Endophytic Phomopsis sp. colonization in Oryza sativa was found to result in plant growth promotion and piperine production

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Endophytic fungi have been reported to have the acquired ability to synthesize host plant specific medicinal natural products. Many fungi with such properties have been characterized and optimized for the conditions which favor maximal production of desired products. However, the inherent plant colonization property of promising endophytic fungi is least studied. Exploiting the transgenome functioning of these fungi have immense applications to add beneficial features to nonhost plants. In the present study, the endophytic fungus Phomopsis sp. isolated from Piper nigrum was confirmed for piperine production by HPLC and LCMS/MS. Further, the fungal isolate was studied for its colonization ability in Oryza sativa. Interestingly, the fungi treated plants were found to have significant plant growth enhancement when compared to the control. Further screening of extract from treated plants by HPLC and LCMS/MS resulted in the confirmation of presence of piperine. The observed result is extremely significant as it opens up novel applications of endophytic fungal colonization in taxonomically diverse plants.

Introduction

Endophytic fungi live within the interior of the plant as harmless agents (Azevedo et al. 2000). They have been reported to have incredible impact on the growth and physiology of host plants. This is because of their ability to produce a wide range of plant beneficial products including phytohormones. When fungi with promising features interact, plants are likely to make use of them as biosystems to manage various physiological and stress conditions. Hence introduction of beneficial fungi can have significant applications in enhancement of crop productivity and disease management. Once the endophytic association process is made successful, the fungi may further be carried in plants from generation to generation.

As a result of extreme adaptation to the endophytic life, some fungi have been reported to have acquired the ability to synthesize plant-specific pharmacologically active compounds. The potential of this has been revealed by the identification of taxol producing endophytic fungi Taxomyces andreanae from Taxus plant. Further, many taxol producing endophytic fungi like Seimatoantlerium nepalense, Monochaetia sp., Pithomyces sp., Chaetomella raphigera, Periconia sp., Alternaria alternata and Pestalotioptis microspora were identified from diverse host plants (Strobel et al. 2004). Even though molecular insight in this process is still in infancy, nat-

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Abbreviations – BAPT, baccatin III-3-amino-3-phenylpropanoyl transferase; BLAST, basic local alignment search tool; HPLC, high performance liquid chromatography; IAA, indole acetic acid; LCMS, liquid chromatography mass spectrometry; MeOH, methanol; NCBI, National Center for Biotechnology Information; PCR, polymerase chain reaction; PDA, potato dextrose agar; PhoA, phomoxanthone A.
Plant-fungal interactions have immense role in the shared biosynthesis of phytochemicals by fungi studied (Strobel et al. 2004). Piperine production by Colletotrichum gloeosporioides and Mycosphaerella sp. from Piper nigrum has been reported in our previous studies (Chithra et al. 2014a, 2014b). The same was also reported for endophytic Periconia sp. from Piper longum (Verma et al. 2011). The distribution of biosynthetic machinery to produce piperine by various plant associated fungi is highly impressive. Exploring the colonization property of these fungi on plants of dietary significance may provide easy and cost effective methods to engineer the plants to have endophyte-mediated features. This may also favor the maintenance of the natural biosynthetic potential of fungi in a competitive form within the live plant system. Such applications have recently been exploited to generate novel compounds and also to enhance the production of desired products (Netzk er et al. 2015). These fungi can have multifaceted applications if it can have plant growth promoting features also in addition to phytochemical biosynthesis. In the study, endophytic Phomopsis sp. with piperine biosynthetic and plant growth promoting features was isolated from Piper nigrum. Further, it was studied for its colonization in rice plant. Very importantly, the experiment has resulted in the generation of rice plants with both piperine production and enhanced growth.

Materials and methods
Isolation of endophytic fungi
The mature stem pieces of Piper nigrum (Karimunda variety) collected from local farms were used for the isolation of endophytic fungi. For this, the selected plant materials were surface sterilized and endophytic fungi were isolated on arginine glycerol agar as described previously (Aravind et al. 2009). The fungi were isolated in the year 2012 and were serially sub-cultured for many generations for various screening studies.

Screening for piperine production
For this, the endophytic fungal isolates were cultured in 200 ml of potato dextrose broth in shaking incubator at 110 rpm for 30 days at 28°C. The filtered fermentation broth was extracted thrice with ethyl acetate, followed by evaporation using a rotary evaporator below 40°C and was finally re-suspended in methanol. This was subjected to HPLC analysis using a mixture of methanol: water in the ratio 70:30 (Verma et al. 2011). The presence of piperine was checked by comparing the peaks in the samples with the respective peak for standard piperine at comparable retention time. The presence of piperine was further confirmed using LC-MS and MS/MS. The electron ionization was used in positive mode to produce mass spectra with a scan range from 100 to 300 for a scan time of 20 min.

Plant growth promoting properties of endophytic fungi
Here, endophytic fungus PF5 with piperine production was selected for the detailed study.

IAA production
The selected isolate PF5 was inoculated into 10 ml of Czapek dox medium (30 g sucrose, 2 g sodium nitrate, 1 g di-potassium phosphate, 0.5 g magnesium sulfate 0.5 g potassium chloride, 0.01 g ferrous sulfate, and 1000 ml distilled water) supplemented with 0.2% (v/v) of L-tryptophan and incubated for 10 days at 28°C. After incubation, the culture was centrifuged at 3000 rpm for 20 min and the supernatant was screened for presence of Indole acetic acid (Syamsia et al. 2015). For this, 1 ml of culture supernatant was mixed with 2 ml of Salkowski reagent (2% of 0.5M FeCl₃ in 35% HClO₄ solution) and the tubes were incubated in the dark for 30 min. This was then observed for development of red color.

The presence of IAA was further confirmed by HPLC using large scale culture extract of PF5. A mixture of
methanol:water (80:20 v/v) was used as the mobile phase which was delivered at a flow rate of 0.3 ml min\(^{-1}\) and the data was collected at wavelength of 280 nm. The peak of the sample was compared with the respective peak for standard IAA at the comparable retention time (Jasim et al. 2014).

**Gibberellic acid production**

Gibberellic acid production in PF5 was analyzed using Czapek broth (120 ml) culture after 7 days of incubation. After incubation, mycelia were separated by centrifuging the culture at 2500 rpm for 15 min. The supernatant collected was acidified and extracted twice with ethyl acetate. The organic layer was evaporated and re-suspended in methanol (MeOH). The presence of gibberellic acid was confirmed by HPLC using a mixture of acetonitrile and 0.01% H\(_3\)PO\(_4\) (in water) (60:40) with a flow rate of 0.6 ml min\(^{-1}\) with detection at a wavelength of 206 nm (Bhalla et al. 2010).

**Identification of the selected endophytic fungus**

The endophytic fungus PF5 isolated from *P. nigrum* was identified on the basis of ITS sequence analysis. For this, genomic DNA from the PF5 was isolated using Chromous Biotech (Bengaluru, Karnataka, India) Fungal gDNA Mini spin kit (category number RKT41) according to the manufacturers recommendation. PCR amplification of the ITS region from the gDNA was done as in the previous report (White et al. 1990). The amplified product was sequenced and the data was analyzed using BLAST analysis.

**Colonization studies of piperine producing PF5 in Oryza sativa**

**Preparation of spore suspension**

Two-weeks-old PF5 culture in potato dextrose agar (PDA) plate was used for this. The conidia were harvested by scraping the surface of sporulating cultures with a sterile scalpel. This was then placed in sterile conical flask with 10 ml sterile distilled water containing 0.05% Triton X-100 and was vortexed for 5 min to produce a homogenous conidial suspension.

**Spore inoculation into seeds of Oryza sativa**

Inoculation of PF5 to rice plant was done as in the previously described method (Syamsia et al. 2015). Here rice seeds were soaked in 1% NaOCl for 1 min to remove surface contaminants. The seeds were then washed several times with sterile water and were soaked in the prepared conidial suspension overnight. In the control, the seeds were soaked in sterile distilled water containing 0.05% Triton X-100. Both control and treated seeds were air dried for 20 min and then transferred into plastic pots containing sterile planting substrate. The substrate was a mixture of red soil and vermi compost in a 3:1 ratio and was sterilized and cooled to ambient temperature before being used. Germinated seedlings were sown 1 cm below the surface of the substrate and maintained at room temperature for 32 days. After this, plant materials were harvested and the growth parameters like shoot length, root length, wet weight and dry weight were analyzed. The dried plant materials were crushed using pulverizer and the powder was extracted twice with ethyl acetate. The crude extract prepared was then checked and confirmed for piperine production by HPLC and LC–MS/MS analysis.

**Results**

**Isolation of endophytic fungi**

The endophytic fungi were isolated on arginine glycerol agar from the stem of *Piper nigrum*. The absence of growth in control plates confirmed the obtained isolates as endophytes. The purified strains were maintained in potato dextrose agar slants for further use (Fig. 1). The crude extracts from obtained fungi were screened for piperine production by HPLC and LC–MS/MS analysis. In the case of extract from PF5, the positive mode ionization in LC–MS/MS analysis showed the presence of mass...
Fig. 2. LC–MS/MS analysis for the confirmation of PF5 for piperine production.

of 286.1 (M+H+) with specific fragments with masses of 201, 171, 143 and 115 (Fig. 2). The obtained mass and the fragmentation pattern is confirmatory to the piperine production by PF5 as reported previously (Chithra et al. 2014b). By HPLC analysis, the concentration of piperine produced was identified as 0.8805 μg ml⁻¹.

The piperine producing isolate PF5 was subjected to screening for IAA/gibberellins production. The red color formed with the addition of Salkowski reagent indicated IAA production by PF5. This was confirmed by formation of a peak at RT 6.365 min in HPLC analysis, which was comparable to that of standard IAA (Fig. 3). The production of gibberellic acid also detected for PF5 by HPLC due to the presence of peak at a retention time of 14.914 min (Fig. 4).

Identification of the selected isolate

The ITS sequence of PF5 was identified and was submitted to NCBI GenBank under the accession number KY321425. Because of the 100% identity of the obtained sequence with that of Phomopsis sp., PF5 was identified as Phomopsis sp. (Fig. 5).

For plant colonization, seeds of rice plant were treated with spore suspension of PF5. The germinating seedlings showed remarkable phenotypic difference from that of the control. The shoot length, root length, fresh weight and dry weight of PF5-treated rice seedlings were significantly higher when compared with negative control (Fig. 6). The changes were statistically significant (P < 0.05) and it clearly confirmed the growth promoting properties of PF5 (Fig. 6, Table 1). To confirm the likely role of fungally produced piperine in observed morphological changes, rice seedling growth was analyzed in the presence of pure piperine. Here, no growth enhancement was observed when compared to the control. Statistical analysis of shoot length of the plant confirmed the absence of significant growth difference in piperine treated and untreated plants.

For analyzing the presence of piperine in PF5-treated rice plants, its crude extracts were prepared and extracted. The extract was found to have a peak at a retention time of 9.803 in HPLC which was comparable to piperine standard (Fig. 7). The concentration of the piperine was calculated from the HPLC and this showed PF5-treated rice to have a piperine concentration of 0.618 μg ml⁻¹ (Table 2). The presence of piperine was also confirmed by LC–MS/MS. Here the presence of mass of 286 (M+H+) with piperine-specific fragmentation masses (m/z 201, 171, 143, 135 and 115) confirmed the product as piperine (Fig. 8).
Fig. 3. HPLC analysis for the confirmation of IAA production by the selected isolate PFS. (A) HPLC chromatogram of standard IAA; (B) HPLC chromatogram of extract of isolate PFS.

Fig. 4. HPLC analysis for the confirmation of gibberellic acid production by the selected isolate PFS. (A) HPLC chromatogram of standard gibberellic acid; (B) HPLC chromatogram of extract of isolate PFS.
Isolate PF 5
Phomopsis sp. PV-S G38 (KJ913819)
Phomopsis sp. MM13 (KJ405639)
Phomopsis sp. MJ1 (KM203568)
Phomopsis sp. R1-6-1 (HM042310)
Diaporthe sp. 75AM/T (GU066649)

Colletotrichum gloeosporioides isolate Cg-246 (HQ264183)
Pleosporales sp. E8006a (HQ008911)
Mycosphaerellaceae sp. E9002f (JN601144)
Pseudocercospora schizolobii isolate TMYN401 (JQ676195)
Fusarium sp. TLAU3 (EU352873)
Saccharomycopsis capsularis strain NRRL Y-17639 (EU057522)

Fig. 5. The phylogenetic analysis of ITS sequence of the selected isolate PF5.

Table 1. Effect of Phomopsis sp. (PF5) on growth promotion of Oryza sativa seedlings. aAverage of three triplicates.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Shoot length (cm)</th>
<th>Root length (cm)</th>
<th>Fresh weight (mg plant(^{-1}))</th>
<th>Dry weight (mg plant(^{-1}))</th>
<th>Leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^a)</td>
<td>22±0.82</td>
<td>10.34±0.62</td>
<td>30.32±0.82</td>
<td>12.34±0.81</td>
<td>3±0</td>
</tr>
<tr>
<td>PF5(^a)</td>
<td>30±2.45</td>
<td>15.77±0.82</td>
<td>42.1±0.82</td>
<td>18.48±0.82</td>
<td>6±0.82</td>
</tr>
</tbody>
</table>

Table 2. Concentration of piperine production by the isolate PF5.

<table>
<thead>
<tr>
<th>Samples used</th>
<th>Piperine concentration (μg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated plants</td>
<td>–</td>
</tr>
<tr>
<td>PF5 treated plants</td>
<td>0.618</td>
</tr>
<tr>
<td>Extract of PF5 supernatant</td>
<td>0.8805</td>
</tr>
</tbody>
</table>

Fig. 6. Plant growth enhancement effect of Phomopsis sp. (PF5) on Oryza sativa (rice) seedlings when compared to control.

Discussion

The use of plants is very popular in traditional systems of medicine and is due to their diverse pharmacological effects. *P. nigrum* is well known for the presence of piperine, which gives its strong and spicy pungent character. Piperine has also been found to increase the absorption and bioavailability of many drugs and nutrients. This has given it an excellent adjuvant function to enhance the therapeutic efficacy of concurrently administered drugs and nutrients. Considering the therapeutic and dietary significance of piperine, its endophytic production can have significant applications. In the present study, a fungus PF5 from *Piper nigrum* was confirmed to have piperine by LC–MS and MS/MS analysis. The masses of fragmentation products obtained was same to the chemical signature of piperine as per previous report (Chithra et al. 2014b). As there are only three other reports on fungal production of piperine, the obtained results are very significant (Verma et al. 2011, Chithra et al. 2014a, 2014b).
By molecular identification, the piperine producing fungus PF5 was identified as *Phomopsis* sp.. This is the first report on piperine production by *Phomopsis* sp.. The reports of Ma et al. (2016) have previously confirmed *Phomopsis* sp. to have remarkable biosynthetic potential due to its production of six different antimicrobial compounds including a novel sesquiterpene. Endophytic *Phomopsis longicolla* isolated from marine algae *Bostrychia radicans*, has been reported for the production of phomoxanthone A (PhoA) with anticancer effect in HL60 cell lines (Pavão et al. 2016). Hence biosynthetic potential of *Phomopsis* sp. in terms of its piperine production can be considered to be the result of its highly specialized phyto-adaptive mechanisms. The isolate PF5 (*Phomopsis* sp.) was also identified to have plant growth promoting properties. Many of the endophytic fungi have been reported to have the ability to produce IAA/gibberellic acid or other plant beneficial features (Strobel et al. 2004). In the current study, the isolated fungus PF5 was found to produce piperine, IAA and gibberellic acid. The heavy accumulation of phytochemical and phytohormone biosynthetic basis in PF5 is highly remarkable. As all these identified features are highly significant to the host plant *P. nigrum*, the PF5 can be considered to be evolved highly favorable to host plant.
For studying the plant growth enhancement and metabolite production in the nonhost plants, rice plants were selected for PF5 colonization. This proved PF5 to have morphologically distinguishable plant growth enhancement effect on rice. As the PF5 is having both piperine and phytohormone biosynthetic basis, piperine was also suspected to be the reason for observed changes. Hence rice seedling growth was also studied using pure piperine which ruled out the fungally formed piperine to have any role in rice plant growth. This is also supportive to the role of phytohormones produced PF5 as the reason for growth changes in rice plant. There are a wide range of reports that suggest the plant probiotic effect of endophytic microorganisms on host as well as nonhost plants. They exhibit these properties either by improving the availability of nutrients or by producing various phytohormones (Jasim et al. 2014).

In the current study, PF5 was found to produce both IAA and gibberellin. Gibberellins have previously been identified to be produced by endophytic Gibberella fujikuroi, Phaeosphaeria sp. L487, Penicillium spp. and Sphaceloma manihoticola. The reports of Khan et al. (2013) have suggested the role of endophytic Penicillium resedanum with gibberellin production to reduce stress in Capsicum annum. The fungus treated plant also showed high biomass, antioxidant capability and salicylic acid content when compared to control (Khan et al. 2013). Hence, the observed morphological changes in rice plant can be due to the phytohormones production by PF5, its synergistic effect or its modulating effect on plant endogenous hormone production.

The PF5-treated rice plants were further analyzed for the presence of piperine by HPLC and LC-MS/MS. From
the results, it could be reported as the piperine production by endophytic fungi in a nonhost plant for the first time. The novel approach used in the study provides opportunity to generate plants with additional metabolite features due to colonized endophytic fungi. Even though the experiment was conducted with various plants like Capsicum annuum, Vigna unguiculata, Oryza sativa and Solanum melongena, only the O. sativa seedlings were found to be colonized by PF5. Due to this colonization, only PF5-treated the rice seedlings showed difference in growth compared to control. The endophytic fungi mediated piperine production in rice opens immense possibilities to explore potential role of endophytic fungi to function as plant transforming agents. In addition to its piperine synthesizing property, the fungus was able to provide a plant growth enhancement effect in rice which makes the current study interesting and promising.

Conclusion

Endophytic fungi from medicinal plants have been considered to have the acquired potential to generate plant-specific compounds as one of its adaptation strategy. The production of host specific metabolites with medicinal applications by the endophytic fungi is highly interesting. Piperine is one among such metabolites which is having high medicinal value. In the present study, an endophytic fungus PF5 was isolated and characterized for the ability for piperine production by LC–MS/MS. Further, colonization studies of PF5 in the rice seedlings have confirmed its plant growth enhancement and piperine production in rice plant. The observed plant growth enhancement effect is identified to be due to the production of phytohormones like gibberellins and indole acetic acid by PF5. The treated plants were confirmed to contain piperine when subjected to HPLC and LC MS/MS analysis. The production of piperine in rice plants is highly novel and important as it provides an insight into the production of medicinal rice which can have significant application in traditional medicinal applications.

Author contributions

Culturing and extraction of isolate for piperine production, culture preparation for plant study and execution of plant study and metabolite analysis was done by C. S.. Isolation and purification of endophyte, HPLC screening of piperine and preparation of manuscript was done by J. B.. Planning of the work, manuscript correction was done by J. M.. Planning and detailed design of the work, result analysis and overall monitoring of the work and manuscript correction was done by R. E. K.

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References

communication leading to the activation of silent fungal secondary metabolite gene clusters. Front Microbiol 6: 299


