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Piperine production by endophytic fungus *Colletotrichum gloeosporioides* isolated from *Piper nigrum*

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ABSTRACT

Many endophytic fungi have been reported with the biosynthetic potential to produce same or similar metabolites present in host plants. The adaptations that might have acquired by these fungi as a result of the long-term association with their host plants can be the possible basis of their biosynthetic potential. The bioactive compounds originated from endophytes are currently explored for their potential applications in pharmaceutical, agriculture and food industries. *Piper nigrum*, a plant of the Piperaceae is very remarkable because of the presence of the alkaloid piperine. Piperine has been reported to have broad bioactive properties ranging from antimicrobial, antidepressant, anti-inflammatory, antioxidative to anticancer activities. Interestingly, piperine also plays a vital role in increasing the bioavailability of many drugs which again is a promising property. The current study was carried out to identify piperine producing endophytic fungus from *Piper nigrum* L. By screening various endophytic fungi, the isolate which was identified as member of *Colletotrichum gloeosporioides* was found to have the ability to form piperine and was confirmed by HPLC and LCMS. Considering the broad bioactive potential of piperine, the piperine producing fungi identified in the study can expect to have much industrial potential.

Introduction

Endophytes are microbes ubiquitously present inside the plant and they occupy either the localized point of entry or spread throughout the plant. They have a very rich biodiversity and unexplored biosynthetic potential to produce an array of bioactive metabolites (Jasim et al. 2013). The metabolite profile of endophytes is greatly influenced by the unique chemical environment of host plant. Some of these endophytes have been reported to possess the biochemical make-up for synthesis of plant specific compounds. So presence of endophytes with the ability to synthesize plant specific metabolite is a significant indication of host endophyte interaction (Verma et al. 2011). As a medicinal plant with various bioactive compounds, many interesting endophytic fungi can be expected from *Piper nigrum*.

The fungal endophytes are considered as a promising source for novel and bioactive metabolites with great pharmaceutical potential (Aly et al. 2010). Paclitaxel producing endophytic fungus *Taxomyces andreanae* isolated from Pacific yew has attracted much scientific interest on exploration of endophytes (Stierle et al. 1993). Camptothecin has also reported to be produced by endophytic fungus *Entrophospora infrequens* obtained from *Nothapodytes foetida* (Puri et al., 2005). Also *Alternaria* sp. isolated from *Sabina vulgaris* has proved to have the ability to produce potent bioactive compound Podophyllotoxin (Yang et al. 2003). Advantages with the identification of fungal biosynthesis of these compounds are their easiness with large-scale production using low cost culture methods. All these suggest the immense biosynthetic potential of endophytic fungi to produce structurally diverse chemical scaffolds with both commercial and pharmaceutical importance.

Piper nigrum L. (black pepper) is the one of the most important spices with pungent pharmaceutical activities. Due to the presence of unique metabolites, black pepper is used as an important ingredient in many traditional medical formulations. The phytochemical studies have demonstrated the components of *Piper nigrum* as Piperine, Piperamine, Sarmentosine, Sarmentine, Trichostachine, etc. (Kiuchi et al. 1988). Among these, the principal metabolite is piperine which is mainly responsible for the spiciness of the pepper. Piperine is present mainly in the fruits of black pepper. It was first discovered by Hans Christian Orsted in 1819 (Orsted, 1820). Piperine consists of a methylenedioxyphenyl (MDP) ring, side chain with conjugated double bonds and a basic piperidine moiety attached to the side chain through a carbonylamide linkage.

Piperine is also reported to have a wide pharmaceutical properties including antibacterial, antifungal, hepato-protective, antipyretic, anti-inflammatory, anticonvulsant, insecticidal (Parmar et al. 1997; Mittal and Gupta 2000), antioxidant (Khajuria

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et al. 1997), antidepressant (Lee et al. 2005), immuno-modulatory, and anti-tumor effects (Sunila and Kuttan 2004). Piperine has also been reported to have stimulatory effect on secretion of digestive enzymes of pancreas and intestines and also biliary bile acid secretion. Because of these array of properties, piperine has been used as active ingredient in most of the traditional medicines and also as flavoring and preservative agent in food preparations (Ahmad et al. 2012).

So by identifying endophytes with potential to form piperine, it can be exploited in various ways including the possibility to generate structurally related compounds by modulating the culture conditions. In the current study, an endophytic fungus *Colletotrichum gloeosporioides* with the potential to produce piperine was isolated and characterized from *Piper nigrum*. This fungus can be exploited for the large scale production of piperine by scaling up the culture conditions.

Materials and methods

Endophytic fungal isolation

Endophytic fungi were isolated from the healthy and mature stem pieces of *Piper nigrum* collected from a local farm. Surface sterilization of the plant material was carried out following the protocol described by Aravind et al. (2009) with slight modifications. Plant samples were washed under running tap water for 10 min followed by immersion in NaOCl (2.5% available chlorine) for 10 min. Then it was further treated with 70% EtOH for 1 min. Finally, the samples were rinsed with sterile distilled water for several times and the final wash was spread plated on arginine glycerol agar (composition (g/L): glycerol, 20; L-arginine, 2.5; NaCl, 1; $CaCO_3$, 0.1; $FeSO_4{\cdot}7H_2O$, 0.1; $MgSO_4{\cdot}7H_2O$, 0.1; agar, 20) as control. Plant samples were then cut aseptically into 1 cm long segments. Then the segments were placed on arginine glycerol agar with 50 μg/ml nalidixic acid. The plates were incubated at 28 °C for 5 days and examined for fungal growth. The fungal isolates obtained were further purified and maintained on PDA (composition (g/L): potato infusion 200; dextrose 20; agar 20) medium.

Screening for the production of piperine using HPLC

For the screening of piperine production the methods described by Verma et al. (2011) was used with some modifications. Initially, the isolated endophytic fungi were inoculated into 200 mL of potato dextrose broth and incubated in a shaker at 30 ± 2 °C for 30 days. After incubation, fermentation broth was filtered using cheese cloth and filter paper to remove the fungal biomass. The culture filtrate was extracted twice with equal volume of ethyl acetate and the organic phase was concentrated by using a rotary evaporator at 50 °C under vacuum to obtain the crude extract powder. The crude extract thus obtained was resuspended in methanol and was analyzed by Agilent Technologies 1290 infinity HPLC system using C18 column (150 mm × 2.00 mm × 5 μm). Mobile phase with a mixture of methanol:water (70:30) was delivered at a flow rate of 0.3 mL min^{-1} with detection at a wavelength of 344 nm and the chromatogram was recorded. Presence of piperine was checked by comparing the peaks in the samples with the respective peaks for standard piperine at the comparable retention time, for which the method described by Shailendra Singh et al. (2012) was used.

Morphological identification of the isolate

For studying the morphological characters, the slide culture of the fungus was prepared and stained using lactophenol cotton blue. For this, the potato dextrose agar blocks (7 × 7 mm) was aseptically transferred on a pre-sterilized glass slide placed inside a sterile petridish. The block was inoculated with the fungal isolate. A cover slip was placed on the top of the agar block and was incubated for 5 days at 30 ± 2 °C. After incubation the coverslip was retrieved and placed on a drop of lactophenol cotton blue stain (Madavasamy and Panneerselvam 2012). This was then observed under bright field microscope (Olympus, Model BX43) with 100× oil immersion objective and the images are processed with Q imaging software. Spore morphology and vegetative structures were compared with a fungal identification manual (Hoog et al. 2000).

Molecular identification of fungal isolate

For the molecular identification of the isolate with piperine production, genomic DNA was purified using the Chromous Biotech Fungal gDNA Mini spin kit (Category number RKT41). The isolated DNA was checked by agarose gel electrophoresis and was used as template for the PCR ampification of ITS region using ITS1 (5′-TCC gTA ggT gAA CCT gCg g-3′) and ITS4 (5′-TCC TCC gCT TAT TgA TAT gC-3′) primers described by White et al. (1990). PCR was performed at a final volume of 50 μL reaction containing 50 ng of genomic DNA, 20 pmoles of each primer, 1.25 units of Taq DNA polymerase (Bangalore Genei), 200 μM of each dNTPs and 1× PCR buffer. The PCR was performed for 35 cycles in a Mycycler™ (Bio-Rad, USA) with an initial denaturation at 94 °C for 5 min, followed by cyclic denaturation at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min with a final extension at 72 °C for 7 min. The PCR product formation was confirmed by agarose gel electrophoresis and the product was further purified for its use as the template for sequencing PCR using Big Dye Terminator Sequence Reaction Ready Mix (Applied Biosystem). The sequence data of ITS fragment thus obtained was further subjected to BLAST analysis. For the phylogenetic analysis, the related sequences were retrieved from NCBI. The selected sequences were first aligned with Clustal W. The aligned data was used for further phylogenetic analysis with Neighbor joining method using MEGA5 with 1000 bootstrap replicates (Tamura et al. 2011).

Large scale fermentation and extraction of piperine

The fungal culture which was positive for piperine production in HPLC analysis was transferred aseptically to 1 L potato dextrose broth and was incubated in a shaker (110 rpm) at room temperature for 30 days. After incubation, fermentation product was extracted as explained earlier. The crude extract thus prepared was resuspended in methanol for further analysis.

Confirmation of piperine production by using LCMS analysis

In order to confirm the presence of piperine in the crude extract, it was subjected to LCMS analysis using Agilent Technologies 1290 infinity UHPLC system with Phenomenex-Luna C18 column (150 mm × 2.00 mm × 5 μm) and an inline Agilent 6460 QQQ (MS/MS) detector. The chromatographic condition was same as that used for HPLC analysis. The electron ionization was used in positive mode to produce mass spectra with a scan range from 100 to 300 and the scan time was 20 min. Nitrogen was used as drying gas at a flow rate of 7 L min^{-1}.

Results and discussion

Isolation of endophytic fungi

Endophytic fungi have been reported to have the well equipped biosynthetic potential to produce an array of bioactive compounds including plant specific compounds. So the identification