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Identification and Molecular Characterization of Endophytic Bacterial Isolates from *Musa* Rhizomes

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Abstract: *Endophytic Bacteria are living in a symbiotic relationship with almost all plant species. These bacteria help the host for survival against pathogens, and several adverse environmental conditions. However, endophytes develop contamination in tissue culture grown plants and resist surface sterilization. To overcome this serious threat to tissue culture plants, we aimed study to decipher the endophytic bacteria present in banana which hinder tissue culture growth of staple food and seek out the effective antibiotics against them. In the current study, four sterilized banana suckers grown in MS medium with other supplements were found to be contaminated with gram negative endophytic bacteria. The bacterial genomic DNA was extracted and amplified for 16S rRNA and cloned for sequencing. The strains were identified as Klebsiella spp. and Erwinia spp. These strains were tested with six different antibiotics to prevent contamination in tissue culture. Three out of these antibiotics gentamicin, rifampicin and cefotaxime were found to be effective. The results obtained for this important nutritional crop can unlock the novel prospects for its improvement.*

Keyword*: Endohytes, Rhizome, Gram staining, 16S rRNA, Antibiotic treatment*

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I. Introduction

Endophytic microorganisms (bacteria, fungi or actinomycetes) are localized within the intracellular or intercellular spaces in plants without causing any harm and live their whole or part of their life cycle in different tissues or organs of plants. Moreover, these endophytes favor the growth of plants in many ways such as by the auxins production, phosphorous solubilization, nitrogen fixation, siderophores production [1, 2]. Endophytes have biological importance in various fields of research and can synthesize many compounds such as alkaloids, terpenoids, steroids, flavonoids and many more. More identification of endophytic presence with the plants will may further lead to synthesize some novel chemicals or compounds which are not synthesized before [3]. It has been reported that both gram-positive and gram-negative endophytes have been isolated from several tissues in numerous plant species and various routes of their entries, primarily through the roots of the plants where they found in abundance [4]. Banana (*Musa* spp.) is an economically important fruit crop that is cultivated in many tropical and sub-tropical countries. These are perennial herbaceous monocots, which can be consumed both as staple food and an export commodity. The banana crop is found to be of two types: sweet "desert" banana and a starchier "cooking" banana. India is a leading producer of Banana accounting for 27.43 % (26.2 million tonnes) of total banana production in the world [5]. This crop is mainly consisting of triploid varieties ($2n = 3x = 33$) resulting from crossing of two wild species *M. acuminate* (genome referred as 'AA') and *M. balbisiana* (genome referred as 'BB'). [6]. In the case of banana, corms suckers and sword suckers are used as starting materials for micropropagation. The tissue culture method consists of four steps namely initiation, multiplication, rooting and acclimatization. Tissue-cultured banana plantlets have higher survival rate in the field and give a significant increase in yield and fruit quality as compare to that of sucker plantlets [6]. Pathogens, endophytes, epiphytes and incidental contaminants interfere with growth of the plant tissue. Almost all plant pathogenic bacteria develop as parasites and partly in plant debris or in the soil as saprophytes [7].

In vitro micro propagation of banana has been observed to suffer from endogenous microorganisms mostly, bacteria and fungi. Tissue culture efficiency is largely affected by endogenous bacteria. So, the prevention of microbial contamination is essential for successful micro propagation. Our aim was to identify the types of endophytic bacteria associated with banana rhizome during micro-propagation which may help the plants agronomically. further, to overcome the contamination, the effective antibiotic against them were identified in banana tissue culture.

II. Materials and Methods

Bacterial Isolation and DNA extraction

The banana explants (suckers) were prepared by removing the outer layer of tissues with a sterile knife. The banana suckers were surface sterilized with0.1% mercuric chloride for 10-15 min and then washed three times with sterile distilled water. After washing, the suckers were inoculated on MS medium hormonal supplements for single shoot regeneration. Suckers showing bacterial contaminants were sorted out and the bacteria were isolated on Luria agar (LA) medium. All the isolated contaminants were purified by serial dilution technique and streaking method respectively. Preliminary identification was done by Gram staining and for DNA extraction each isolate was grown in Luria broth (LB) for 24 h at 37 °C under constant mixing at 220 rpm. Bacterial genomic DNA was extracted with a DNA extraction kit (Sigma, USA).

PCR Amplification

The isolated DNA was analyzed on a 0.8% agarose gel for its integrity and was quantified using spectrophotometer. The 16S region was amplified using primers; Forward 8-27F (5'AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-TAGGGYTACCTTGTTACGACTT-3'). The reaction consisted of 5µl of 5X phire buffer-0.625ul of 10pm dNTP, 0.5µl of each primer (10pm), 0.5 µl of DNA polymerase enzyme, 50 ng of template DNA and sterile MilliQ water in a final volume of 25ul. The PCR conditions includes an initial denaturation at 98 °C for 3 min, followed by 30 cycles of denaturation, primer annealing and extension at 98 °C for 30 s, 50 °C for 30 s and 72 °C for 1.30 min respectively,and a final extension at 72 °C for 5 min. The amplified fragments were visualized in 1% agarose gel and documented using gel doc instrument. The amplified fragments were removed from the gel by using a sigma gel extraction kit.

Cloning and sequencing

The PCR products were cloned in a SK^+ pbluescript vector. For cloning, the ligation reaction mixture consisted of 30 ng PCR product, 3U T4 DNA ligase, 2X Ligation Buffer, sterilized water to a final volume of 10µl. The ligation reaction was carried out by incubating it for 16 hour at 16^0C temperature. Then the ligated product was transformed into *E. coli* strain DH5α. After 30 minutes, cells were given a heat shock heat shocked at 42^0C for 90 seconds and immediately placed on ice for 10 minutes. The bacterial culture was incubated at 37^0C with constant shaking at 220 rpm for 1 hour. After incubation, culture mixture was centrifuged at 2000rpm for 2 min and the cells were placed on LB/ampicillin/IPTG/X-Gal plates (double selection, with ampicillin on one hand, and X-Gal on the other hand) which were incubated overnight at 37˚C. The cultures were used for plasmid extraction and isolated plasmids were digested and sequenced for confirmation. The plasmids from positive clones were sequenced with ABI sequencer. The PCR reaction for sequencing were performed using same oligonucleotide primers and a Promega Sequencing Kit, the purified plasmid serving as template DNA.

Phylogeny analysis

The phylogenetic tree was constructed using Mega 5.2 software. The protein sequences of banana endophytic bacteria and other different bacterial species were used for tree construction. Rooted tree was prepared using 1000 bootstrap value.

Antibiotic testing

For antibiotic susceptibility, the bacterial culture in LB was tested with different antibiotics at same concentration i.e. 250 mg/L. rifampicin, augmentin, cefotaxime, streptomicin and gentamycin were taken for the analysis. Absorbance of selected bacteria was recorded after 24h and 48h at 600nm in 96 well plate and further reduce the concentration of effective antibiotics to check its minimum inhibitory concentrations.

III. Results

Bacterial Identification and Characterization

Morphological Analysis

Banana sucker multiplication hindered by endophytic bacterial contamination which led us to look forward for its elimination. white color bacterial contamination was observed around the banana suckers on multiplication medium (Figure1). Four bacterial strains were isolated from Rasthali variety of banana using serial dilution on Luria agar plates and pure culture was isolated by streaking method. Initially, bacterial identifications were carried out using Gram staining. All isolates were gram negative, showing pink colonies of cocci and rod shape (fig. 2).

Genomic DNA Extraction and PCR Amplification

Total genomic DNA of bacterial samples (1, 2, 3 and 4) was isolated and visualized on agarose gel electrophoresis (Figure3). Purified genomic DNA of bacterial samples was amplified for 16S rRNA sequencing

using 8-27F and 1492r universal primer of 16SrRNA. Amplified fragments of 1500bp size were observed on 1% agarose (Figure 3).

Sequencing and Phylogenetic Analysis

16S amplified fragments were cloned in SK+ vector. Blue and White colonies were observed on the LA plates. The transparent colonies were selected and plasmid were further confirmed with digestion. Sequence results were analyzed for homology using BLAST search. The Phylogenetic results revealed that V1, V2, V3 and V4 showing close resemblance with *Klebsiella* spp. and *Erwinia* spp. (Figure 4)

Antibiotic Testing

Antibiotic effective against bacterial culture were tested in LB cultures with the same antibiotic concentration. Gentamicin, cefotoxime, streptomycin, rifampicin, augmentin and ampicillin were tested and among them Gentamicin, cefotaxime and rifampicin depicted susceptibility for antibiotics (Tables 1(a) and 1(b)).

Minimum Inhibitory Concentration (MIC) of Selected Antibiotic

Based on these preliminary results, we optimized the minimum inhibitory concentration of gentamicin and rifampicin for *In vitro* test of the culture plates. We tested the concentration of antibiotics in the range 30mg/L to 250mg/L. The results showed culturing of the sterilized explants in MS medium containing 50mg/L rifampicin, 250 mg/L cefotaxime and 40 mg/L gentamicin produced healthy shoots (Table 2).

IV. Discussion

Four explants showing bacterial contamination were used for the isolation of endophytic bacteria. The preliminary identification of bacteria was done by gram staining. Their microscopic morphological analysis depicted that all the isolates were rod or cocci shaped gram-negative bacteria. 16S rRNA gene amplification, sequencing and BLAST analysis shown sequence similarity of isolates with Klebsiella and Erwinia spp. These two gram negative endophytes have also been isolated in various studies of endophytic bacteria in plants. (Habiba et al; 2002) isolated *Klebsiella* spp., *Erwiniacypripedii* and *Pseudomonas* with other gram positive bacteria *Cellulomonasuda*, *C. flavigena, Corynebacterium paurometabolum* and *Bacillus megaterium* form Musa Species Table banana [8]. Similarly, Msogoya et al; 2012 also isolated these gram-negative bacteria with additional isolation of *Proteus sp*. [8]. This shows that among gram negatives bacteria Erwinia and Klebsiella spp. are quite common among different cultivars of banana but the different strains and different types of endophytes are reported which are due to the factors of plant age, different geographical conditions, and tissue type and host specificity [2]. Identification of endophytes presence in the Rasthali cultivar of banana lead to unravel the concepts for the improvement in this important cultivar of banana by knowing the symbiotic mechanism between them. Further, these endophytic bacterial contamination is a challenge for plant tissue culture grown plants. To resolve this issue, different antibiotics are required for its elimination as previous studies have revealed that a single antibiotic is not sufficient for the elimination of endophytic contamination [9]. In the preliminary antibiotic testing, among six antibiotics, rifampicin was the most effective against all bacterial culture whereas augmentin and streptomycin were found to be least effective. Van den Houwe and Swennen (2000) have also revealed that the rifampicin has an ability to remove the contamination in banana tissue culture [10]. In our study, we found that the cefotaxime and gentamycin also shows high degree of susceptibility against bacteria. So, we used all three antibiotics together to kill the bacteria efficiently as the combinations of antibiotics has been reported to diminish the risk of antibiotic resistance in bacteria [11]. Msogoya et al; 2012, have also reported the effectiveness of Chloramphenicol, rifampicin and gentamicin at a concentration of 150mg/ in the suppression of *Klebsiella* spp., *Proteus* spp., *Erwinia* spp. and *Staphylococcus* spp. Moreover, other culture susceptibility studies have revealed that vancomycin at different concentrations have different effectiveness against different bacteria especially against the gram positive bacteria, at 200mg/l was only effective against *Erwinia, Proteus* and *Staphylococcus* whereas at 250mg/l combination with cefotaxime at 250mg/l effectively eliminated *Erwinia, Proteus, Staphylococcus* and *Agrobacterium tumefaciens* in soybean embryogenic tissues without any significant toxic effects to plant cells [12]. So, we have used the three antibiotics i.e. Rifampicin, Gentamicin and Cefotaxime with the same concentration of 250 mg/l initially. gentamicin has been reported to have toxic effects to plant cultures for at a dose of 100mg/l and has been reported to inhibit shoot initiation from tobacco callus and cause reduction *in vitro* shoot growth of tansy (*Tanacetum vulgare*) plants. [13, 14] Rifampicin can also suppress the bacterial contaminants at 50mg/l in artichoke explants cultures without having any adverse effects on plant cell division, differentiation and DNA synthesis [15]. Finally, Minimum Inhibitory Test was performed for the evaluation of concentration of antibiotics effectivity against bacteria. In this study, we not only identified the endophytic bacteria but along with remedies for contamination caused by them on tissue culture plants. we found that *Klebsiella* and *Erwinia* species in banana were Gram negative and effective antibiotic against them are Rifampicin, Cefotaxime and Gentamicin.

V. Conclusion

Bacterial Contamination remains a continuing threat to plant tissue culture, which can be prevented by taking several logical steps during tissue cultures such as aseptic environment, proficient handling and surface sterilization but endophytic contamination resists these logical precautions. In this study, we isolated four bacterial strains i.e. *Klebsiellavaricolastrain SK01, Klebsiella pneumoniae subsp., Ozaenae strain, Klebsiella sp. XC-08, Erwinia sp. JLX06* which belongs to two genera *Klebsiella* and *Erwinia*. To disinfect this contamination, we have performed culture susceptibility test with six antibiotics i.e. Ampicillin, Augmentin, Cefotaxime, Gentamicin, Rifampicin and Streptomyces with same concentration (250mg/l) and among them, three antibiotics i.e. Rifampicin, Gentamicin, Cefotaxime were effective against contamination and further minimum inhibitory test was performed to check the minimum concentration of these effective antibiotics by reducing the concentration. We have found that Rifampicin, Cefotaxime and Gentamicin at the concentration of 50mg/l, 250mg/l and 40 mg/l were effectively suppresses all the identified bacterial contaminants. These two identified bacteria will further characterized for their growth promoter mechanism as endophytic bacteria in the plants such as Nitrogen fixation, IAA production, Phosphorous solubilization, and production of siderophores (iron chelators).

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Figure Caption

Figure 1: Bacterial contaminated Banana Tissue Cultures

Figure 2: Morphological characteristics of bacterial isolates.

Figure 3: Gel image of 16S rRNA amplified regions of bacterial strains in lane 1,2,3,4 lane 5 showing 1kb marker.

Figure 4: Phylogenetic analysis between identified endophytic bacterial strains based on 16S rRNA gene sequence using MEGA 5.2 software.

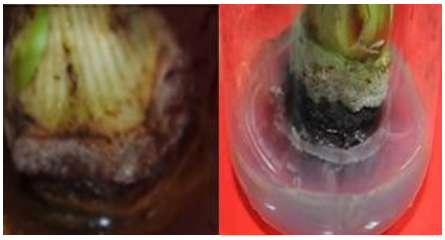

Figure 1:

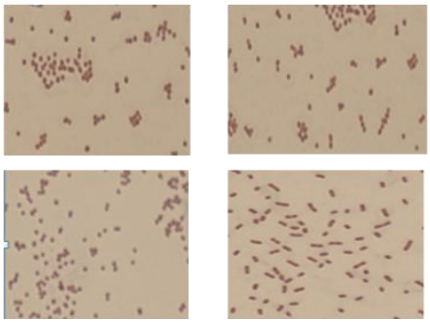

Figure 2:

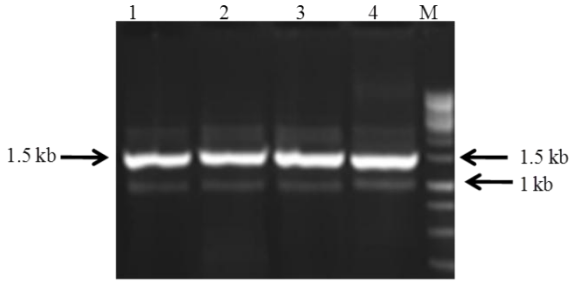

Figure 3:

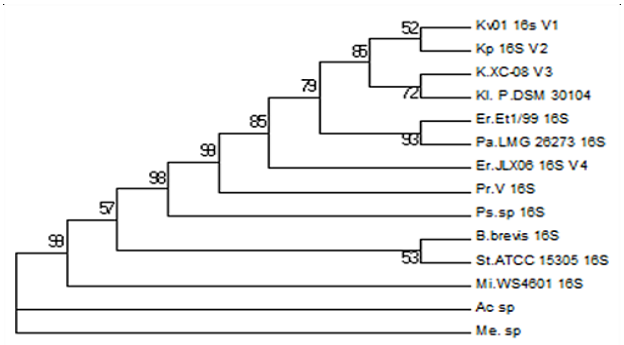

Figure 4:

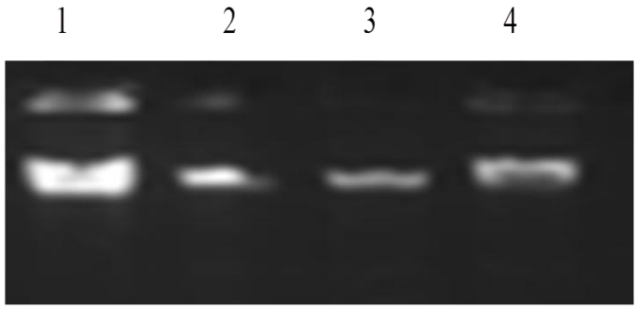

Figure 5: Genomic DNA of four bacterial strains in lane 1, 2, 3 and 4.

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