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ISOLATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA PRESENT IN THE CASHEW USING 16S rDNA AMPLIFICATION AND SEQUENCING

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ABSTRACT

Endophytic bacteria reside within plant hosts without causing disease symptoms. Endophytic bacteria, named as KAU-EC1 was isolated from shoot cultures derived from field plants as well as *in vitro* seedlings of cashew (*Anacardium occidentale* L.). Isolation of endophyitic bacteria on nutrient agar media yielded large mucoid, opaque, circular convex colonies on nutrient agar medium. Variability of this bacterium was studied by cultural and morphological tests. PCR was performed for the amplification of 16S rDNA gene. 16S rRNA molecules contain both highly conserved regions and variable regions. The highly conserved regions provide priming site suitable for polymerase chain reaction and sequencing applications. 16S rDNA gene was amplified using two universal bacterial primers: $16S_{43\text{-}63}$ and $16S_{1404\text{-}1387}$. The PCR product when checked on agarose gel indicated the presence of band 1.3 Kbp. 16S rDNA gene from KAU-EC1 was cloned in pGEMT vector and sequenced and analyzed after vector and adapter editing. *In silico* analysis using bioinformatics tools revealed that sequence of KAU-EC1 showed 99 per cent homology with *Klebsiella pneumoniae* strain SA-D6-7 16S ribosomal RNA gene. This is the first study reporting endophytic bacteria in tissue culture cashew plants. The possible role for this bacterium in the biology of cashew plant remains unknown.

INTRODUCTION

The endophytic niche offers protection from the environment for those bacteria that can colonize and establish in planta. These bacteria generally colonize the intercellular spaces, and they have been isolated from all plant compartments including seeds (Posada & Vega, 2005). Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants, ranging from woody tree species, such as oak and pear, to herbaceous crop plants such as sugar beet and maize. A review by Lodewyckx *et al*. (2002) highlights the methods used to isolate and characterize endophytic bacteria from different plant species. Molecular approaches for the isolation and characterization of bacterial endophytes and plant-associated bacteria and communities have been reviewed by Franks *et al*. (2006). Microbial communities inhabiting stems, roots and tubers of various varieties of plants were analyzed by 16S rRNA gene-based techniques such 16S rRNA gene cloning and sequencing. Five taxa exhibiting the most promising levels of colonization and an ability to persist were identified as *Cellulomonas*, *Clavibacter*, *Curtobacterium*, *Pseudomonas* and *Microbacterium* by 16S rRNA gene sequence, fatty acid and carbon source utilization analyses (Elvira-Recuenco and Vuurde, 2000; Zinniel *et al*., 2002). 16S rDNA was amplified by two universal primer: $16S_{43\text{-}63}$ and $16S_{1404\text{-}1387}$ (Radeva and Selenska-Pobell, 2005). High densities of endophytic bacteria were found in plant material from poplar, larch and spruce that had been micropropagated for at least 5 years. The majority of these bacteria were assigned to the genus *Paenibacillus* based on the sequencing of the 16S rRNA genes (Ulrich *et al*., 2008). Fourteen distinct bacterial clones were isolated from surface sterilized shoot tips of Papaya (*Carica papaya*) plated on MS during 2-4 week period following *in vitro* culturing. These isolates were ascribed to six Gram-negative genera namely *Pantoea* (*P. ananatis*), *Enterobacter* (*E. cloacae*), *Brevundimonas* (*B. auriaca*), *Sphingomonas*, *Methylobcterium* (*M. esteraromaticum*) and *Bacillus* (*B. benzoevoeas*) based on 16S rDNA sequence analysis (*Thomas et al*., 2007a). Identification of endophytic bacteria *Enterobacter*, *Klebsiella*, *Ochrobactrum*, *Pantoea*, *Staphylococcus* and *Bacillus* spp. isolated from shoot tip cultures of banana cv. Grand Naine based on partial 16S rRNA gene sequence homology analysis (Thomas *et al*., 2008a; Thomas *et al*., 2008b). In this paper an attempt has been made to isolation and identification of endophytic bacteria present in micropropagated plants of cashew. The objective was to identification of endophytic bacteria by 16S rDNA amplification and sequencing.

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MATERIALS AND METHODS

The procedure as reported by Dinakaran *et al* (2003) was used for the isolation of endophytic bacteria from shoot cultures derived from field plants as well as *in vitro* seedlings. Sample materials were cut into small pieces. Surface disinfection was done by treating with 20 per cent hydrogen peroxide for 10 minutes followed by 5 rinses in 0.02M potassium phosphate buffer at pH 7. To confirm the surface sterilization, 0.1 ml of final washing solution of each sample was transferred to a petri plate with nutrient agar media and incubated at 28°C for 3 days. The tissue (1g) was ground in sterilized pestle and mortar in 9.9 ml of the final buffer and serial dilutions up to 10^{-3}. From each dilution, 1 ml was transferred to petri plates and poured in nutrient agar medium and mixed thoroughly. Replica for each sample was also maintained. Plates were incubated for 24 to 48 hr at 28+/- 2°C. The colony count was taken and endophytic population per gram of tissue was calculated. Bacterial colonies developed were picked and transferred to nutrient agar media, purified and stored.

For PCR the genomic DNA was isolated from the bacteria following Schleif and Wensink (1981). Quality of DNA was assessed by agarose gel elecrophoresis on 1% agarose in Genei electrophoresis system and quantity of DNA calculated from optical density in a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA) based on calculation.

$OD_{260} = 1$ is equivalent to 50 ng double stranded DNA.
1 OD at 260 nm = 50 ng ml^{-1} DNA

16S rDNA gene of isolates was amplified using two universal bacterial primers: $16S_{43-63}$ (5'-CAGGCCTAACACATGCAAGTC-3') and $16S_{1404-1387}$ (5'-GGGCGGWGTGTACAAGGC-3') (Radeva and Selenska-Pobell, 2005). The primers were obtained from Carmina Renteria, Labware Scientific, INC, USA. Each reaction was set up in 25µl mixture consisting of 2.5µl 10X Taq assay buffer with 2.5 mM $MgCl_2$, 200 mM dNTP, 1U Taq DNA polymerase, 10 pM primer and 50 ng of template DNA. DNA amplification consisted of 35 cycles of denaturation at 94°C for 90 seconds, primer annealing at 55°C for 40 seconds, primer extension at 72°C for 90 seconds in a thermal cycler (Master Cycler personal -Eppendorf). The amplified products were separated on 1% agarose gel in 1X TAE buffer at 100V and visualized on a UV transilluminator and documented using gel documentation system (UVP, GelDoc It ™ Imaging system, UK) and amplified band in bacteria was eluted using Axy Prep DNA Gel Extraction Kit (Axygen, Bioscences). The eluted product was cloned in pGEM-T vector using pGEM-T Easy vector system supplied by Promega, USA. Plasmid DNA was isolated from white colonies using alkaline mini prep procedure as described by Birnboim and Doly (1979). The recombinant plasmid DNA isolated by alkali lysis method was amplified by PCR (Eppendorf Master Cycler Personnel) in 25 µl reaction mix using M13 primer. The PCR product was checked on 1% agarose gel and documented.

The stab of the recombinant clone of bacteria was sent to DNA sequencing facility, Bangalore Genei (www.Bangaloregeni.com) for sequencing. Details of the vector cloned and size of the insert were provided. Sequencing was done with SP6 primer to obtain 5'- 3' sequence information of the insert from the reverse region, using automated sequencer, ABI-31100 Genetic Analyzer, that used fluorescent labeled dye terminators and fluorescent labeled primers. The Sanger's method of sequencing was reported to be adopted by the firm. The sequence information obtained from the firm was further analyzed for its characterization.

RESULTS AND DISCUSSION

Isolation of endophyitic bacteria on nutrient agar media yielded large mucoid, opaque, circular convex colonies on nutrient agar medium. It was detected in all the *in vitro* cultures (10 cultures). Hereafter it was named as KAU-EC1. It appeared red after Gram staining reaction indicating Gram negative nature which was further confirmed by the KOH test.

The possible role for endophytic bacteria in the biology of cashew plant remains unknown. The reported functions of endophytic bacteria include (1) promotion of plant growth and increased diseased resistance (Sturz *et al*. 1997, 1999; Chanway, 1998; Welbaum *et al*, 2004), (2) N_2-fixation, e.g. sugarcane (Nogueira *et al*., 2001; Boddey *et al*., 2003) and maize (Estrada *et al*., 2002), and (3) protection against plant pathogenic microorganisms via production of antibiotics (Broadbent *et al*., 1971; Weller, 1988; Sturz *et al*., 1999) or synthesis of secondary metabolites (Long *et al*., 2003).

Bacterial genomic DNA of endophytic bacteria was isolated, purified and subjected to PCR for 16S rDNA amplification. Genomic DNA extraction yielded good quality and quantity of DNA as revealed by spectrophotometry and agarose gel electrophoresis (Table 1 and Plate 1). 16S rDNA of different isolates were amplified by using universal primer $16S_{43-63}$, $16S_{1404-1387}$. Analysis of PCR amplification was carried out on 1.2 per cent agarose gel. Size of the amplified product was 1.3 kb. Distinct bands obtained in PCR amplification were eluted and checked on 1.2 per cent agarose gel. The band was later eluted and cloned in pGEM-T vector. By using the 16S primers a single amplification product having molecular size of about 1.3 kb was obtained for KAU-EC1.

Table 1 Quality and quantity of DNA isolated from endophytic bacteria

Sample	Absorbance at 260 nm	Absorbance at 280 nm	OD $_{260}$/OD $_{280}$	Quantity (ng/µl)
KAU-EC1	4.83	2.72	1.77	241.3

KAU-EC1: KAU - endophytic cashew1

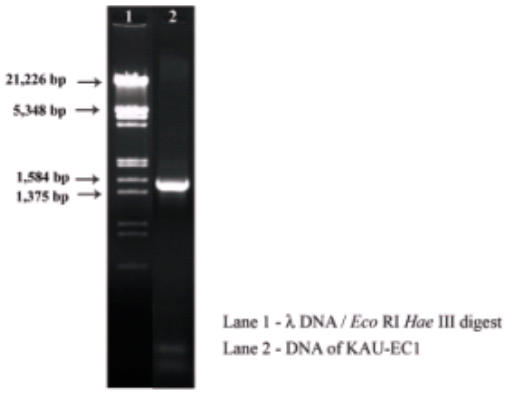


Plate 1 DNA of KAU-EC1 isolated by Schleif and Wensink method

Amplified DNA fragments of KAU-EC1 was ligated into pGEM-T vector (Fig. 1) and transformed into *E. coli*. JM 109 cells. Competence of JM 109 *E. coli* cells was confirmed by transforming the cells with plasmid (pUC 18) having ampicillin resistance. *E. coli* cells alone could not grow on ampicillin containing media, as they lack the gene for ampicillin resistance. But the competent cells harbouring the plasmid could grow in the media. In the present study, large number of blue colonies was observed on LB/ampicillin plate after overnight incubation at 37°C, confirming the competence of *E. coli* (JM 109) cells for transformation.

For cloning of 16S rDNA gene, pGEM-T vector of approximately 3.1 Kbp size was used. It contains T7 and SP6 DNA polymerase promoters that flank a multiple cloning region within the α peptide-coding region of the enzyme β-galactosidase.

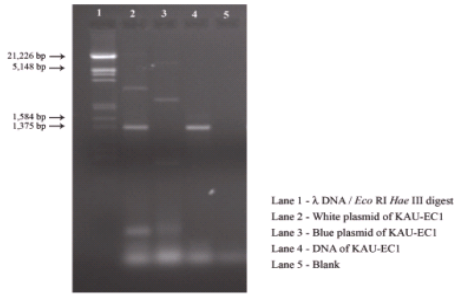


Plate 2 Confirmation of recombination in plasmid DNA of KAU –EC1 with M13

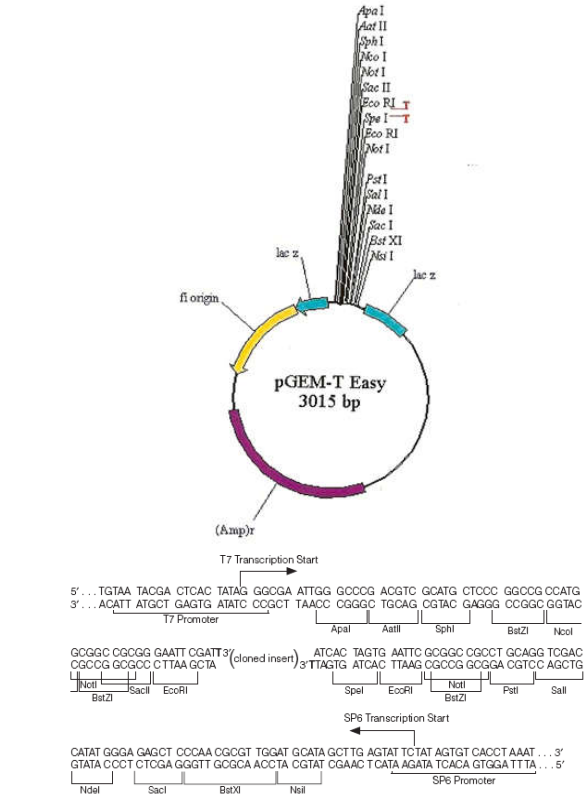
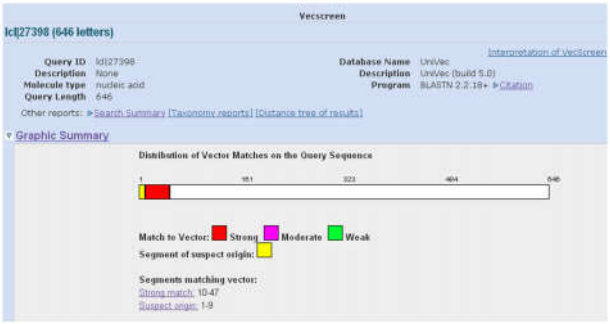
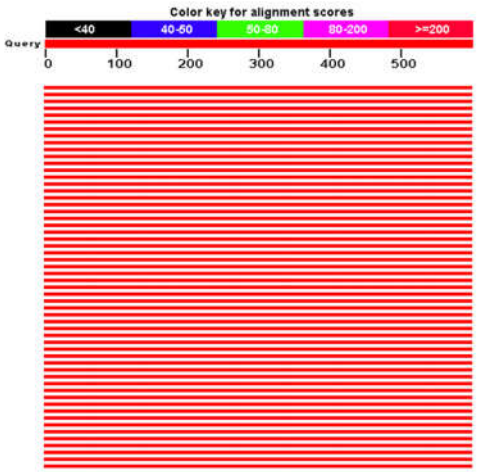


Fig. 1 pGEM-T Easy Vector (Promega) used for cloning PCR product. The *lacZ* region, promoter and multiple cloning sites are shown in the figure. The top strands of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase and bottom strands corresponds to the RNA synthesized by SP6 RNA polymerase.

Thus, due to insertional inactivation of the α peptide region, the recombinants can be directly identified by the blue-white screening of indicator plates. The vector contains multiple restriction sites within the multiple cloning regions thus facilitating easy ligation of insert and its release by digestion with the restriction enzyme. The presence of the insert was further confirmed by PCR amplification of the plasmids with M13 primers (Plate 2). Amplification was present in the case of white colonies. There was no amplification in blue colonies. Since there is no automated sequencing facility at KAU and it would be highly laborious to sequence the clones manually, the autosourcing facility providing by Bangalore Genei, was utilized. The sequences obtained after cloning, were subjected to vector screening to delete the sequences of vector if any present. Vector screening of KAU-EC1 showed significant similarity with vector from the region starting from 10-47 base pairs (Fig. 2a). Hence these regions showing similarity to vector were deleted and only the sequences of gene were retrieved.



A Vecscreen output



B Blast output

Figure 2 Results of sequence analysis for the KAU-EC1

Homology search through BLAST is a heuristic method to find the highest scoring locally optimal alignments between a query sequence and a database sequence (Altschul *et al*., 1997). The BLAST analysis can determine the sequence homology to predict the identity and function of the query sequence. When the cloned sequences of KAU-EC1 was subjected to Blastn homology search, it was identified as *Klebsiella pneumoniae* strain SA-D6-7 (Fig. 2b). Sequence of KAU-EC1 showed 99 per cent homology with *Klebsiella pneumoniae* strain SA-D6-7

16S ribosomal RNA gene present in NCBI data bank (Wang and Dang, 2008).

CONCLUSIONS

A endophytic bacterium, refered to as KAU-EC1 was isolated from shoot cultures derived from field plants as well as *in vitro* seedlings of cashew (*Anacardium occidentale* L.). Isolation of endophytic bacteria on nutrient agar media yielded large mucoid, opaque, circular convex colonies on nutrient agar medium. PCR was performed for the amplification of 16S rDNA gene. The PCR product when checked on agarose gel indicated the presence of band 1.3 Kbp. The 16S rDNA gene from KAU-EC1 was cloned in pGEMT vector, sequenced and analysed after vector and adapter editing. *In silico* analysis using bioinformatics tools revealed that sequence of KAU-EC1 showed 99 per cent homology with *Klebsiella pneumoniae* strain SA-D6-7 16S ribosomal RNA gene. The possible role for this bacterium in the biology of cashew plant remains unknown

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