Pharmacogn. Mag.
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ORIGINAL ARTICLE

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

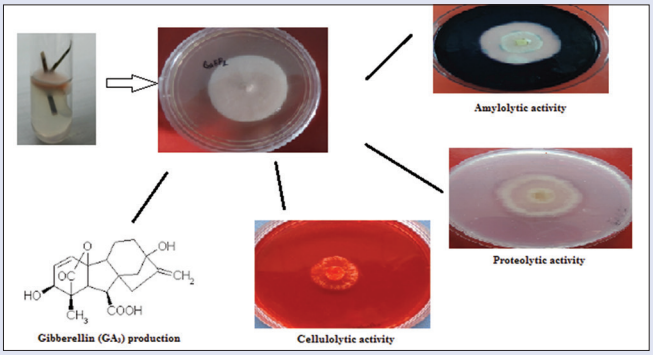
ABSTRACT

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. KP274872. The endophytic fungus had shown substantial production of amylase, cellulase, and protease media. Gibberellic acid (GA_3) production by *F. equiseti* was maximum on the 7th day on inoculation.



Abbreviations used: *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, GA_3: Gibberellic acid

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DOI: 10.4103/pm.pm_20_17

Access this article online
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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.[4,5] 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.

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 attitude 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% $HgCl_2$ 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.[11] 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/m^2/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

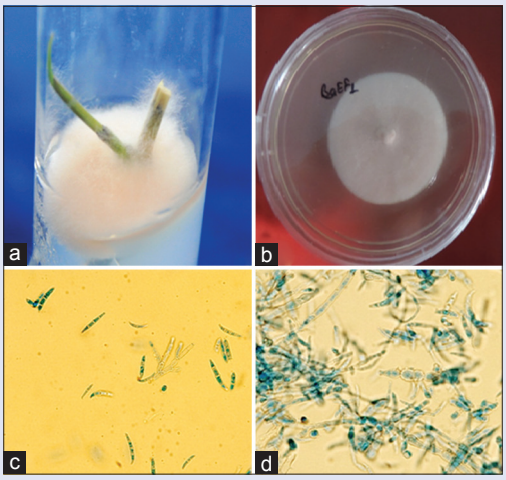

Figure 1: (a) Fungus contaminated explant. (b) Isolated pure culture of fungus. (c) Macroconidia. (d) Conidiophores and conidia

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

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% $HgCl_2$	Bavistin for 1 h and $HgCl_2$ for 5 min	No removal of fungus
IIIa	Bavistin (0.1%) followed by sodium hypochlorite (0.1%), Vitavax (0.1%), $HgCl_2$ and ethanol (70%)	Bavistin for 1 h followed by sodium hypochlorite for 5 min, Vitavax for 5 min, half of sample were treated with $HgCl_2$ 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%), $HgCl_2$ and ethanol (70%)	In this experiment, Vitavax is followed by ethanol washing for 45 s after it, the explants were treated with $HgCl_2$ for 2 and 4 min	No growth of explants

of the fungal species were universal primers for fungal amplification: ITS1 (5'–TCC GTA GGT GAA CCT GCG G-3') which hybridizes at the end and ITS4 (5'–TCC TCC GCT TAT TGA TAT GC-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.[13] 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.[14] 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 Bankit 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 (GA_3) in the ethyl acetate phase was measured by UV spectrophotometer at 254 nm.[18]

Ultraviolet-visible spectroscopy absorption spectra of secretory 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 (v/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 (GA_3) production by *F. equiseti* was estimated. GA_3 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 GA_3 initiated by the 3rd day and maximum on the 7th day.[24] The UV-visible spectroscopy

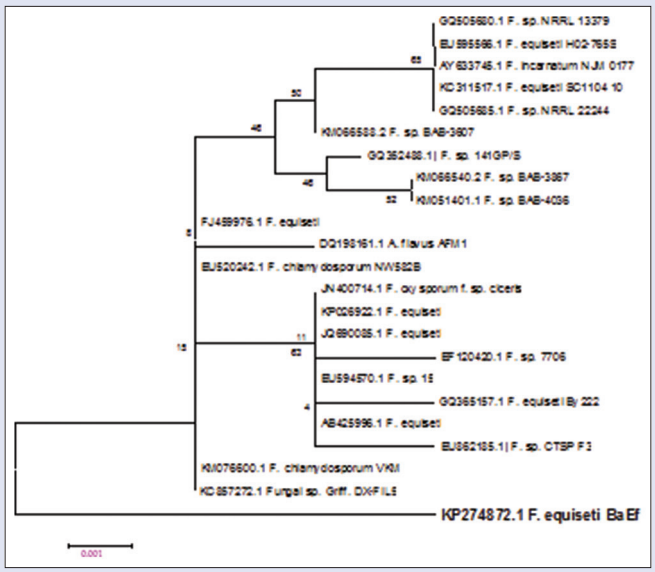

Figure 2: Phylogenetic tree based on ITS1-5.8S-ITS4 sequences of endophytic fungi. The number of each branch point represents percentage bootstrap support from maximum parsimony bootstrap support and neighbor-joining bootstrap support

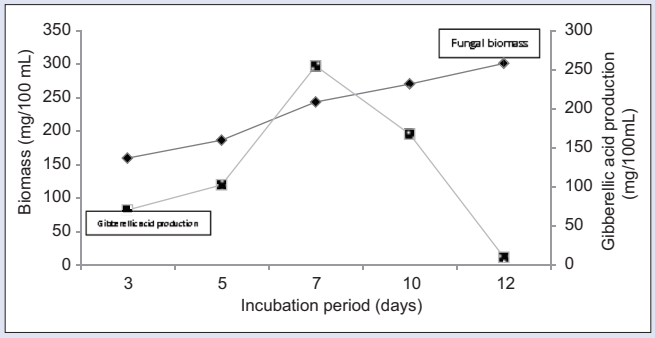

Figure 4: Effect of incubation period on fungus biomass with gibberellic acid production

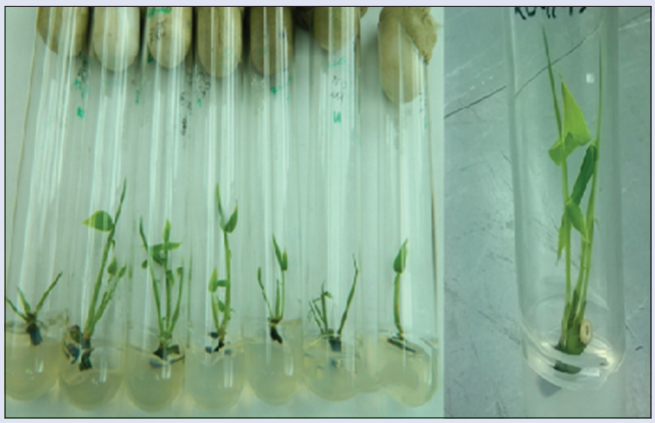

Figure 3: The growth of healthy plant was not affected after supplementation with antimicrobial supplement (500 µl/l)

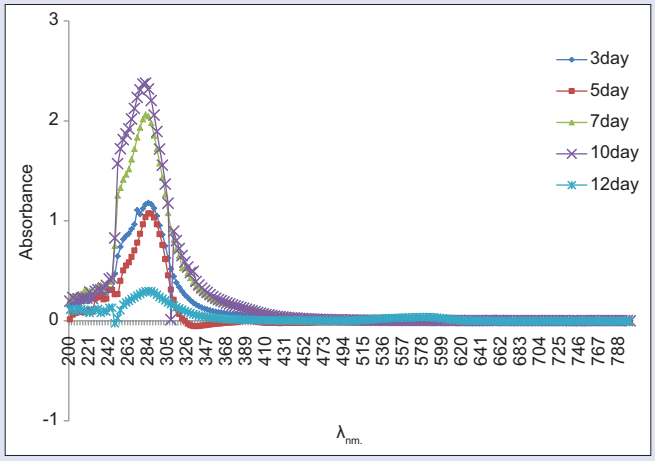

Figure 5: Ultraviolet-visible spectroscopy spectra of secretory product of fungus isolate

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 λ_{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 *F. 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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