Biochemical Characterization of Fungus Isolated during In vitro Propagation of Bambusa balcooa

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Submitted: 20-01-2017 Revised: 03-03-2017 Published: 31-01-2018

ABSTRACT

Background: Bambusa balcooa (Poaceae: Bambusoideae) is a multipurpose bamboo species, which is native of the Indian subcontinent. B. balcooa is regarded as one of the best species for scaffolding and building purposes because of its strong culm. Other uses include paper pulp, handicrafts, and products of the wood chip industry. Due to these various uses in industries, this species has been identified as one of the priority bamboos by the National Bamboo Mission. Objective: This study is designed to analyze the identification of fungus and develop the strategy to eliminate the contamination during in vitro establishment of B. balcooa through nodal part. Fungus contamination is a problem which is encountered during in vitro establishment of B. balcooa cultures.

Materials and Methods: In the present study, fungus contamination from in vitro cultured plant has been isolated and subjected to partial sequence analysis of the 18S rRNA gene to identify the fungus strain. Experiments were designed to develop a strategy for removal of the fungus contamination with the help of antifungal compounds and commercial antimicrobial supplement supplied by HiMedia.

Results: Fusarium equiseti was identified as endophytic fungus. It was observed that antimicrobial supplement at concentration of 500 µl/l was more effective concentration to remove fungus contamination and not showed any detrimental effect on growth parameters of shoot.

Conclusion: This experiment would help in identification and to get rid of fungal contamination and improve the in vitro establishment of B. balcooa cultures for large-scale propagation.

Key words: 18S rRNA gene sequencing, bamboo, endogenous fungus, Fusarium equiseti, in vitro propagation

SUMMARY

- Endogenous fungus was isolated from contaminated culture of B. balcooa, and it was identified as Fusarium equiseti and submitted to NCBI under accession no. KP248727. The endophytic fungus had shown substantial production of amylase, cellulase, and protease media. Gibberellic acid (GA₃) production by F. equiseti was maximum on the 7th day on inoculation.

ABBREVIATIONS

- B. balcooa: Bambusa balcooa, F. equiseti: Fusarium equiseti, PDA: Potato dextrose agar, PCR: Polymerase chain reaction, MS: Murashige and Skoog's, BAP: 6-Benzylaminopurine, ITS1/4: Internal transcribed spacer region 1/4, GA₃: Gibberellic acid

INTRODUCTION

Bambusa balcooa, tropical clumping bamboo from family Poaceae, is a multipurpose bamboo species that originates from Northeast India. This bamboo species is often used as a food source, in scaffolding, paper craft. It has maximum girth of culms and thickness among all species of the genus Bambusa. Seed setting is not recorded in B. balcooa and clump dies after gregarious flowering cycle of 55–60 years.[1] Large quantity of this bamboo species is consumed in pulp and paper industry.[2] B. balcooa can be propagated through vegetative propagation from different parts such as culm cuttings, branch cuttings, or rhizomes. The propagation of B. balcooa through branch cutting forms only 66.7% roots and rhizomes.[3] Lower success rates of 18.5% and 40% with branch cuttings were found.[4] Thereby, vegetative propagation through asexual mean is unsuitable for large-scale propagation of this species. Although many protocols have been reported the micropropagation of B. balcooa, the production of aseptic culture is main problem associated with it as high fungal diversity has been associated with Bambusa species.[6–10] Contaminants compete for the media for nutrients and bring to an end the growth of plant. Continuously persisting microbial contamination has been monitored. For removal or minimizing the contamination, different procedures and chemicals are used. The present study describes identification of endogenous fungus and optimization of various experimental conditions for an efficient in vitro protocol.

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Cite this article as: Tyagi B, Tewari S, Dubey A. Biochemical characterization of fungus isolated during In vitro Propagation of Bambusa balcooa. Phcog Mag 2017;13:S775-9.
MATERIALS AND METHODS

Collection of explants

Tender nodes of 2–4 cm in length from a 6-year-old plant were collected from Agroforestry Research Centre, Pantnagar. Geographically, the site lies in Tarai plains about 30 km southward of foothills of Shivalik ranges of Himalayas at 29°N latitude, 79.3°E longitude, and as altitude of 243.8 M above from the mean sea level.

Disinfestation process

The explants were washed repeatedly after removing the leaf sheath, and the node containing axillary bud was dipped in Tween-20 for half an hour for the removal of all the adhering dust particles and microbes from the surface, and then, explant was treated with Bavistin (0.1%) for 1 h. Under sterile conditions in a laminar airflow bench, these explants were additionally sterilized with 70% ethanol (v/v) for 1 min and soaked in 0.01% HgCl₂ for 3 min. After each step of sterilization, the explants were washed 3–4 times autoclaved water.

Establishment of in vitro propagation protocol

The sterilized explants were inoculated in culture tubes containing the Murashige and Skoog’s (MS) medium supplemented with 6-Benzylaminopurine (BAP, 0.75 mg/l), sucrose (3%), and agar. Antimicrobial supplementation (Himedia) was also added to media for the removal of fungal contamination in different volume as described in Table 1. The pH of the culture media was adjusted to 5.8 ± 0.02 before autoclaving. The cultures were incubated at a photosynthetic photon flux density of 70 ± 5 µmol/m²/s from cool, white, fluorescent lamps at 25°C ± 2°C. Furthermore, the day length was maintained at 16 h in a 24-h light/dark cycle.

Isolation and identification of endogenous microbial contaminant

Fungus appeared as small mycelial growth in the MS medium around the node within 7 days invariably in all the cultures. The fungus was isolated from node region from contaminated culture directly on potato dextrose agar medium (PDA) and incubated at 28°C for 3 days. During the incubation period, fungus growth was observed. Pure culture of this fungus was maintained on PDA plate at 4°C for DNA isolation and polymerase chain reaction (PCR) amplifications of 18S rRNA gene. Morphological and microscopic characteristics of isolated fungus were demonstrated [Table 2 and Figure 1].

Antifungal treatment standardization of explants

For the standardization of antifungal treatment, the surface sterilized explants were immersed in various antifungal compounds such as Bavistin and Vitavax for different duration of time with or without supplementation of antimicrobial supplement in MS medium to ensure contamination-free cultures [Table 3]. The antimicrobial supplement was added to the multiplication medium, i.e., the liquid MS medium containing BAP (0.75 mg/L) in the dosages as given in Table 1. Shoots with less contamination were observed. Growth and plant appearance were continuously observed to determine whether the antimicrobial supplement had any phytotoxic effect on plants during in vitro establishment.

Genomic DNA extraction and polymerase chain reaction amplification

We isolated DNA from pure culture of fungus according to slightly modified method of Cenis.[12] The primers used for the identification

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**Table 1:** Antifungal compounds supplemented in Murashige and Skoog’s media for the removal of fungus contamination

| Antifungal compounds | Concentration (µL/L) | Removal of fungus | Effect on health of shoot |
|----------------------|---------------------|-------------------|--------------------------|
| Antimicrobial supplement | 0                   | -                 | Overgrowth of fungus inhibits the survival rate of plant |
|                       | 100                 | -                 | Overgrowth of fungus inhibits the survival rate of plant |
|                       | 250                 | +                 | Overgrowth of fungus inhibits the survival rate of plant |
|                       | 500                 | ++                | Shoots and leaves were fresh, green, and healthy |
|                       | 750                 | +++               | plant leaves and shoots become yellowish |
|                       | 1000                | +++               | Necrosis |

+: Sign denotes a positive response to a small extent; ++: Sign denotes a positive response to a moderate extent; +++: Complete positive response; -: No response

**Table 2:** Morphological and microscopic characteristics of isolated fungus

| Isolate number | Colonial morphology | Microscopic appearance |
|----------------|---------------------|------------------------|
| F1             | Colony on PDA developed rapidly with white pink aerial mycelium at first, becoming tan to brown as the culture ages [Figure 1b] | Macroconidia septate, falcate, with a distinctive curvature, and the foot-shaped basal cell, with the apical cell very elongated. Conidiophores are either branched or unbranched monophialides. Chlamydospores are produced in abundance, smooth or roughened walls, formed in clumps or chains. Microconidia were absent [Figure 1c and d] |

PDA: Potato dextrose agar
BHAWNA TYAGI, et al.: Endophytic fungus in Bambusa balcooa

Table 3: Surface sterilization for the removal of fungus contamination

| Treatments | Sterilization procedures | Time duration for sterilization | Effect on growth of explant |
|------------|--------------------------|---------------------------------|-----------------------------|
| I          | Bavistin (0.05%)          | 6 h                             | No growth of explants       |
| II         | Bavistin (0.1%) followed by 0.1% HgCl₂ | Bavistin for 1 h and HgCl₂ for 5 min | No growth of explants       |
| IIIa       | Bavistin (0.1%) followed by sodium hypochlorite (0.1%), Vitavax (0.1%), HgCl₂ and ethanol (70%) | Bavistin for 1 h followed by sodium hypochlorite for 5 min, Vitavax for 5 min, half of sample were treated with HgCl₂ for 2 min, and half of them for 5 min | No growth of explants |
| IIIb       | Bavistin (0.1%) followed by sodium hypochlorite (0.1%), Vitavax (0.1%), HgCl₂ and ethanol (70%) | In this experiment, Vitavax is followed by ethanol washing for 45 s after it, the explants were treated with HgCl₂ for 2 and 4 min | No growth of explants |

of the fungal species were universal primers for fungal amplification: ITS1 (5'-TCC GTA GGT AAG GCT CCG G-3') which hybridizes at the end and ITS4 (5'-TCC TGG TAT TGA TTA GTC G-3') which hybridizes at the beginning. The PCR conditions for gene amplification were: initial denaturation 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 1 min, and final extension at 72°C for 5 min. Take 5 μl volume of the above PCR amplified product was used for electrophoresis using 1.0% agarose gel in 1.0X TAE buffer. The PCR product was performed and analyzed on an agarose gel. The gel was stained in ethidium bromide and was observed under ultraviolet (UV) illumination. The PCR product was directly used for nucleotide sequencing of the 18S rRNA gene using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). For identification of fungus, preliminary searches in the NCBI database were performed with BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD, USA).

Phylogenetic analysis
BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/), using ITS1-5.8S rDNA-ITS2 as query sequences, were conducted on all the sequences to check their closest known relatives.[15] The isolates were arranged as the closest to a certain genus, and when identified in a database, the matches were about 95%. However, when the similarity was <95%, the strain was considered unidentified.[16] The construction of the phylogenetic tree was generated by MEGA 5.0.1. The amplified internal transcribed spacer region 1/4 sequence was deposited in the NCBI Gene Bank nucleotide sequence database.

Biochemical analysis
Plate-based assay for extracellular enzymes
The endophytic fungus isolated from the in vitro culture Bambusa balcooa explant was tested for cellulose and pectinase production using 1% carboxymethyl cellulose and 1% pectin as carbon source, respectively. An agar diffusion method incorporating methyl red dye was used as a qualitative assay modified from Downie et al., 1994.[15] Amylase activity was tested using starch agar plates and lipase activity by Tween-20 (10%) incorporated agar plates. Protease activity was assayed using casein hydrolysis medium, which contained 1% skimmed milk and laccase activity by 1-naphthol (0.005%). After incubation at 25°C for 5 days, the diameter of the clear zone was measured.[16]

Gibberellic acid production by endogenous fungus
Culture media were filtered, and then, samples were acidified to pH 2.5 with HCl and extracted using liquid-liquid (ethyl acetate/NaHCO₃) extraction.[17] Gibberellic acid (ΓA₃) in the ethyl acetate phase was measured by UV spectrophotometer at 254 nm.[18]

Ultraviolet-visible spectroscopy absorption spectra of secretary products of endogenous fungus
The fermentation was carried out in Erlenmeyer flasks using a complex medium consisting of potato dextrose broth. The flasks containing 200 ml. fermentation medium were inoculated with fungus mycelia, the flask cultures allowed for inoculum development and fermentation at 28°C ± 2°C, and pH 7.0 with orbital shaking at 120 rpm.[19] After 14 days of fermentation, the fungus biomass was separated with Whatman No. 1 filter paper from fermented broth, and filtered broth was allowed to liquid-liquid separation with EtOAc (1:1 ratio) in a separatory funnel. The spectra of secretory products by Fusarium equiseti were observed on different time intervals (from day 3 to day 12).

RESULTS
B. balcooa is a promising and multipurpose species. Endophytic contaminant hindered the successful establishment of in vitro cultures B. balcooa. Many rigorous attempts have been made for the removal of endophytic fungus using different antifungal compounds and commercial antimicrobial supplement (Himedia). Many previous studies have been shown the association of endophytic contaminants in different parts of Bamboo.[20,21] Bavistin, Vitavax, and many combinations of these fungicides were also tested for surface sterilization for different time durations, but surface sterilization was failed to remove contamination [Table 3]. Our testing revealed that only antimicrobial supplement in MS media was more effective against contamination at concentration of 500 μl/l (w/v) without showing any detrimental effect on plant health. Higher concentration of antimicrobial caused yellowing of plant [Table 1]. Further molecular mechanism of antimicrobial supplement is not known. These shoots were not showing any contamination of fungus and bacteria also. After 2 weeks, such shoots were transferred on fresh media without antimicrobial supplement. Fungus contaminant was identified by 18S rRNA gene sequence analysis [Figure 2] in B. balcooa. The fungus contaminant was highly similar to F. equiseti (NCBI# KP274872). Endophytic Fusarium species recovered from the tissue-cultured B. balcooa. From the present study, it can be concluded that F. equiseti was endogenously present at the nodal region of tissue-cultured B. balcooa that can be controlled by antimicrobial supplement supplied by Himedia at concentration of 500 μl/l. Antimicrobial supplement had not showed any phytotoxic effect on plants during in vitro establishment [Figure 3]. Significant variation was not found in the production of extracellular enzymes by the endophytic fungus isolate. The endophytic fungus in the current study had shown substantial growth on amylase, cellulase, and protease media but not on laccase, lipase, and other media. Endophytic fungus had not shown any growth on lipase media as bamboo is not a good source for fat.

F. equiseti was suspected to be causal of Bamboo blight, culm rot disease.[22,23] However, in the present study, the F. equiseti did not show any disease symptom on B. balcooa. In the present study, gibberellin (ΓA₃) production by F. equiseti was estimated. ΓA₃ production was investigated for 3–12 days, and the maximum production was observed on day 7th [Figure 4]. The same results were reported by Uthandi et al. that Fusarium fujikuroi SG2 showed the production of ΓA₃, initiated by the 3rd day and maximum on the 7th day.[24] The UV-visible spectroscopy
absorption (VIS) spectra of *F. equiseti* ethyl acetate extract showed that the secretory products absorption was in the range of 200–300 nm. Maximum absorption was observed on 284 nm except on day 10. On the 10th day, the maximum absorption was on 281 nm [Figure 5].

**DISCUSSION**

The association of fungus with in vitro cultures of different plants, such as Aglaonema and potato, has been encountered. This has been the cause of decline in the performance of cultures, degeneration of long-term maintained stocks, and lack of reproducibility of tissue culture protocols.[25,26] Similarly, in previous studies, many of the fungal strains were isolated from the nodal region of Sasa and Take species of bamboo.[20] *Fusarium*, *Phyllachora*, and *Sclerotium* species are facultative parasites on bamboo. Thirty-seven taxa have also been isolated as endophytes of bamboo.[27] Most of the taxa identified were typical of endophytes of other monocotyledonous hosts. Bamboo isolates were highly diverse within several fungal groups. Bamboo may represent a huge resource in the search for novel strains, including novel metabolites. Consequently, taxonomic studies involving both morphological and molecular approaches should be intensively performed.

Proteolytic enzymes play an important role in fungal physiology and development. External digestion of protein substrates by secreted proteases is required for survival and growth of both saprophytic and pathogenic species. The amylase activity exhibited by endophytic fungus may help the host plant to degrade starch during plant senescence before other new colonies appear. The extracellular enzyme production by the endophytic fungi suggests their ecological roles as endophytes/latent pathogens or saprobes in their natural environment.[28,29] Endophytes enter the plant by local cell wall degradation and/or fractures in the root system and are involved in the promotion of plant growth and protection against pathogens.[30,31] The plant growth-promoting capacity of fungal endophytes is partly due to the production of phytohormones, such as indole-3-acetic acid (IAA), cytokines, and other plant growth-promoting substances and/or partly owing to the fact that endophytes enhance the host uptake of nutrients such as nitrogen and phosphorus.[32–35] UV-VIS scanning (shoulder peak with prominent) of secretory products revealed that this \( \lambda_{max} \) corresponds to some proteinaceous material which may help gibberellic acid transport from endogenous fungus to host.

**CONCLUSION**

Further studies of fungi-bamboo association are needed for in vitro establishment of aseptic culture of bamboo. Isolation and identification
of fungi from bamboos is limited. The most significant inference from the study was identification of endogenous fungus, which was encountered during in vitro culture of B. balcooa. The common problem of in vitro culture of B. balcooa was growth of E. equiseti on nodal part. This problem was solved out by the use of antimicrobial supplement commercialized by Himedia in 500 µl/l(v/v). This would improve in establishment of in vitro propagation of B. balcooa. The role of this fungus in B. balcooa is still unknown, so further studies are required to know about the relationship between fungus and host plant.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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