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ORIGINAL ARTICLE

A diverse community of jute (*Corchorus* spp.) endophytes reveals mutualistic host–microbe interactions

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Abstract Endophytes are plant-associated microbes that live within plants as an integral part of the host metabolism and function. This study aimed to identify the molecular and physiological characteristics of both culturable and non-culturable endophytic bacteria and fungi present in different parts of the jute (*Corchorus olitorius*) plant. Using universal primers used to amplify hypervariable bacterial 16S rDNA and fungal internal transcribed spacer (ITS) regions of 18S rDNA, we identified five different culturable and 20 non-culturable endophytic bacteria as well as 14 different fungal endophytes from various parts of jute. Biochemical and physiological tests suggest that these microbes may bring a wide range of benefits to their hosts. For example, all five culturable endophytic bacteria were positive for auxin and catalase activity, which may lead to improved root elongation and stress resistance, respectively. These bacteria also have metal uptake, haemolytic and hydrolytic activities that could be useful in medical, environmental and industrial applications. The fungal endophytes were positive for lignin peroxidase, cellulase and xylanase activities, all of which may influence jute physiology. Another important finding was the antifungal activity of one of the fungi against a devastating pernicious fungus that affects hundreds of plant species.

Keywords *Corchorus* · Endophyte · Host–microbe crosstalk · Ribotyping · 16S rDNA · 18S rDNA

Introduction

Symbiosis is one of the driving forces shaping interspecies interactions in an ecosystem. Plants and microbes appear to be invariably connected, with all plants in nature harbouring a diverse community of microbes (Bodenhausen et al. 2014). These microscopic organisms can colonize plant rhizospheres as well other organs, such as flowers, fruits, leaves, stems, roots and seeds (Kobayashi and Palumbo 2000). Several recent studies have shown that these intrinsic microbes bring significant benefits to their hosts (Mack and Rudgers 2008). These plant-associated microbial communities, called endophytes, are integral to plant growth and development without having any apparent negative effects in the plant (Kobayashi and Palumbo 2000). Endophytes have been shown to stimulate growth (Kuklinsky-Sobral et al. 2004), increase nitrogen fixation (Boddey et al. 2003), enhance protection against pathogens (Madhaiyan et al. 2004), increase drought resistance (Malinowski and Belesky 2000), help plants obtain nutrients (Malinowski et al. 2000) and regulate phytohormone production (Hardoim et al. 2008). This symbiotic mutualism can be species- and environment-specific (Long et al. 2008). One remarkable contribution of endophytes is in the production of bioactive secondary metabolites, molecules promising for the development of new drugs for human, plants and animals (Strobel et al. 2004).

The use of natural fibres for new biobased products is a growing industry (Carole et al. 2004). Researchers are turning to plants that grow quickly and from which cellulose-rich fibres can be easily extracted. Jute (*Corchorus olitorius*) is one example of a plant that meets these criteria. Jute is important globally because of its tough but environmentally

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friendly, biodegradable properties (Ahmed et al. 2011). It is one of the cheapest and strongest of all natural fibres and is considered the fibre of the future.

Despite many studies on different aspects of jute, very little is known about the endophytes associated with this plant. This work is a first step towards characterizing the natural microbial community of jute. We have isolated and characterized endophytic bacteria and fungi from jute seeds, seedlings and other organs (roots, stems, leaves and flowers) of mature jute plants. We used universal primers to amplify the hypervariable regions of bacterial 16S rDNA and the ITS region of fungal 18S rDNA, as these sequences vary from strain to strain and thus act as a signature for certain microbes (Martin and Rygiewicz 2005; Saxena et al. 2005). A range of biochemical, physiological and morphological assays suggest these endophytes may provide a broad range of benefits to their hosts.

Materials and methods

Isolation of endophytic bacteria from jute

Seed collection

Healthy *Corchorus olitorius* seeds were collected from the Bangladesh Jute Research Institute. All seeds were collected during the harvesting period (mid-September) from 3-month-old jute plants that were in good physical condition.

Isolation and molecular characterization of culturable endophytic bacteria from seeds

A modified protocol for seed sterilization as illustrated by Long et al. (2003) was followed. The sterilized seeds were crushed in an autoclaved mortar-pestle in 3 mL phosphate-buffered saline (PBS). The suspension was collected by carefully avoiding seed debris. Distilled water used in the final rinse of the seeds served as a control to check sterility. The seed extract and the control were spread on tryptic soy agar (TSA) plates and incubated at 37 °C for 36 h under aerobic conditions (Thomas et al. 2008). Colonies were picked and cultured by streaking again on fresh TSA plates. Colonies were categorized based on morphology after 2–3 days of incubation. Selected colonies were picked, incubated in 5 mL Luria Broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) overnight at 37 °C in a shaker (200 rpm) and were used for DNA extraction. From each plate, colonies were picked and stored in 15 % glycerol solution at −80 °C for further use.

The UltraClean Microbial DNA isolation kit (MoBio Laboratories, USA) was used to isolate bacterial DNA from the incubated LB cultures. PCR was performed against the 16S rDNA sequence, and each PCR tube had a total volume of 15 μL (1× PCR buffer, 5 mM $MgCl_2$, 0.2 mM dNTP, 3 μL 20 % DMSO, 200 ng DNA template and 1 unit Taq DNA polymerase (Invitrogen, USA). Specific forward (AGAGTT TGATCCTGGCTCAG) and reverse (GACTACCAGGGTAT CTAAT) primers from 16S rDNA were used in 1 μL of each 10 mM concentration in a 35-cycle PCR reaction on a GeneAmpR PCR System 9,700 (Applied Biosystems). The program was set as follows: 95 °C for 5 min; 35 cycles at 95 °C for 40 s, 51 °C for 50 s and 72 °C for 50 s; final extension at 72 °C for 5 min and holding temperature of 4 °C. After gel electrophoresis, the desired products to be sequenced were excised from agarose gels and extracted using QIAquick Gel Extraction Kit (QIAGEN, Germany). Samples were sequenced by 1st Base Laboratories, Malaysia.

Isolation of DNA from non-culturable endophytic bacteria in jute seed, PCR amplification, cloning and sequencing

Seeds from the same source were surface-sterilized by the process mentioned above. The PowerPlant DNA Isolation Kit (MoBio Laboratories) was used to extract DNA from clean seeds following the manufacturer's protocol. The extracted DNA was quantified and then amplified using 16S rDNA specific primers as described. The PCR products were cloned in the pCR 2.1 vector (Invitrogen) following the protocol previously described. Plasmids from the successful inserts were isolated by a modified protocol for plasmid isolation from Green and Sambrook (2012). PCR was carried out using the same program used for amplifying 16S rDNA except for the annealing temperature, which was 57 °C. Plasmid DNA (200 ng) was used as the template, and the plasmid-specific primers were as follows: M13 Forward (TGTAAAACGACGGCCAGT) and M13 Reverse (CAGG AAACAGCTATGACC). PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN) and sequenced commercially by 1st Base Laboratories.

Isolation and molecular characterization of endophytic bacteria from jute seedlings

Another batch of seeds from the same collection was germinated in Murashige and Skoog (MS) medium inside a plant growth chamber at 28 °C. Three-day-old seedlings were collected and surface-sterilized by the process previously described. DNA was extracted using the PowerPlant DNA Isolation Kit (MoBio Laboratories) and then cloned (pCR 2.1 vectors). The isolated plasmids were subjected to M13-based PCR amplification and sent for sequencing. The same protocols were followed for all the procedures.

Isolation and molecular characterization of endophytic fungi from different parts of the jute plant

The seeds, seedlings, roots, leaves, flowers and stems of *C. olitorious* were carefully surface-sterilized following the modified method of Goveas et al. (2011). Samples were washed under running tap water, soaked in 100 % ethanol for 1 min and then treated with 4 % sodium hypochlorite for 3 min. Finally, samples were rinsed with autoclaved nanopure (Milli-Q) water and dried on sterile tissue paper (Goveas et al. 2011). The sterilized samples were then placed on Sabouraud Dextrose Agar plates. The process was carried out aseptically in a laminar air flow cabinet. After 7 days of incubation, a mixed fungal culture appeared on the plates. Pure culture plates for individual fungal colonies were generated through repeated subculturing. Fungal DNA was isolated from a pure colony using the ZR Fungal/Bacterial DNA MiniPrep (Catalog No. D6005) DNA isolation kit. ITS (ITS-1 and ITS-4) primers, universal for fungal DNA amplification and synthesized from the conserved region of 18S rDNA, were used for PCR amplification of the fungal DNA (McKendrick et al. 2000; Anderson and Cairney 2004; Martin and Rygiewicz 2005; Rungjindamai et al. 2008). Subsequent cloning, sequencing and bioinformatic analyses were used to identify the different endophytic fungi at the molecular level.

Phylogenetic analysis

rDNA sequences of all of the culturable and non-culturable bacteria and fungi were analysed using BLAST (www.ncbi.nlm.nih.gov/BLAST). The 16S and 18S rDNA nucleotide sequences of the top five BLAST hits were used for phylogenetic analyses. Sequences were aligned using the maximum likelihood (ML) method (Douady et al. 2003) and MEGA 6.0 software (Tamura et al. 2013).

Auxin (Indole-3-acetic acid) production by endophytic bacteria

The isolated culturable bacterial endophytes were quantitatively investigated for indole-3-acetic acid (IAA) production using $FeCl_3$ and $HClO_4$ following the method of Khan and Doty (2009).

Determination of enzymatic activity for bacterial endophytes

Representative isolates were further analysed for the production of different hydrolytic enzymes and metabolites. For amylase, cellulase, protease and lipase assays, respectively, addition of 0.2 % soluble starch (Hankin and Anagnostakis 1975), 0.5 % carboxymethyl cellulose (CMC) (Teather and Wood 1982), 1 % skimmed milk (Mosca et al. 2003) and olive oil (Bulder 1955) was made to separate aliquots of LB media. To detect haemolytic activity of endophytes, a modified protocol as described by Ruiz et al. (2009) was used. All the bacteria were grown for 14–72 h at 37 °C, depending on the media used.

CMC agar, skimmed milk agar and blood agar plates contained halo zones around positive colonies for cellulase, protease and haemolytic activities, respectively; degradation of tiny lipid droplets in olive-oil-containing plates indicated lipolytic activity. Amylolytic plates were flooded with Iodine (I_2) solution (prepared with potassium iodide) to visualize halo zones around positive colonies.

To detect catalase activity, an inoculation loop full of bacterial culture was added to 2–3 drops of 30 % H_2O_2. Production of bubbles indicated a positive result.

Heavy metal uptake by endophytic bacteria

Bacteria were grown for 24 h in screw-capped tubes with 5 mL liquid broth containing metal salt (3.0 mg/L). The supernatant was collected by centrifugation at 4,000 rpm for 20 min. After overnight incubation at 37 °C, the supernatant was subjected to nitric acid (65 %) digestion following the protocol of Zarcinas et al. (1987). The final concentration of metal was 1.8 mg/mL, which was measured by atomic absorption spectra (AAS); results were analysed using the SOLAAR Data Station V11.02.

Experiments on fungal endophytes in jute

Physical characterization of endophytic fungi

Several assays for enzymatic activities (amylolytic, proteolytic and lipolytic) were performed to physiologically characterize the fungi present in jute. In Sabouraud Dextrose Agar medium, 4 % starch, 2 % skimmed milk and 1 % olive oil were used as substrates for assays of amylolytic, proteolytic and lipolytic activities, respectively (Anderson and Cairney 2004).

Cellulolytic activity assay

Both qualitative and quantitative approaches were undertaken to study the cellulolytic activity of endophytic fungi using carboxymethyl cellulose (CMC) as a substrate and dinitrosalisylic acid (DNS) as an indicator in Sabouraud Dextrose Agar medium (Miller et al. 1960; Fujii et al. 2010).

Xylanolytic activity assay

For the xylanolysis assay, 4 % xylan was used as a substrate in Sabouraud Dextrose Agar media, and iodine was used as an indicator (Lowe et al. 1987).

Ligninolytic activity

Isolated jute endophytic fungi were assayed for lignin peroxidase, a major lignin-degrading enzyme. The assay was performed using modified Boyd and Kohlmeyer (B&K) agar medium containing 4 mM guaiacol along with 0.001 % azure B dye (Islam et al. 2012).

Antifungal activity of endophytic fungi

A lawn culture of *Macrophomina phaseolina* was prepared by pouring a concentrated liquid culture of the fungus in potato dextrose broth onto potato dextrose agar (PDA) plates and incubating at 28 °C for 2 days. Jute endophytic fungi were inoculated into 500 μL sterile distilled water and mixed by vortexing. Sterile discs (with no antibiotic) were dipped into this endophytic fungal preparation for 2 min, then placed on the lawn culture plate of M. *phaseolina* and incubated overnight.

Results

Culturable endophytic bacteria

Inoculation of TSA culture plates with seed extract produced five morphologically different colonies. Colonies were named after their colour as White1 (W1), White2 (W2), White3 (W3), Yellow1 (Y1) and Yellow2 (Y2). Optimum pH and temperature profiling (data not shown) for bacterial growth indicated the bacteria grow well at a pH near 7.0 and at an average temperature of 37 °C.

Molecular detection of culturable endophytic bacteria from seeds

PCR with primers specific for 16S rDNA amplified a region of approximately 800 bp of DNA extracted from bacterial colonies. Sequences of these amplified regions were run through BLASTn against the 16S ribosomal RNA sequences in the database. The colony isolates demonstrated homology with *Staphylococcus*, *Micrococcus* and *Bacillus* strains. Table 1 provides the list of the top BLAST hits against the colony-derived sequences.

Non-culturable bacteria

Non-culturable bacteria were collected from both seeds and seedlings. The approximately 800-bp 16S rRNAs were cloned in plasmids and the inserts sequenced in a number of random clones. Only the non-redundant sequences of the positive colonies were kept. Such screening led to the identification of 6 bacteria from seeds and 14 from seedlings. BLASTn results of these sequences are shown in Table 2.

Maximum parsimony (MP) (not shown) and ML trees were built by combining the sequence data of endophytic bacteria from jute seeds and seedlings. Both trees show similar topologies. The ML tree (Fig. 1) was consists of four major clades. Each clade represents a genus. In the ML tree, Seed Endophytic Bacteria (SEB) 9 and Seedling Endophytic Bacteria (SLEB) 20 form a single clade consisting of *Micrococcus*. This finding is consistent with the BLASTn result for these clones. Similarly, SEB 5 and SLEB 22 emerge from a single node that is paracladic to *Bacillus*. This finding is also in harmony with the BLAST result, reinforcing the possibility that these clones belong to the genus *Bacillus*. The pairs SEB 9/SLEB 20 and SEB 5/SLEB 22 appear to represent the same bacteria inhabiting both the seed and seedling stages of jute. SEB 17, 30, 78 and SLEB 18, 28, 36 and 39 formed the largest clade, consisting of many branches with several species of *Staphylococcus*. Within this clade, SEB 17, SLEB 36 and SEB 78 emerge from the same node and thus are possibly the same strain present in both developmental stages of jute. Even in the phylogenetic analysis, SLEB 28 and 39

Table 1 Identification of culturable jute endophytic bacteria from seeds

Isolate	Top hit against colony isolate	Accession no.	Maximum score	Maximum identity
Colony Y1	*Micrococcus lylae*	EU379260.1	1,229	98 %
Colony Y2	*Staphylococcus arlettae*	KF436533.1	1,294	99 %
Colony W1	*Staphylococcus hominis* subsp. *novobiosepticus* strain GTC 1,228	NR_041323.1	1,083	95 %
Colony W2	*Staphylococcus haemolyticus* strain BQN2T-01d	FJ380980.1	1,124	99 %
Colony W3	*Bacillus firmus* strain AU9	EF032672.1	1,269	98 %

Table 2 Identification of non-culturable bacterial isolates from jute seeds and seedlings

Clone	Strain	Accession no.	% Query	% Identity
SEB 5	*Bacillus* sp. H-278	KF021914.1	100	99
	Bacillus sp. H-159	KF021838.1	100	99
SEB 9	*Micrococcus* sp. 08XMSZT-3	HM565983.1	100	99
	Micrococcus lylae strain ES 142con	EU934093.1	100	99
SEB 25	Non-culturable bacterial clone 11-95	JF276745.1	85	99
	Bacillus sp. TB12	JX188082.1	85	99
SEB 3	*Staphylococcus haemolyticus* strain BQN2T-01d	FJ380980.1	99	99
	Non-culturable bacterial clone nbw923h11c1	GQ029304.1		
SEB 42	*Bacillus* sp. SS14.5	KC160777.1	99	98
	Bacillus niabensis strain G3-1-20	KC494318.1	99	98
SEB 78	*Staphylococcus arlettae* strain 3 m-3	JX188021.1	97	100
	Staphylococcus sp. I3-CRBM-T3P21	JN853047.1	97	100
SLEB 14	*Bacillus niabensis* strain HGN10	KF444384.1	100	100
	Bacillus niabensis strain BAB-2457	KC443089.1	100	100
SLEB 17	Non-culturable bacterial clone 6A61	JN882123.1	100	99
	Non-culturable bacterial clone 2A54	JN882105.1	100	99
SLEB 18	*Staphylococcus haemolyticus* strain SH6	KF150639.1	100	100
	Staphylococcus haemolyticus strain 11W6FAP22	KF193934.1	100	100
SLEB 20	Non-culturable bacterial clone ncd2577c01c1	JF226301.1	100	99
	Micrococcus sp. 08XMSZT-3	HM565983.1	100	99
SLEB 22	*Bacillus* sp. NF20130129	KC916741.1	100	99
	Bacillus firmus strain OWS-F5	AY536538.1	100	99
SLEB 28	Non-culturable bacterial clone ncd44e08c1	HM253435.1	100	100
	Non-culturable bacterial clone nbw986d05c1	GQ045383.1	100	100
SLEB 36	*Staphylococcus arlettae* strain 3 m-3	JX188021.1	99	99
	Staphylococcus sp. I3-CRBM-T3P21	JN853047.1	99	99
SLEB 39	Non-culturable bacterial clone ncd44e08c1	HM253435.1	100	100
	Non-culturable bacterial clone nbw986d05c1	GQ045383.1	100	100
SLEB 46	Non-culturable bacterial clone 11–94	JF276744.1	99	99
	Non-culturable bacterial clone 11–75	JF276727.1	99	99
SLEB 49	Non-culturable bacterium	AB696500.1	100	99
	Bacillus niabensis strain SCSAAB0013	JQ647878.1	100	99
SLEB 50	*Bacillus* sp. L41	DQ249996.1	100	99
	Bacillus sp. TB12	JX188082.1	100	99
SLEB 54	*Bacillus* sp. TB12	JX188082.1	100	99
	Uncultured organism clone ELU0024-T375-S-NIPCRAMgANb_000450	HQ748161.1	100	99
SLEB 60	Non-culturable bacterial clone 11–95	JF276745.1	100	99
	Bacillus sp. TB12	JX188082.1	100	99
SLEB 65	*Bacillus* sp. TB12	JX188082.1	100	99
	Bacillus sp. SS14.5	KC160777.1	99	99

retain their bias towards uncultured bacteria, making their molecular identification ambiguous. These data suggest *Staphylococcus* is the most abundant endophytic bacterial population present in jute. Additionally, SEB 25 and SLEB 60 may also be the same species of bacteria present in both jute seeds and seedlings. Their identification was not possible, but they were found to be paracladic to *Bacillus*. A similar situation was observed for SLEB 46, although it is not in the same clade as *Bacillus*. The molecular identities of these three species remain ambiguous, even in the phylogenetic tree, due to the presence of both *Bacillus* and non-culturable bacteria in their corresponding clades. Only one clade was found to act

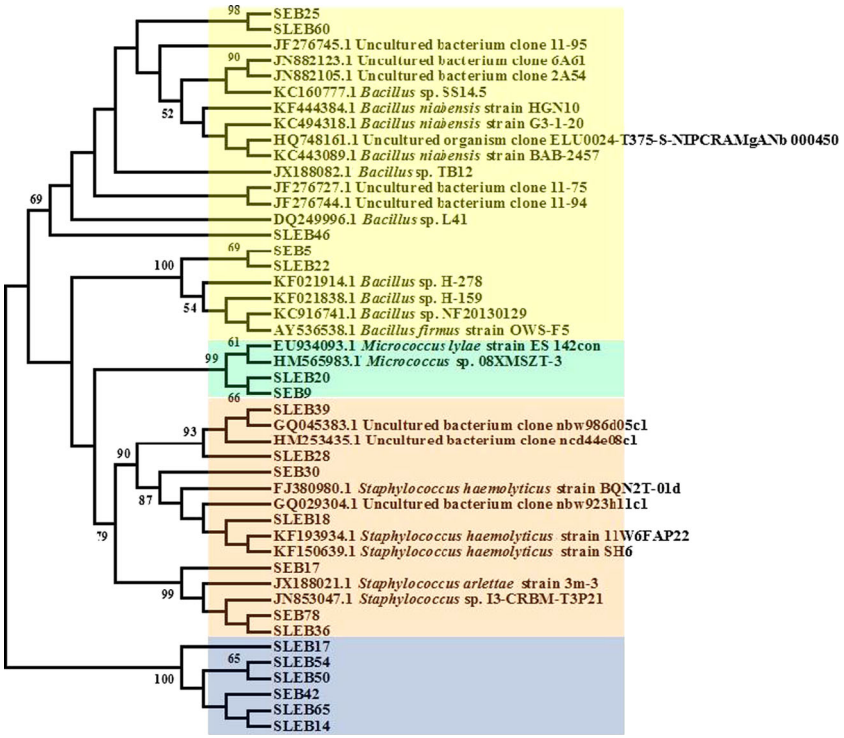

Fig. 1 Maximum likelihood (ML) tree of non-culturable endophytic bacteria. The tree was constructed using Mega 6.0 software and the ML method with a bootstrap value of 1,000. Four major clades shown in different colours represent four different genera. The numbering on the branches of the tree represents the bootstrap value of the corresponding branches

like an out-group, consisting of SEB 42 and SLEB 14, 17, 50, 54 and 65. Multiple sequence alignment (MSA) of these five operational taxonomic units (OTU) shows a strong sequence similarity, and indicating they are strains of the same bacterial species.

Endophytic fungi

Fourteen endophytic fungi specimens were isolated from various parts (seeds, seedlings, roots, stems, leaves and flowers) of the jute plant. Isolated fungal colonies were distinguished based on colour, texture, colony morphology and other physical appearances. Sequence characterization of the ITS regions confirmed the existence of some unique fungi at the genus and species level. These fungal populations are known to exist as endophytes in other plants, thus supporting our data and confirming these observations were not the result of contamination or false positives (Kour et al. 2008; Rungjindamai et al. 2008). BLASTn results of all the fungal endophytic sequences are shown in Table 3.

Although BLASTn can predict the genus of an organism through sequence comparison, we performed phylogenetic analyses of the sequences to lend further accuracy to the predictions. Both ML and MP trees (data not shown) built for jute endophytic fungi show similar topologies. In the ML tree (Fig. 2), a total of eight major clades were found, where each species was found to belong to a clade representing its genera (except endophytes 1, 2, 4, 5, 8, 13 and 14). Endofungi 3 and 6 fell into the same clade as *Schizophyllum*, which belongs to the phylum Basidiomycota. Endofungi 11 and 12 formed clades with *Eutypella* and *Ascotricha*, respectively, supporting their blast output. Taxonomically, these two genera have the common phylum Ascomycota, and up to the order Xylariales, their classifications are the same. Endofungus 7, identified as a *Cochliobolus*, also belongs to the same phylum as that of endophytes 11 and 12. Endofungus 9 fell into the clade of *Aspergillus*, which itself is an Ascomycota. However, Endofungus 10, which is supposed to be *Xylaria*, forms another distant clade and remains paracladic to a clade consisting of endophytes 1, 2, 4, 5, 8, 13 and 14. Endophytes 1, 2, 4, 5, 8, 13 and 14 formed another clade with some branching, giving rise to making their molecular identification ambiguous. This pattern remains the same even when the tree is re-built with the top five BLAST hits of the corresponding clones.

Plant-growth-promoting activity of endophytic bacteria

With respect to the negative control, all five culturable bacterial strains produced IAA at 1 % significance according to the 2-sided Dunnett *t* test. Of these bacteria, W1 had the highest IAA activity (Fig. 3; Supplementary Tables 1, 2). Root elongation of jute was also assessed in the presence of IAA-producing bacterial endophytes. IAA-producing bacteria were found to promote root

Table 3 Identification of fungal endophytes from various parts of jute

ID	Source	Top two blastn hit	Accession no.	% Query coverage	% Identity
Endofungus 1	Seed	*Fusarium subglutinans* isolate UAS017	FJ158133.1	65	98
		Fusarium sp. KUMBF1201	KF113884.1	65	98
Endofungus 2	Seedling	*Aspergillus awamori* strain 13/51	KF154413.1	98	99
		Fungal sp. W-3	KC354375.1	98	99
Endofungus 3	Flower	*Schizophyllum commune* isolate VPCI 240/P/13	KF291014.1	94	99
		Schizophyllum commune strain P11-B	KF293890.1	94	99
Endofungus 4	Seedling	*Aspergillus aculeatus* isolate A3S1 40	JX501394.1	97	99
		Aspergillus aculeatus isolate A3S1 16	JX501393.1	97	99
Endofungus 5	Root	*Aspergillus niger* strain UBOCC-A-101076	KF225022.1	84	97
		Aspergillus niger strain UBOCC-A-101072	KF225021.1	84	97
Endofungus 6	Stem	*Schizophyllum commune* isolate MD21 13	JQ697551.1	94	99
		Schizophyllum commune isolate Z171	JN628212.1	94	99
Endofungus 7	Flower	*Cochliobolus verruculosus*	HE861831.1	94	99
		Cochliobolus verruculosus	HE861830.1	94	99
Endofungus 8	Seed	*Byssochlamys nivea* strain KUC5008	GQ241275.1	97	99
		Byssochlamys nivea strain BCC 14366	AY753338.1	94	99
Endofungus 9	Flower	*Aspergillus flavus* strain KAR-8	KF433946.1	89	96
		Aspergillus flavus strain JP44MY8	KF031021.1	89	96
Endofungus 10	Seed	*Xylaria* sp. CU-1	EU593767.1	95	98
		Xylariaceae sp. B14	FJ517764.1	95	98
Endofungus 11	Seedling and seed	*Eutypella* sp. CCG-2012 isolate PanB1A0064SNA2CC928	JQ922173.1	85	99
		Eutypella scoparia isolate GYYL G002	HM751800.1	96	94
Endofungus 12	Flower	*Ascotricha* sp. ZJ-M-5	JX088707.1	92	96
		Ascotricha sp. OUCMB0156	KC503897.1	92	94
Endofungus 13	Leaf	*Byssochlamys nivea* strain KUC5008	GQ241275.1	62	99
		Byssochlamys nivea strain BCC 14366	AY753338.1	59	99
Endofungus 14	Seed	*Paecilomyces* sp. ATCC 20766	FJ389951.1	94	97
		Paecilomyces saturatus strain CBS 251.55	GU319995.1	95	96

elongation over that of controls at comparable amounts of inoculum (data not shown).

Endophytes show a wide range of enzymatic activities

To assess the physiochemical characteristics and beneficial roles of the fungal and bacterial endophytes, several enzyme assays were performed (Fig. 4; Supplementary Table 3; Supplementary Figs. 1, 2).

Antifungal activity of endophytic fungus

Jute endophytic fungi were assessed for antifungal activity against a devastating plant pathogenic fungus, *M. phaseolina* (Islam et al. 2012). Disc assay results indicate *Schizophyllum commune* has antifungal activity against this pathogenic fungus (Fig. 4).

Heavy metal uptake by endophytic bacteria

Jute endophytic bacteria having a constant OD (approximately 1.80) were grown in a solution containing cadmium sulphate (3 mg/mL). Atomic absorption spectrum results of metal uptake activity indicate significant Cd uptake by W1, W2 and Y1 ($p = 0.05$) with respect to negative controls (*E. coli*). The test was repeated six times and the level of sigificance was calculated using the two-sided Dunnett *t* test (Fig. 5; Supplementary Tables 4, 5).

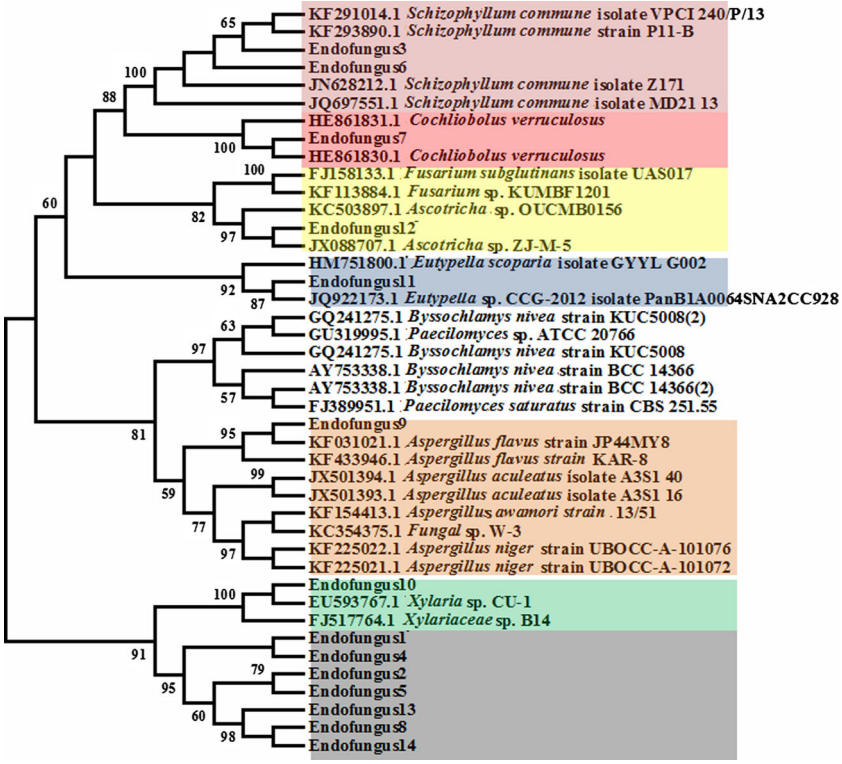

Fig. 2 Maximum likelihood (ML) tree of endophytic fungi. The tree was constructed using Mega 6.0 software and the ML method with a bootstrap value of 1,000. In the tree, eight major clades represent eight different genera. The numbering on the branches of the tree represents the bootstrap value

Discussion

We have detected and indentified endophytic bacterial and fungal populations in seeds, seedlings and other mature parts of jute *C. olitorius* and obtained a first glimpse of the different microbiota inhabiting a jute plant throughout its life cycle. 16S and 18S rDNA genomic sequences were used for molecular characterization of bacterial and fungal endophytes, respectively, as these sequences are known to act as signatures for particular microbes (Martin and Rygiewicz 2005; Saxena et al. 2005). Since this characterization was based on total DNA isolated from different tissues of the jute plant, the possibility of the presence of microorganisms causing seed-borne diseases cannot be ignored. To rule out this possibility, we used certified healthy seeds that generated healthy seedlings, eliminating the risk of seed-borne pathogenic microbes in the endophytic pool. None of the endophytes identified are known to be pathogenic for jute, and the isolation procedure followed in this study is widely used for analysing seed endophytes (Okunishi et al. 2005).

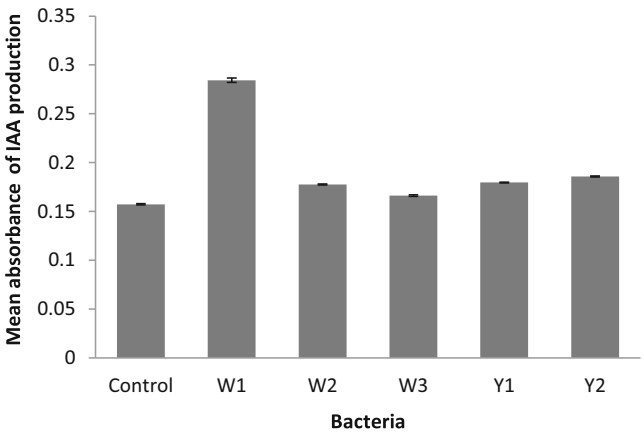

Fig. 3 Indole-3-acetic acid production by bacterial endophytes. Production of IAA (as mean absorbance) is compared between the bacterial colonies. All bacterial endophytes produce a significant amount of IAA at 1 % significance as determined by the 2-sided Dunnett *t* test. IAA production is highest in W1

As expected, most of the endophytes reported in this study (Table 1) have also been reported as endophytes by others. For example, *Bacillus* spp. is one of the most prominent endophytes reported in other plants (Misaghi and Donndelinger 1990; Fisher et al. 1992; Brooks et al. 1994; Araújo et al. 2001; Ferreira et al. 2008; Barros et al. 2010; Sturz et al. 1997) as well as *Micrococcus* (Germida et al. 1998; Boer and Copeman 1974; Garbeva et al. 2001) and *Staphylococcus* (Krishnan et al. 2012). Endophytes were identified by comparing the harmony between phylogenetic clades and BLAST outputs. Phylogenetic analysis using a maximum likelihood tree strengthened the molecular identification of each species. Almost all endophytic fungi and about half of the non-culturable bacteria could be identified from the phylogenetic tree.

Metabolic and physiological characterization together with molecular categorization revealed the amylolytic, proteolytic, lipolytic and cellulolytic activities of the jute endophytic

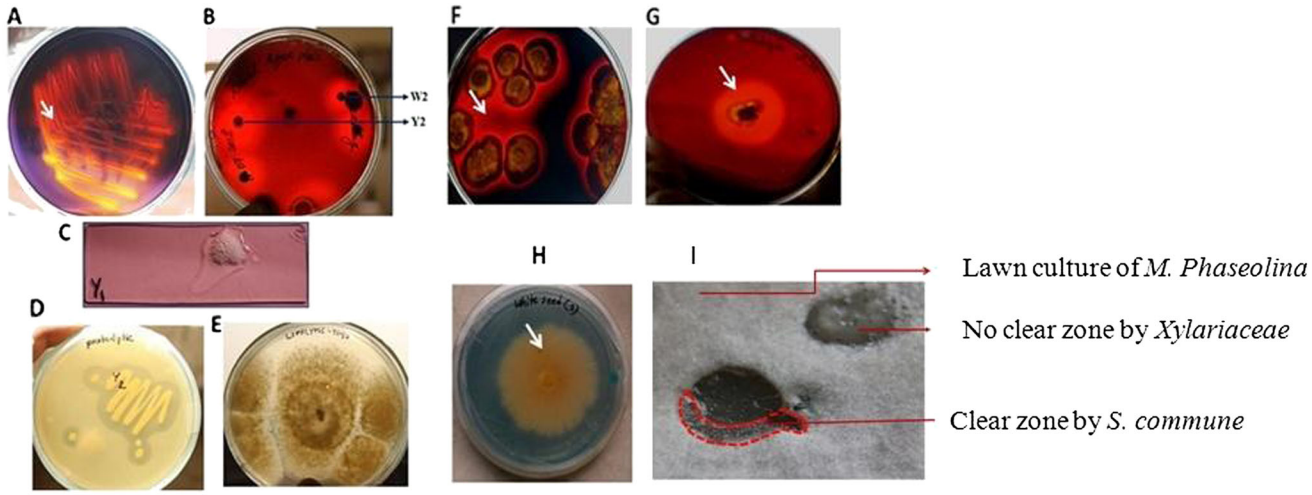

Fig. 4 Positive enzyme and antifungal assays of fungal and bacterial endophytes. **a** Amylolytic activity in Y2 bacteria, where disappearance of the iodine–starch blue colour complex (indicated with a *white arrow)* indicates positive amylolytic activity. **b** Haemolytic activity of W2 and Y2, where formation of a lysis zone on blood agar plates by the bacteria indicates positive haemolytic activity. **c** Catalase activity, where bubble production from H_2O_2 upon treatment with endophytic bacteria (Y1) indicates positive activity. **d** Proteolytic activity in Y2 bacterial endophytes, where formation of a transparent area by bacteria on turbid media indicates positive activity. **e** Lipolytic activity of one of the fungal endophytes, where lysis of olive oil lipid droplets by *Xylaria* sp. indicates positive activity. **f**. **g** Xylanolytic and cellulolytic activity of *Schizophyllum commune* and *Aspergillus niger,* respectively, where formation of a clear zone on xylan- and cellulose-containing media (indicated by a *white arrow*) indicates positive activity. **h** Lignin peroxidase assay, where change in the colour of the substrate (guaiacol+azure B dye) from blue to yellow by *Eutypella scoparia* indicates positive ligninolytic activity. **i** Antifungal property of endophytic fungus. On a lawn culture of *M. phaseolina,* formation of a hollow zone after overnight incubation by a disc impregnated with *S. commune* culture indicates antifungal activity against *M. phaseolina.* No such lysis zone was observed with *Xylaria* spp.

population. Endophytes with such activities have been reported by others (Abouzied and Reddy 1986; Fujii et al. 2010; Kilcawley et al. 2002; Cihangir and Sarikaya 2004; Sandhya et al. 2005; Carrim et al. 2006; Hung and Annapurna 2004). Such enzymatic activities may assist the seeds through germination (Rosenblueth and Martínez-Romero 2006) and help with the transmission of these bacteria into the plant tissues. *Micrococcus* has been reported to have defensive activity against plant pathogens (Quadt-Hallmann et al. 1997). Bacterial endophytes were also tested for catalase activity. Symbionts with catalase activity are known to help plants in scavenging reactive oxygen species (ROS) and thereby assist in better management of stress responses (Polidoros et al. 2001).

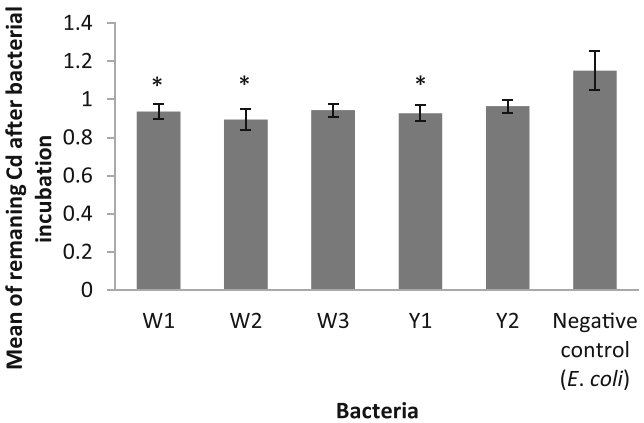

Fig. 5 Cadmium metal uptake by jute endophytes as measured by atomic absorption spectra. The endophytic bacteria were tested for heavy metal uptake in a 1.8 mg/mL Cd solution. The mean concentration of remaining Cd (mean of six trials) after bacterial incubation (*Y*-axis) was plotted against the bacterial samples (*X*-axis); *E. coli* was used as a negative control. Of the five endophytic bacteria, W1, W2 and Y1 showed significantly increased Cd uptake ($p<0.05$) (*) with respect to the negative control. All values were normalized to a blank sample with no bacteria and the significance level was tested using the two-sided Dunnett *t* test

Xylanolytic and cellulolytic enzymes are particularly important for of the degradation of tissues surrounding the bast fibre, an essential process for harvesting jute fibres (Ali 1958; Munshi and Chattoo 2008). Fungal endophytes are well known for such degradative activity (Oses et al. 2008). These two enzymes, present in almost all endophytic fungi of jute, may be helpful for manipulating processes to improve jute retting (Bateman and Basham 1976; Sinsabaugh et al. 2002).

Jute is an annual plant with long bast fibres that make it suitable for the paper and packaging industries. Delignification of these fibres is essential in the pulping and bleaching processes, which requires the use of harsh chemicals. The use of environmentally friendly methods involving enzymes would improve these processes. All the jute endophytic fungi we isolated were found to be positive for lignin peroxidase activity, a major lignin-degrading enzyme. Such fungi may play a

role in lignin degradation (Kirk and Farrell 1987). Similar roles may also be inferred for fungi positive for xylanolytic and cellulolytic activities.

One of the most important findings of this study is the production of IAA by several jute bacterial endophytes. IAA is a common auxin that is involved in the regulation of stem and root growth (Khan and Doty 2009). Plants exploit the IAA synthesis pathway by using tryptophan as a precursor. Production of IAA by different air- and soil-borne bacteria is pervasive (Verma et al. 2001), and such growth regulators can even fertilize the soil (Jha and Kumar 2007). All five culturable endophytic bacteria produce significant amounts of IAA, with W1 producing the most.

Endophytes not only play a role in host growth but also aid in stress alleviation (Azevedo et al. 2000; Hallmann 2001). Root shortening is a consequence of cadmium metal stress in jute (Tamas et al. 2008). This deleterious effect of the metal was alleviated to some extent when seeds were grown in the presence of W2 bacteria. Growth-promoting bacteria are known to reduce metal toxicity in plants (Burd et al. 2000). Nagata et al. (2014) reported the endophytes *P. putida* and *Rhodopseudomonas* sp. isolated from *C. virosa* seedlings facilitated heavy-metal accumulation and enhanced plant growth by solubilizing the metals from the sediment and possibly by producing IAA (Nagata et al. 2014). This observation prompted us to assay for metal uptake by culturable endophytes that produce IAA. Under cadmium stress conditions, W1, W2 and Y1 showed significant metal uptake activity compared to controls ($p = 0.05$). The host plant and bacteria may both play roles in the uptake of metals; studies have shown the uptake of metals by a host plant increases with the addition of endophytic inoculum (Hasnain and Sabri 1997; Ma et al. 2011). Another notable phenomenon was the difference in metal uptake of W1 as compared to that of W2 and Y2, even though they belong to the same genus. Similar results have been reported for fungal endophytes (Li et al. 2012), where endophytes from the same taxa were shown to have different metal uptake abilities. Because seeds develop in contact with microbe-rich soil, such defence-related characteristics of endophytes are essential for the seeds to cope with stress or damage (Mundt and Hinkle 1976; Dalling et al. 2011). Antifungal activity detected in one of the jute endophytic fungi (Fig. 4) illustrate a possible defence against the pernicious fungus *M. phaseolina*, an important finding towards elucidating mechanisms of biotic stress control in plants.

One interesting finding of this study is the high similarity (98–100 %) of some isolates with human-associated pathogenic bacteria such as *S. hemolyticus*, *Shigella* and *E. coli*. However, this is not the only study reporting human pathogens as endophytes; enteric bacteria that are not pathogenic to plants are now known to be common inhabitants of plants (Tyler and Triplett 2008). Fibers are extracted from harvested jute plants by retting, a process in which the jute stalks are tied into bundles and kept submerged in water for about 20 days. Open water sources are generally used for this process; therefore, the possibility that pathogenic endophytes could contaminate these bodies of water cannot be ignored. Severe deterioration of water quality is known to occur during jute retting (Debjit and Anilava 2008). Even though the peak in diarrhea cases (September–November) (Faruque et al. 2004) marginally overlaps with the time when jute is retted (July–September), no studies have investigated whether there is a link between diarrhoea and jute retting. The ability of enteric bacteria to colonize non-animal hosts has to be seriously considered in the context of jute retting. A comprehensive study together with metagenomic analysis of retting water endophytes would aid in addressing this issue.

Conclusion

This study is the first molecular and functional characterization of the microbial community living inside a jute plant. We identified a diverse microbiota of bacteria and fungi in jute. This community of microbes may allow jute to better cope with stress and damage. Functional assays performed on these microbes open new doors for their potential applications in phytoremediation, organic farming, biocontrol and accelerated retting. Given the debates associated with transgenic plants, manipulation of jute endophytes could provide an alternative for developing improved jute varieties. Studies on jute endophytes will not only enrich our understanding of host–endophyte interactions but will also assist in designing agricultural processes that are commercially and environmentally viable.

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