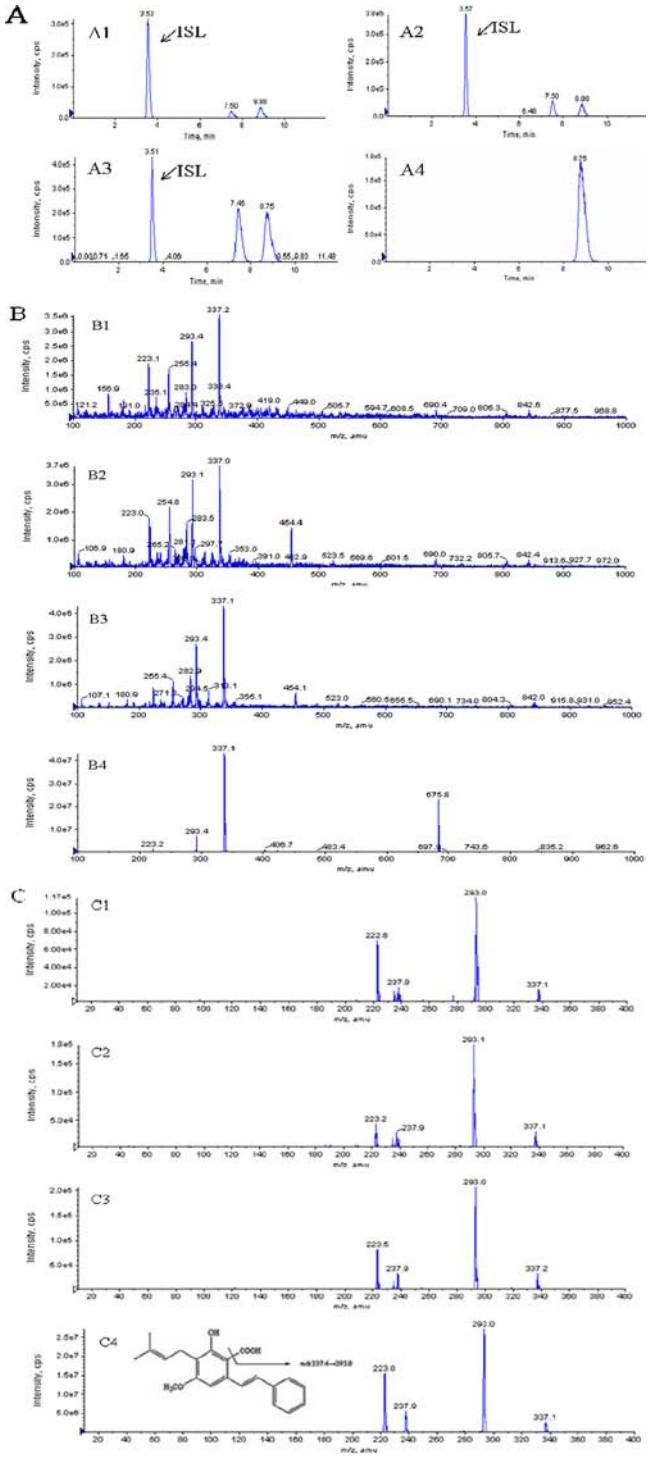

Figure 4. (A) Representative LC-MS/MS chromatograms of ethyl acetate extracts of endophytic fungi fermentation (ERP-07 (A1), ERP-10 (A2), ERP-13 (A3), and reference CSA (A4)). Isoliquiritigenin (ISL) was used as internal standard. (B) Mass spectrometry analysis of the fungal CSA of ERP-07 (B1), ERP-10 (B2), ERP-13 (B3), and reference CSA (B4). The diagnostic mass spectral fragment ion was at m/z 337.1 $(M - H)^-$. (C) MS/MS analysis of the fungal CSA of ERP-07 (C1), ERP-10 (C2), ERP-13 (C3), and reference CSA (C4). The MS/MS of CSA was characterized by transition at m/z 337.1 → 293.0.

produce CSA. Although the majority of CSA-producing endophytic fungi were closely related to *Fusarium* sp., these three strains produced different amounts of CSA. Among these, ERP-13 produced the maximum amount of CSA, which was in the range of 504.8 ± 20.1 μg/L of culture filtrate or 100.5 ± 9.4 μg/g dry weight of mycelium, respectively (Table 1). With longer times, CSA can still be detected, indicating that the screened endophytic fungi could produce CSA (unpublished data). Therefore, this study unequivocally established de novo production of CSA by the endophytic fungi.

Assay of Antioxidant Activity. The antioxidant activity of ethyl acetate extracts from ERP-13 was determined in vitro using the DPPH radical-scavenging assay. The DPPH method is based on the reduction of DPPH in the presence of a hydrogen-donating antioxidant due to the formation of the nonradical form DPPH-H by the reaction.[26] The antioxidant activity of CSA has recently been reported.[4] Here, we have extended the study to understand the antioxidant efficacy of fungal CSA (from the endophytic fungal source) in comparison to standard CSA. The result of LC-MS/MS analysis showed that endophytic fungi can produce CSA. Furthermore, the structure of the fungal CSA was the same as the standard CSA. Therefore, we speculated that the fungal CSA should have an antioxidant activity similar to that of standard CSA. We further performed activity experiments to verify this speculation. Fungal CSA could be extracted and used instead of plant CSA from natural plant resources, on the basis of the hypothesis that the purpose of plant resource protection could be achieved. The obtained results are presented in Figure 5. Free radical-scavenging activity of fungal CSA (as mentioned

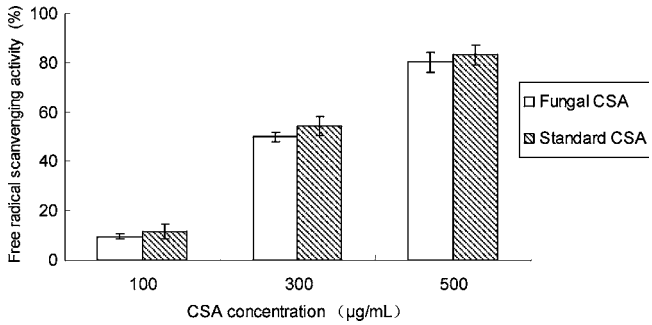

Figure 5. Antioxidant activities of fungal CSA in comparison to standard CSA by DPPH radical-scavenging assay. Values are the mean ± SDs (n = 3); $p < 0.05$.

mentioned in Antioxidant Activity Assay), at a concentration of 500 μg/mL, inhibition percentage was arrived 80%. It was slightly lower than standard CSA (83%), the reason may be other compounds in the fungal extracts had antagonistic effect to CSA antioxidant activity.

DISCUSSION

The morphologies of the three CSA-producing endophytic fungi were most closely related to *Fusarium* sp. Phylogenetic analysis of ITS gene sequences supported the identifications of ERP-07, ERP-10, and ERP-13 as *F. solani*, *F. oxysporum*, and *F. proliferatum*, respectively. As the ITS region is generally considered to be less conserved than either small or large subunits of rRNA genes,[27] the ITS analysis may be more informative in distinguishing closely related species. The genus *Fusarium* is well-known to produce a wide range of secondary metabolites including mycotoxins, organic acids, and pigments.[28] In our study, endophytic fungi produced CSA,

dx.doi.org/10.1021/jf205097y | J. Agric. Food Chem. 2012, 60, 4314–4319

which may act as a biologically active constituent to help its host plant resist invasions of fungal pathogens and pests.

More literature surveys demonstrated that therapeutic compounds could be produced by endophytic micro-organisms. An endophytic strain of *F. oxysporum* produced podophyllotoxin from *Juniperus recurva*,[29] and *Cryptosporiopsis* cf. *quercina* was isolated and characterized as an endophyte from *Tripterigeum wilfordii*. This endophyte could produce a unique peptide antimycotic, termed cryptocandin.[30] A taxol-producing endophytic fungus, *Taxomyces andreanae*, was discovered in *Taxus brevifolia*.[31] It is interesting to note that endophytic fungi associated with pigeon pea can accumulate the same metabolite which is also found in the host. We inferred that the CSA detected in plants may be due to fungal biosynthesis, but there is also a possibility that CSA can be produced by the host plant and the endophytic fungi together. The CSA-producing fungi were isolated from pigeon pea root. However, in the host plant, CSA production in the leaves was significantly higher than that in the roots. An explanation of this phenomenon is that metabolic regulation of endophytic fungi in the host plant is likely to be substantially different from that which occurs in axenic culture.[20,32] We infer that the presence of CSA was affected by the environment. CSA is relatively stable under acid environment but can be easily transferred to other structures in an alkaline environment.[33] The roots of pigeon pea have nitrogen fixation and showed an alkaline environment; hence, CSA presented lower level in roots.[33] Endophytic fungi are conducive to the synthesis of CSA in culture medium having adequate nutrition, appropriate temperature, and stable pH value.

From the genetic standpoint, the production of bioactive metabolites such as CSA by endophytic fungi increasingly lends support to the possibility of horizontal gene transfer between pigeon pea and its corresponding endophytic organisms. The theory is that, in evolutionary time, endophytic fungi have adapted to their special microenvironments by genetic variation, including uptake of some plant DNA into their own genomes, which has been proposed by Germaine et al.[34] This could lead to the ability of certain endophytic fungi to biosynthesize some phytochemicals that are characteristic of the host plants.[35] These gene transfer mechanisms have already been proposed in endophytic fungi isolated from other plant species, such as *N. fetida*, *C. acuminata*, and others.[36] Further studies of endophytic fungi producing CSA should provide insights into the genomic and metabolomic studies between pigeon pea and its endophytic fungi.

We also found that fungal CSA showed strong antioxidant activity. This can be attributed to the characteristics of its structure. (i) All stilbene compounds reveal antioxidant properties due to the conjugation between rings A and B via a planar C2 unsaturated structure, which allows an electron delocalization across the molecules for stabilization of the radical.[37] (ii) The presence of a 5-hydroxyl group in the A ring also may boost the antioxidant activity.[38] (iii) There are some reports that methoxy groups at the 3-position can increase the antioxidant activity of stilbene compounds.[12,39,40] Therefore, CSA from endophytic fungi ethyl acetate extracts may be developed as a novel natural antioxidant.

AUTHOR INFORMATION

Corresponding Author

*Phone: +86-451-82190535. Fax: +86-451-82190535. E-mail: yujie_fu2002@yahoo.com.

Funding

We gratefully acknowledge the financial supports by the Program for Importation of International Advanced Forestry Science and Technology, National Forestry Bureau (2012-4-06), Special Fund of Forestry Industrial Research for Public Welfare of China (201004040), Heilongjiang Province Science Foundation for Excellent Youths (JC200704), Project for Distinguished Teacher Abroad, Chinese Ministry of Education (MS2010DBLY031), and NEFU Foundation for Excellent Master's Degree Project (STIP10).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge Prof. Thomas Efferth for critically reviewing the manuscript.

REFERENCES

(1) Misra, A.; Kumar, R.; Mishra, V.; Chaudhari, B. P.; Tripathi, A.; Das, M.; Dwivedi, P. D. Partial characterization of red gram (*Cajanus cajan* L. Millsp) polypeptides recognized by patients exhibiting rhinitis and bronchial asthma. *Food Chem. Toxicol.* **2010**, *48*, 2725−2736.

(2) Liu, W.; Fu, Y. J.; Zu, Y. G.; Kong, Y.; Zhang, L.; Zu, B. S.; Efferth, T. Negative-pressure cavitation extraction for the determination of flavonoids in pigeon pea leaves by liquid chromatography−tandem mass spectrometry. *J. Chromatogr., A* **2009**, *1216*, 3841−3850.

(3) Zhang, D. Y.; Zhang, S.; Zu, Y. G.; Fu, Y. J.; Kong, Y.; Gao, Y.; Zhao, J. T.; Efferth, T. Negative pressure cavitation extraction and antioxidant activity of genistein and genistin from the roots of pigeon pea [*Cajanus cajan* (L.) Millsp.]. *Sep. Purif. Technol.* **2010**, *74*, 261−270.

(4) Wu, N.; Fu, K.; Fu, Y. J.; Zu, Y. G.; Chang, F. R.; Chen, Y. H.; Liu, X. L.; Kong, Y.; Liu, W.; Gu, C. B. Antioxidant activities of extracts and main components of pigeonpea [*Cajanus cajan* (L.) Millsp.] leaves. *Molecules* **2009**, *14*, 1032−1043.

(5) Zu, Y. G.; Liu, X. L.; Fu, Y. J.; Wu, N.; Kong, Y.; Wink, M. Chemical composition of the SFE-CO_2 extracts from *Cajanus cajan* (L.) Huth and their antimicrobial activity *in vitro* and *in vivo*. *Phytomedicine* **2010**, *17*, 1095−1101.

(6) Duker-Eshun, G.; Jaroszewski, J. W.; Asomaning, W. A.; Oppong-Boachie, F.; Christensen, B. S. Antiplasmodial constituents of *Cajanus cajan*. *Phytother. Res.* **2004**, *18* (2), 128−130.

(7) Kong, Y.; Fu, Y. J.; Zu, Y. G.; Chang, F. R.; Chen, Y. H.; Liu, X. L.; Stelten, J.; Schiebel, H. M. Cajanuslactone, a new coumarin with anti-bacterial activity from pigeon pea [*Cajanus cajan* (L.) Millsp.] leaves. *Food Chem.* **2010**, *121*, 1150−1155.

(8) Khadem, S.; Marles, R. J. Monocyclic phenolic acids; hydroxy- and polyhydroxybenzoic acids: occurrence and recent bioactivity studies. *Molecules* **2010**, *15*, 7985−8005.

(9) Zheng, Y. Y.; Yang, J.; Chen, D. H.; Sun, L. Effects of the stilbene extracts from *Cajanus cajan* L. on ovariectomy-induced bone loss in rats. *Acta. Pharm. Sin.* **2007**, *42*, 562−565.

(10) Sen, S.; Chakraborty, R.; Sridhar, C.; Reddy, Y. S. R.; De, B. Free radicals, antioxidants, diseases and phytomedicines: current status and future prospect. *Int. J. Pharm. Sci. Rev. Res.* **2010**, *3*, 91−100.

(11) Kong, Y.; Fu, Y. J.; Zu, Y. G.; Liu, W.; Wang, W.; Hua, X.; Yang, M. Ethanol modified supercritical fluid extraction and antioxidant activity of cajaninstilbene acid and pinostrobin from pigeonpea [*Cajanus cajan* (L.) Millsp.] leaves. *Food Chem.* **2009**, *117*, 152−159.

(12) Wu, N.; Kong, Y.; Fu, Y. J.; Zu, Y. G.; Yang, Z. W.; Yang, M.; Peng, X.; Efferth, T. *In vitro* antioxidant properties, DNA damage protective activity, and xanthine oxidase inhibitory effect of cajaninstilbene acid, a stilbene compound derived from pigeon pea [*Cajanus cajan* (L.) Millsp.] leaves. *J. Agric. Food Chem.* **2011**, *59*, 437−443.

(13) Yanishlieva, N. V.; Marinova, E. M. Stabilisation of edible oils with natural antioxidants. *Eur. J. Lipid Sci. Technol.* **2001**, *103*, 752−767.
(14) Maciá-Vicente, J. G.; Jansson, H. B.; Abdullah, S. K.; Descals, E.; Salinas, J.; Lopez-Llorca, L. V. Fungal root endophytes from natural vegetation in Mediterranean environments with special reference to *Fusarium* spp. *FEMS Microbiol. Ecol.* **2008**, *64*, 90−105.
(15) Silva, G. H.; Teles, H. L.; Zanardi, L. M.; Young, M. C. M.; Eberlin, M. N.; Hadad, R.; Pfenning, L. H.; Costa-Neto, C. M.; Castro-Gamboa, I.; Bolzani, V. S.; Araújo, Â. R. Cadinane sesquiterpenoids of *Phomopsis cassiae*, an endophytic fungus associated with *Cassia spectabilis* (Leguminosae). *Phytochemistry* **2006**, *67*, 1964−1969.
(16) Huang, W. Y.; Cai, Y. Z.; Xing, J.; Corke, H.; Sun, M. A potential antioxidant resource: endophytic fungi from medicinal plants. *Econ. Bot.* **2007**, *61* (1), 14−30.
(17) Aly, A. H.; Edrada-Ebel, R. A.; Wray, V.; Müller, W. E. G.; Kozytska, S.; Hentschel, U.; Proksch, P.; Ebel, R. Bioactive metabolites from the endophytic fungus *Ampelomyces* sp. isolated from the medicinal plant *Urospermum picroides*. *Phytochemistry* **2008**, *69*, 1716−1725.
(18) Huang, Y. F.; Zhao, J. L.; Zhou, L. G.; Wang, M. A.; Wang, J. G.; Li, X. L.; Chen, Q. Antimicrobial compounds from the endophytic fungus *Fusarium* sp. Ppf4 isolated from the medicinal plant *Paris polyphylla* var. *yunnanensis*. *Nat. Prod. Commun.* **2009**, *4*, 1455−1458.
(19) Priti, V.; Ramesha, B. T.; Singh, S.; Ravikanth, G.; Ganeshaiah, K. N.; Suryanarayanan, T. S.; Shaanker, R. U. How promising are endophytic fungi as alternative sources of plant secondary metabolites? *Curr. Sci. India* **2009**, *97*, 477−478.
(20) Strobel, G.; Daisy, B.; Castillo, U.; Harper, J. Natural products from endophytic microoranganisms. *J. Nat. Prod.* **2004**, *67*, 257−268.
(21) Hua, X.; Fu, Y. J.; Zu, Y. G.; Wu, N.; Kong, Y.; Li, J.; Peng, X.; Efferth, T. Plasma pharmacokinetics and tissue distribution study of cajaninstilbene acid in rats by liquid chromatography with tandem mass spectrometry. *J. Pharm. Biomed.* **2010**, *52*, 273−279.
(22) White, T. J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Application*; Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp 315−22.
(23) Li, Q. Z.; Wang, G. Y. Diversity of fungal isolates from three Hawaiian marine sponges. *Microbiol. Res.* **2009**, *164*, 233−241.
(24) Wu, N.; Zu, Y. G.; Fu, Y. J.; Kong, Y.; Zhao, J. T.; Li, X. J.; Li, J.; Wink, M.; Efferth, T. Antioxidant activities and xanthine oxidase inhibitory effects of extracts and main polyphenolic compounds obtained from *Geranium sibiricum* L. *J. Agric. Food Chem.* **2010**, *58*, 4737−4743.
(25) Wipornpan, P.; Paul, W. J. T.; Rebecca, F.; Kevin, D. H.; Saisamorn, L. Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. *Fungal Divers.* **2005**, *18*, 117−133.
(26) Shon, M. Y.; Kim, T. H.; Sung, N. J. Antioxidants and free radical scavenging activity of *Phellinus baumii* (*Phellinus of Hymenochaetaceae*) extracts. *Food Chem.* **2003**, *82*, 593−597.
(27) Scorzetti, G.; Fell, J. W.; Fonseca, A.; Statzell-Tallman, A. Systematics of Basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Res.* **2002**, *2*, 495−517.
(28) Estrada, A. E. R.; Hegeman, A.; Kistler, H. C.; May, G. *In vitro* interactions between *Fusarium verticillioides* and *Ustilago maydis* through real-time PCR and metabolic profiling. *Fungal Genet. Biol.* **2011**, *48*, 874−885.
(29) Kour, A.; Shawl, A. S.; Rehman, S.; Sultan, P.; Qazi, P. H.; Suden, P.; Khajuria, R. K.; Verma, V. Isolation and identification of an endophytic strain of *Fusarium oxysporum* producing podophyllotoxin from *Juniperus recurva World*. *J. Microb. Biotechnol.* **2008**, *24*, 1115−1121.
(30) Strobel, G. A.; Miller, R. V.; Miller, C.; Condron, M.; Teplow, D. B.; Hess, W. M. Cryptocandin, a potent antimycotic from the endophytic fungus *Cryptosporiopsis cf. quercina*. *Microbiology* **1999**, *145*, 1919−1926.
(31) Strobel, G. A.; Stierle, A.; Stierle, D.; Hess, W. M. *Taxomyces andreanae* a proposed new taxon for a bulbilliferous hyphomycete associated with Pacific yew (*Taxus brevifolia*). *Mycotaxon* **1993**, *47*, 71−80.
(32) Hardoim, P. R.; Overbeek, L. S.; Elsas, J. D. Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* **2008**, *16*, 463−471.
(33) Wang, H.; Liu, L.; Guo, Y. X.; Dong, Y. S.; Zhang, D. J.; Xiu, Z. L. Biotransformation of piceid in *Polygonum cuspidatum* to resveratrol by *Aspergillus oryzae*. *Appl. Microbiol. Biot.* **2007**, *75*, 763−768.
(34) Germaine, K.; Keogh, E.; Garcia-Cabellos, G.; Borremans, B.; Lelie, D.; Barac, T.; Oeyen, L.; Vangronsveld, J.; Moore, F. P.; Moore, E. R. B.; Campbell, C. D.; Ryan, D.; Dowling, D. N. Colonisation of poplar trees by gfp expressing bacterial endophytes. *FEMS Microbiol. Ecol.* **2004**, *48*, 109−118.
(35) Aly, A. H.; Debbab, A.; Proksch, P. Fungal endophytes: unique plant inhabitants with great promises. *Appl. Microbiol. Biot.* **2011**, *90*, 1829−1845.
(36) Kusari, S.; Lamshöft, M.; Spiteller, M. *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* L. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. *J. Appl. Microbiol.* **2009**, *107*, 1019−1030.
(37) Teguo, P. W.; Fauconneau, B.; Deffieux, G.; Huguet, F.; Vercauteren, J.; Merillon, J. M. Isolation, identification, and antioxidant activity of three stilbene glucosides newly extracted from *Vitis vinifera* cell cultures. *J. Nat. Prod.* **1998**, *61*, 655−657.
(38) Queiroz, A. N.; Gomes, B. A. Q.; Moraes, W. M., Jr.; Borges, R. S. A theoretical antioxidant pharmacophore for resveratrol. *Eur. J. Med. Chem.* **2009**, *44*, 1644−1649.
(39) Hasiah, A. H.; Ghazali, A. R.; Weber, J. E. F.; Velu, S.; Thomas, N. F.; Inayat Hussain, S. H. Cytotoxic and antioxidant effects of methoxylated stilbene analogues on HepG2 hepatoma and Chang liver cells: implications for structure activity relationship. *Hum. Exp. Toxicol.* **2010**, *30* (2), 138−144.
(40) Wilson, M. A.; Rimando, A. M.; Wolkow, C. A. Methoxylation enhances stilbene bioactivity in *Caenorhabditis elegans*. *BMC Pharmacol.* **2008**, *8*, 15−26.