Bacopaside N1 biosynthetic potential of endophytic *Aspergillus* sp. BmF 16 isolated from *Bacopa monnieri*

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Abstract Chemically unique environment of endophytes makes them to have various adaptive mechanisms for survival. One of such mechanisms involves the production of pharmacologically significant plant-specific metabolites. In the present study, 26 endophytic fungi were isolated from stem of *Bacopa monnieri* (L.) Wettst. plants. All the isolates were screened for bacopaside production property by HPLC. Among these, the fungal isolate BmF 16 which was identified as *Aspergillus* sp. was confirmed for bacopaside N1 production (m/z 796) by LC–MS/MS analysis. As the extract of BmF16 used in the study was prepared from the fifth generation of culture, the obtained result can be confirmed as due to fungal production of bacopaside. In addition, this property was identified only for one among the 26 fungi

screened. As bacopaside N1 production in fungi has not yet been reported, the results of the study are novel.

Keywords *Bacopa monnieri* (L.) Wettst. · *Aspergillus* sp. · Bacopaside N1 · LC–MS/MS analysis · Plant secondary metabolite

Introduction

Chemical ecology provided by the host plant is considered to have tremendous impact on biosynthetic potential of endophytic microorganisms. This has been proved by increasing reports on production of plant-specific metabolites by endophytes (Jasim et al. 2013). Compounds that have been identified to be produced by plant and associated fungi include azadirachtin production by endophytic *Eu- penicillium parvum* from *Azadirachta indica*; cajanol by endophytic *Hypocrea lixii* from *Cajanus cajan*; hypericin from fungus endophytically associated with *Hypericum perforatum*, etc. (Kusari et al. 2008, 2012; Zhao et al. 2013). In addition, a huge number of endophytic fungi have been characterized for the production of taxol, camptothecin, and podophyllotoxin (Stierle et al. 1993; Puri et al. 2005; Eyberger et al. 2006). These demonstrate the significance of studies on fungi associated with medicinal plants for drug discovery and development.

Bacopa monnieri (L.) Wettst. (Brahmi) is an important medicinal herb used in the traditional medicinal preparations to treat brain-related diseases and to enhance memory and learning. The bioactivity of *B. monnieri* is due to the presence of high concentration of saponins bacoside A and bacoside B (Deepak and Amit 2004). The compounds coming under bacoside A include bacoside A3, bacopaside II, jujubogenin, and bacopa-saponin C. However, in the

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case of bacopside B, it consists of bacopaside N1, bacopaside N2, bacopaside IV, and bacopaside V (Pareek and Kumar 2014). Due to the presence of a large number of bioactive metabolites, *B. monnieri* is having significant applications in diverse clinical conditions (Aguiar and Borowski 2013). The array of metabolites also makes *B. monnieri* to be a potent source for investigating the presence of fungal endophytes capable of synthesizing host plant-specific metabolites.

As the in vitro study of endophytic fungi is conducted in an ecologically different condition, identification of fungi with biosynthetic basis for plant-specific compound is highly challenging. However, advances in analytical instrumentation offer methods to identify potential fungi from medicinal plants. Current study has focused on the isolation and identification of endophytic fungi from *B. monnieri*. The isolation has resulted in the purification of 26 endophytic fungi which were further screened and analyzed for the biosynthesis of bacopaside using LC–MS and MS/MS analyses. Among various isolates, one was found to have bacopaside producing potential.

Materials and methods

Isolation of endophytic fungi

The plant material (*B. monnieri*) which was used for endophytic fungal isolation was obtained from Kerala Agricultural University. The stem portions were cleaned with running tap water and surface sterilized using sodium hypochlorite (2% available chlorine) for 10 min which was followed by treatment with 70% (v/v) ethanol for 1 min (Jasim et al. 2013). The plant material was then washed five times with sterile distilled water. The efficacy of the sterilization procedure was checked by spread plating samples from the final wash onto potato dextrose agar (PDA) plate (Potato infusion form 200 gm/L, Dextrose 20 gm/L, Agar 15 gm/L, pH 5.6) which served as the control. For endophytic fungal isolation, the treated plant materials were placed on PDA medium. All the plates (including control plates) were incubated for 5–10 days and were observed periodically for microbial growth. After incubation, morphologically distinct organisms were selected, purified, and were sub-cultured for further studies.

Screening of endophytic fungi for bacopaside production by LC–MS

For screening the fungal isolates for bacopaside production, small-scale fermentation was performed in

150-mL potato dextrose broth (PDB). All the isolated fungi were inoculated into 150-mL PDB and incubated at 28 °C for a period of 30 days. After this, the broth was collected and filtered, and its pH was adjusted to 3.5. This was followed with solvent extraction using equal amount of ethyl acetate. Then, it was evaporated in a rotary evaporator and the residual powder was reconstituted in methanol. For screening of bacopaside production, all the extracted samples were subjected to HPLC analysis. From this, those with expected bacopaside production were selected for the LC–MS analysis. LC–MS/MS was carried out using the crude extract after filtering through a 0.22- μ m syringe filter (Jasim et al. 2016).

Morphological and molecular identification of BmF 16 with bacopaside production

For studying the morphological characters, lacto phenol cotton blue staining was used (Chithra et al. 2014) only for those isolate which was confirmed for bacopaside production by HPLC and LC–MS analysis. For molecular identification, the selected fungal isolate was subjected to genomic DNA isolation. This was followed by PCR amplification of ITS region (White et al. 1990). PCR was performed using the conditions described by Anisha and Radhakrishnan (2015). The ITS sequence was then aligned with similar sequences using Clustal W and the aligned data were used for phylogenetic analysis using neighbor-joining method of MEGA6 with 1000 boot strap replicates (Tamura et al. 2011; Zhang et al. 2000).

Results

Isolation of endophytic fungi

Based on distinct colony characteristics, 26 endophytic fungi have been purified from stem of *B. monnieri* and were named as BmF 1 to BmF 26. The purified isolates were sub-cultured periodically and stored for further screening and identification.

Screening for bacopaside production by LC–MS analysis

By LC–MS analysis, the extract from the isolate BmF 16 was found to have the molecular ion mass corresponding to bacopaside derivative as indicated by the presence of m/z 795.4352 ($M-H^+$) at the retention time of 3.00 (Fig. 1). More specifically, BmF 16 showed m/z 795.4352 on negative mode ionization and the mass was corresponding to

Fig. 1 Screening of the methanolic extract of isolate BmF 16 for bacopaside production. **a** LC analysis of the extract of isolate BmF 16 which shows a peak at RT 3.00. **b** Mass spectrum analysis

that of bacopaside N1 (actual mass 796). Further confirmation of bacopaside production was done by LC–MS/MS-based fragmentation analysis.

Confirmation of bacopaside production by LC–MS/MS

When the extract of BmF 16 was subjected to LC–MS/MS-based fragmentation analysis, it showed the presence of both molecular ion mass and the fragmentation masses specific to bacopaside N1. The detailed analysis of the fragmented masses confirmed bacopaside N1 to have six possible fragmentation product ion masses of m/z 728.45, 548, 545.31, 477.39, 339.19, and 295.18 (Fig. 2). All the masses were in agreement with the possible fragmentation pattern of the compound. The presence of specific mass spectrum is confirmatory to the presence of bacopaside N1.

Identification of fungi with bacopaside production

The fungal isolate which was confirmed for bacopaside production was selected for identification. The stained mycelia of BMF 16 were found to have vegetative hyphae and conidia borne in chains (Fig. 3). The morphologic characters were specific to *Aspergillus* sp. The identification was confirmed by molecular methods, where PCR–based sequence similarity analysis of ITS region was done. NCBI BLAST of ITS sequence (KY994100) revealed the isolate BmF 16 to have 92% identity towards *Aspergillus* sp. (KP292571). The BLAST analysis was followed by phylogenetic analysis where BmF 16 showed distinct clustering with sequences of *Aspergillus* sp. (Fig. 4).

using negative mode ionization of RT 3.00 which was found to have the mass 795.4352 ($M-H^+$) which is the specific mass of bacopaside N1

Discussion

Endophytic organisms reside in a metabolite rich environment which can have significant impact on their biosynthetic potential. There are a large number of studies which suggest the role of endophytic microbes in plant growth regulation, phyto-protection and also in the production of host plant-specific metabolites. In the present study, sodium hypochlorite (2% available chlorine)-mediated surface sterilization was followed for endophytic isolation. Similar methods have been used for the isolation of diverse endophytic microorganisms from different species of plants with varying metabolic compositions (Verma et al. 2011). Recent studies of Katoch et al. (2014) confirmed the presence of endophytic fungi like *Fusarium* sp. with cytotoxic and antimicrobial activities in *B. monnieri*, but fungal production of bacopaside has not been reported. However, role of microorganisms in modulation of secondary metabolites of *B. monnieri* has been reported. Studies with *Chitiniphilus* sp. MTN22 and *Streptomyces* sp. MTN14 singly as well as in combination revealed their modulatory effect on *in planta* bacopaside A production. Here, the expression of bacopaside biosynthetic pathway genes (3-hydroxy-3-methylglutaryl coenzyme A reductase, mevalonate diphosphate decarboxylase, and squalene synthase) has also been shown to be upregulated in plants treated with the microbial combination (Gupta et al. 2017). Our recent report also indicated the promises of plant growth promoting rhizobacteria in modulation of bacopaside content in *B. monnieri* (Jimtha and Radhakrishnan 2016).

The mass m/z 795 obtained in the negative mode ionization of LC–MS was analyzed in detail and it indicated the presence of bacopaside N1 as per previous report

Fig. 2 Confirmation of bacopaside N1 by MS/MS analysis which shows mass of bacopaside N1 along with specific fragments 728.45, 548.30, 545.31, 477.39, 339.19, and 295.19 in the crude extract of BmF 16

Fig. 3 Microscopic image of the isolate BmF 16 after staining with lactophenol cotton blue; showing branched vegetative hyphae, erect conidiophores, and ovate conidia

(Sivaramakrishna et al. 2005). Further confirmation of the compound was done after the mass fragmentation analysis using LC-QToF tandem-mass spectrometer. Various endophytic fungi with the potential to synthesize plant-specific metabolites have previously been reported. The reports of Kumaran et al. (2010) have suggested the presence of endophytic fungi *Pestalotiopsis versicolor* and *P. neglecta* in the leaves and bark of *Taxus cuspidata* (Japanese Yew tree) with the ability to produce taxol as identified by HPLC and LC-MS analysis. The endophytic *Periconia* sp. isolated from *Piper longum* has also been reported to have the ability to synthesize piperine under liquid culture conditions (Verma et al. 2011). These reports suggest the likely presence of such promising endophytic fungi in other metabolically enriched plants like *B. monnieri*. Use of fungi after fourth generation for bacopaside production analysis has made it sure that the observed property is due to fungal biosynthesis only. As microbial biosynthesis of bacopaside N1 has not been previously reported, it highlights the novelty of the report. The isolate

Fig. 4 Phylogenetic analysis of ITS sequences of the selected endophytic fungal isolate BmF 16 isolated from *Bacopa monnieri* along with sequences from NCBI using MEGA 6 with neighbor-joining method using 1000 bootstrap replicates, expressed as

percentage. Only values equal to or greater than 95% are shown at the branch point. The scale bar represents 0.1 nucleotide exchanges per 100 nucleotide sites

with bacopaside production (BmF 16) was further identified as *Aspergillus* sp. Endophytically associated *Aspergillus fumigatus* LN-4 with multipotent bioactivity has already been isolated and characterized from the stem bark of *Melia azedarach* (Li et al. 2012). Endophytic *A. flavipes* with promising plant growth promoting as well as bioactive properties has also been reported from leaf of medicinal plant *Stevia rebaudiana* Bertoni (Verma et al. 2011). These suggest the endophytic nature of *Aspergillus* sp. and its promising biosynthetic potential.

In conclusion, the study has resulted in the isolation of 26 endophytic fungi from the stem of *B. monnieri*. Detailed screening by HPLC, LC–MS and LC–MS/MS analyses confirmed the isolate BmF 16 which was identified as *Aspergillus* sp. to have the capability to produce bacopaside N1. This is the first report on the ability of the endophytic fungi from *B. monnieri* to produce its own metabolite bacopaside which highlights the novelty of the work.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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