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Identification of microbial contaminants in tissue culture media, its antibiotic sensitivity and molecular characterization by 16s ribosomal DNA

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Abstract
Banana is an edible fruit produced by the genus *Musa*. It is an important fruit crops due to its great economic importance as well as nutritional value and high availability throughout the year. Banana plants have been growing in different climatic conditions however commercial banana plantations are primarily found in equatorial regions of the globe. Tissue culture is used for the mass production of planting material of banana. Using this technique, viral free and large numbers of clones are produced. Clonal propagation is a complex process. There are many problems in banana tissue culture. They are microbial contamination and phenolic exudation, during explant propagation, phenolics are synthesized and released in sufficient quantities under the influence of polyphenol oxidases. The present investigation aimed to identify the microbial contamination in the banana tissue culture medium which includes isolation and identification of the microbes such as fungi, bacteria *etc.* using conventional methods as well as molecular methods including 16S DNA study. Antibiotic sensitivity test against the bacteria to suggests better antibiotic. On identification it was found that *Penicillium* was the fungus present in the medium. Three types of bacteria could be isolated from the media and it could not be identified by conventional methods hence subjected for 16S DNA assay. It revealed that the bacteria present in the medium was *Staphylococcus warneri* (Sequence ID: MH014968.1) and the bacteria B identified as *Pseudomonas nitroreducens* (Sequence ID: MF627712.1). We could not identify one of the isolates using universal primers. An antibiotic sensitivity study was conducted and found that the antibiotic Tetracycline was more effective for all the bacteria isolated. However more combinations and phytotoxic studies should be conducted to ascertain its suitability.

Keywords: tissue culture, banana, antibiotic, sequencing

1. Introduction
Plant tissue culture refers to the aseptic growth, multiplication and maintenance of cells, tissues or organs of Plants isolated from the mother plant, on a solid or liquid media under controlled environment. The technique consists of taking a bit of a plant referred as the explant and placing it in a sterile nutrient medium where it multiplies [1]. The success of design of a culture media is based on whether it is intended to produce callus, multiply plantlets or embryos for synthetic seed production. The advantage of tissue culture method lies in the production of superior quality and uniform propagules that can be multiplied round the year under disease free condition [2].

Banana, being popular fruit, consumed in fresh or cooked form both as ripe and raw. It is a rich source of carbohydrate and is rich in vitamins particularly vitamin B. It is also a good source of Potassium, Phosphorus, Calcium and Magnesium. Due to its high nutritive value and easy digestibility it forms the food for all ages. It helps in reducing risk of heart diseases when used regularly and is recommended for patients suffering from high blood pressure, arthritis, ulcer, gastroenteritis and kidney disorders. There are different cultivars viz., Dwarf Cavendish, Robusta, Safed Velchi, Basrai, Ardhapuri, Raskathali, Monthan, Poovan, Nendran, Red banana, Nyali, Karpurvalli, Kathali and Grand Naine etc. Among the different cultivars Grand Naine, become the most preferred variety due to its tolerance to abiotic stresses and good quality bunches.

The commonly used propagating material is the sword suckers with well-developed rhizome with spherical shape and having actively growing conical bud and weighing approximately 450-700gm. The insect pests mostly observed are root stock/rhizome weevil (*Cosmopolites sordidus*), stem borer (*Odioporus longicollis*), thrips, banana beetle (*Nodostoma subcostatum*), banana aphid (*Pentalonia nigronervosa*) and nematodes (http://www.agrifarming.in/banana-farming).

The main diseases reported are panama wilt (*Fusarium oxysporum*), anthracnose (*Gleosporium musarum*), leaf spot (Sigatoka) (*Mycosphaarella musicola* and *Cercospora musae*], shoot rot (*Ceratostomella paradoxa*) and viral diseases [3]. Tissue culture is used for the mass production of planting material of banana. Using this technique, viral free and large numbers of clones are produced. Clonal propagation is a complex process. It completed in five major steps, namely, mother plant selection, establishment of aseptic culture, shoot proliferation phase, root proliferation phase, hardening phase. There are many problems in banana tissue culture. They are microbial contamination and phenolic exudation, during explant propagation, phenolics are synthesized and released in sufficient quantities under the influence of polyphenol oxidases.

In the culture of plant cells, tissue, and organs, the main reasons for the loss of plant material are contamination

caused by fungi, bacteria, yeast, arthropods (mites and thrips) have been identified as contaminants in plant tissue culture. These microbial contaminations in growth media in tissue culture attributed to un effectiveness in the process of explant disinfection or to inefficient aseptic practices in the handling of the culture.

Contamination in tissue may be caused by endogenous bacteria that escape initial disinfection or by microorganisms introduced during tissue culture manipulation. Microorganisms may survive in the plant material for several subculture cycle and over extended periods of time without expressing symptoms in the tissue or visible signs in medium. The common bacterial contaminants are *Bacillus licheniformis, Bacillus subtilis and Erwinia sp.* [4]. Bacterial growth appeared as a cloudy zone in the agar medium around the shoot base. These bacterial contaminants can be avoided by using antibiotics, which can suppress bacterial growth.

The present investigation aimed to identify the microbial contamination in the banana tissue culture medium which includes isolation and identification of the microbes such as fungi, bacteria *etc.* using conventional methods as well as molecular methods including 16S DNA study. Antibiotic sensitivity test against the bacteria to suggests better antibiotic.

2. Material and Methods

2.1 Collection of plant materials

The banana suckers (Robusta) were collected from healthy plants aged about three months, from the plantations of Panaikulam located at Alangad Gramapanchayat of Paravur Thaluk of Ernakulam district.

2.2 Laboratory preparation

The inoculation and culture room was maintained in aseptic condition by standard procedure. The laminar flow hood was sterilized with 95% ethanol. The UV light was used to sterilize the laminar flow hood as well as culture racks before the initiation of culture works.

2.3 Media preparation

MS supplementary medium was used in the present study. 1000ml of Stock solution, dissolved the salts one after another in 800 ml of distilled water and then made up the desired volume. The solution was filtered and stored in refrigerator (10 -16^{0}C) for further use.

2.4 Sterilization of media and explant

The culture media and glass wares autoclaved at 15 psi for 15 minutes. Suckers were collected from the field were washed in tap water and sun dried for one day. Then the suckers were treated with 0.1% Bavistin. The upper middle portion and the outer leaf sheaths of the suckers were removed with sharp knife and the remaining basal portion was washed with Bavistin (systemic fungicide). The explants were excised and surfaced sterilized with teepol (soap solution) for ten minutes. Again it was washed with tap water. The next layers of leaf sheath removed to obtain a block measuring 6 to 8 cm long, 3 to 5 cm in diameter and soaked in Mercury chloride solution for 20 minutes. After treatment explant was washed with distilled water for several times. Under aseptic condition, inside the laminar flow, four sides of explant was removed by trimming and exposing the meristematic cells. Then divided into four quarters and inoculated into the initiation medium. The cultures were labeled, transferred to the culture room and incubated at 22 ^{0}C with 12 hours light/ dark cycle.

2.5 Isolation of microbial contaminant

On the fifth day after inoculation microbial contamination were observed nearly about 50% of tubes of culture. Detectable microbial contaminants were isolated and observed under the light microscope. The observed contaminant was identified using the standard diagnostic keys [5].

After the isolation of samples, contaminated tubes were autoclaved at 15 psi for 15 minutes. After the infestation of fungi, other microbial contamination was observed at probably the end of initiation period and beginning of multiplication period. Bacterial contamination observed as white and orange cloudy mass on surface of medium of the explant after 30 days. Bacterial contaminants were isolates from culture media. Pure bacterial isolates were obtained by serial dilution and repeated sub culturing. Isolates were gram stained for identification. Initially the isolated bacteria were named as Bacteria A, Bacteria B, and Bacteria C.

Microbial contaminated media were collected and transferred into a sterile conical flask containing 10 ml distilled water, shake the flask gently for 10 minutes by using magnetic stirrer to get homogeneous suspension. Three sterile test tubes were taken and 9 ml of distilled water was transferred aseptically into it and labeled it as 10^{-1}, 10^{-2}, 10^{-3}. 1 ml of the sample from homogenous suspension were taken and transferred to the test tube containing 9 ml distilled water and shaked the flask gently. It gave the dilution 1:10 (10^{-1}), similarly transferred 1 ml suspension from 10^{-2} dilution to test tube 3 (10^{-3}) to get dilution of 10^{-3} and mix the suspension gently.

Aseptically streak the microbial contaminant containing dilution 10^{-3} using inoculation loop to sterilized petri plates containing nutrient agar. After inoculation incubated at 35^{0}C for 24 hours.

2.6 Preparation of agar plate.

Nutrient agar contains 1% peptone, 1% meat extract 0.5% NaCl it's pH range of 7.4 - 7.5 & 2.30% agar. 14 gram of nutrient agar dissolved in 500ml distilled water and boiled to the medium completely dissolved. Then plugged each test tube using cotton plug. Both medium and Petri plate autoclaved separately at 15 psi for 15 minute. After sterilization the autoclaved medium was aseptically transferred into sterilized petri plates.

2.7 Preparation of nutrient broth

Nutrient broth contain 1 % peptone, 1 % meat extract, 0.5% NaCl It's pH ranges of 7.4 to 7.5. 7.5 grams of nutrient broth was dissolved in 500 ml distilled water and mix the suspension gently. Then transferred into the test tubes and plugged with cotton plug. Then the nutrient broth was autoclaved at 15 psi for 15 minutes.

2.8 Gram staining

Gram staining was performed as per the procedure described elsewhere [6].

2.9 Antibiotic susceptibility testing

The susceptibility of bacterial cultures to antibiotic was

Tested by disc diffusion method [7]. Three locally available antibiotics viz., Taxim – O, Ampicillin and Tetracycline was selected. Prior to the antibiotic's susceptibility test, inoculated the plates with the bacterial culture (bacteria A, B and C) using sterile cotton swab and spread well so as to form a uniform lawn of culture, labeled the plates with the name of organisms as well as the antibiotic disc to be placed. After 2hrs antibiotic disc were placed in the center of inoculated plates. slightly pressed the disc down with the sterile forceps so that it adheres to the surface to the medium. Incubated the plate at 37°C in an inverted position for 24 hours and measured the zone of inhibition between the growth of the organisms with a scale and recorded the result.

2.10 Extraction of DNA and 16S RNA assay

Bacterial culture centrifuged at 5000 rpm for 10 minutes. After that, 5.6ml of TE buffer added into the pellet.100ul of lysozyme solution added into the pellet and kept it in the water bath for 1.5 hours at 37^{0}C. 1000ul of 10% SDS and 2.5ul of Proteinase K added into the tube with pellet solution and water bathed for 1hour at 37^{0}C.3.5 ml of phenol added into the microfuge tube. Centrifuged at 1000 rpm for 15 minutes. After centrifugation, 2.5ml of phenol, 2.4ml of Chloroform and 100ul of isoamyl alcohol is added to the new tube containing the supernatant. Centrifuged at 1000 rpm for 15 minutes. After centrifugation, 4.8ml of chloroform and 200ul of isoamyl alcohol was added to the new tube containing the supernatant. Centrifuged at 1000 rpm for 10 minutes. After centrifugation, 100ul of sodium acetate and 1000ul of absolute ethanol added to the new tube containing supernatant solution. Transferred the thread like DNA structures to a new sterile microfuge tube.

2.10.1 Polymerase Chain Reaction (PCR)

Fully automated machine (Agilent) and universal primers were used for PCR. There were three steps involved in PCR, namely denaturation at 95^{0}c for 5 minutes. Annealing at 58.5 for 30 seconds. Elongation at 72 ^{0}C for 1 to 20s. 30 cycles were carried out for this process. Soon after PCR cycle, the amplified DNA copies were purified by gel electrophoresis.

2.10.2 Agarose Gel Electrophoresis

50 x stock solution of TAE buffer prepared in 1000ml of distilled H_2O, then the electrophoresis tank filled with TAE buffer and gel was casted. For gel casting 3.7 g agarose dissolved in 25 ml TAE buffer. When the molten gel was cooled,2µl of Bromo phenol blue xylene cyanol FF added. 8 wells were prepared by using comb then a small amount of electrophoresis buffer poured on the top of the gel, and carefully removed the comb. Pour off the electrophoresis buffer. The gel mounted in the electrophoresis tank. Sample mixture and ladder of 100 bp slowly loaded into the slots of the submerged gel using a disposable micropipette. After electrophoresis samples were eluted. Purified samples were sequenced. After that the isolated 16S DNA sequence blasted in NCBI. The output was compared with nucleotide data bank of NCBI.

3. Results

On the fifth day after inoculation microbial contamination was observed nearly about 50% of tubes of culture. Detectable microbial contaminants were isolated and observed under the light microscope. The observed contaminant was identified using the standard diagnostic keys [5]. The observed white cottony mass was showed the following characters. Microbial contaminant observed was flat, filamentous, velvety, woolly or cottony in texture, initially white but later becoming blue green or gray green at center surrounded by white cottony appearance. Isolates microscopically appear simple or branched with conidiophores, metulae, phialides and conidia. Metulae carry flask shaped phialides which form brush like clusters under the microscope.

Bacterial contamination observed as white and orange cloudy mass on surface of medium of the explant after 30 days. Bacterial contaminants were isolated from culture media. Pure bacterial isolates were obtained by serial dilution and repeated sub culturing. Isolates were gram stained for identification. Initially the isolates were named as Bacteria A, Bacteria B, and Bacteria C.

Table 1: Colony characters of bacterial isolates

Sl. No.	Name of Bacteria	Features of bacteria
1	Bacteria A	Colony usually had a slightly elevated center, and was circular, entire, smooth, and opaque. They were small and had a diameter of 0.3 to 0.5 mm. The consistency of colonies or culture streaks was usually sticky. Pigment occurred as a characteristic bright orange in colour.
2	Bacteria B	Colony usually had a slightly raised center, and was circular, undulate, rough. They were small and had a diameter of 0.1 to 0. 2 mm. colony appeared as cream in colour.
3	Bacteria C	Colony usually had a slightly raised center, and were circular, elevated, smooth. They were small and had a diameter of 0.2 to 0.3 mm. colony appeared as white in colour.

Table 2: Gram features of bacterial isolates.

Sl. No.	Name of Bacteria	Features of bacteria
1	Bacteria A	Gram positive bacteria with spherical cell appearing in clusters.
2	Bacteria B	Gram negative with rod shaped cells.
3	Bacteria C	Gram positive with rod shaped cells.

Table 3: Antibiotic sensitivity towards Bacteria A. Values are expressed in cm (diameter).

Name of antibiotic	Concentrations of antibiotics											
	0.0833 gm				0.1666 gm				0.3332 gm			
	Plate1	Plate2	Plate3	Mean value	Plate1	Plate2	Plate3	Mean value	Plate1	Plate2	Plate3	Mean value

Taxim-O	0	0	0	0	0	0	0	0	0.8	0.8	0.8	0.8
Ampicillin (amp)	0.5	0.5	0.5	0.5	0.6	0.6	0.6	0.6	2.3	2.3	2.3	2.3
Tetracycline (tet)	0.5	0.5	0.5	0.5	4.5	2.5	4.5	3.8	2.3	2.2	2.2	2.25

Table 4: Antibiotic sensitivity towards Bacteria B. Values is expressed in cm (diameter).

Name of antibiotic	Concentrations of antibiotics											
	0.0833 gm				0.1666 gm				0.3332 gm			
	Plate1	Plate2	Plate3	Mean value	Plate1	Plate2	Plate3	Mean value	Plate1	Plate2	Plate3	Mean value
Taxim-O	0	0	0	0	0	0	0	0	0	0.9	0	0.9
Ampicillin (amp)	2.1	0	0	2.1	1.1	1.1	0	1.1	1.2	1.2	1.2	1.2
Tetracycline (tet)	1	1	1	1	1	1	1	1	1	1.5	1.5	1.5

Table 5: Antibiotic sensitivity towards Bacteria C. Values are expressed in cm (diameter).

Name of antibiotic	Concentrations of antibiotics											
	0.0833 gm				0.1666 gm				0.3332 gm			
	Plate1	Plate2	Plate3	Mean value	Plate1	Plate2	Plate3	Mean value	Plate1	Plate2	Plate3	Mean value
Taxim-O	0	0	0	0	0	0	0	0	0.5	0.5	0.5	0.5
Ampicillin (amp)	0	0	0	0	1.5	1.5	1.5	1.5	1.3	1.2	1.2	1.23
Tetracycline (tet)	1.6	1.6	1.6	1.6	1.9	2	2	1.96	1.5	1.5	1.5	1.5

Fig.5. Forward sequence of bacteria A, A >A_F_10768-1_P1642, Trimmed Sequence (888 bp)

GTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGAC
GTTAGCGGCGGACGGGTGAGTAACACGTGGATAAC
CTACCTATAAGACTGGGATAACTTCGGGAAACCGG
AGCTAATACCGGATAACATATTGAACCGCATGGTT
CAATAGTGAAAGGCGGCTTTGCTGTCACTTATAGA
TGGATCCGCGCCGTATTAGCTAGTTGGTAAGGTAA
CGGCTTACCAAGGCAACGATACGTAGCCGACCTGA
GAGGGTGATCGGCCACACTGGAACTGAGACACGGT
CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCGCAATGGGCGAAAGCCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCT
GTTATCAGGGAAGAACAAATGTGTAAGTAACTGTG
CACATCTTGACGGTACCTGATCAGAAAGCCACGGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT
GGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCG
CGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCC
CACGGCTCAACCGTGGAGGGTCATTGGAAACTGGA
AAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAT
GTGTAGCGGTGAAATGCGCAGAGATATGGAGGAA
CACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACT
GACGCTGATGTGCGAAAGCGTGGGGATCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGT
GCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGA
GTACGACCGCAAGGTTGAACTCAAAGGAATTGACG
GGGACCCGCACAAGCG

Fig 6. Reverse sequence of bacteria A >A_R_10768-2_P1634, Trimmed Sequence (866 bp)

AATGGTTACTCCACCGGCTTCGGGTGTTACAAACT
CTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGG
GAACGTATTCACCGTAGCATGCTGATCTACGATTA
CTAGCGATTCCAGCTTCATGTAGTCGAGTTGCAGA
CTACAATCCGAACTGAGAACAACTTTATGGGATTT
GCTTGACCTCGCGGTTTAGCTGCCCTTTGTATTGTC
CATTGTAGCACGTGTGTAGCCCAAATCATAAGGGG
CATGATGATTTGACGTCATCCCCACCTTCCTCCGGT
TTGTCACCGGCAGTCAACTTAGAGTGCCCAACTTA
ATGATGGCAACTAAGCTTAAGGGTTGCGCTCGTTG
CGGGACTTAACCCAACATCTCACGACACGAGCTGA
CGACAACCATGCACCACCTGTCACTTTGTCCCCCG
AAGGGGAAAACTCTATCTCTAGAGCGGTCAAAGGA
TGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGA
ATTAAACCACATGCTCCACCGCTTGTGCGGGTCCC
CGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTAC
TCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGC
ACTAAGGGGCGGAAACCCCCTAACACTTAGCACTC
ATCGTTTACGGCGTGGACTACCAGGGTATCTAATC
CTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAG
TTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTT
CCTCCATATCTCTGCGCATTTCACCGCTACACATGG
AATTCCACTTTCCTCTTCTGCACTCAAGTTTTCCAG
TTTCCAATGACCCTCCACGGTTTGAGCCGTGGCTTT
CACATCAGACTTAAAAAA

Fig.7. Forward sequence of bacteria B, >B_F_10768-3_P1634, Trimmed Sequence (862 bp)

TGCAGTCGAGCGGATGAGTGGAGCTTGCTCCATGA
TTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATC
TGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAAC
GCTAATACCGCATACGTCCTACGGGAGAAAGCAGG
GGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAG
GTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACC
AAGGCGACGATCCGTAACTGGTCTGAGAGGATGAT
CAGTCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGGACAATG
GGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGA
AGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGG
GAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTG
ACGTTACCAACAGAATAAGCACCGGCTAACTTCGT
GCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCG
TTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGG
TGGTTTGGTAAGATGGATGTGAAATCCCCGGGCTC
AACCTGGGAACTGCATCCATAACTGCCTGACTAGA
GTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCG
GTGAAATGCGTAGATATAGGAAGGAACACCAGTG
GCGAAGGCGACCACCTGGACTGATACTGACACTGA
GGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCGACTA
GCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCT
AACGCGATAAGTCGACCGCCTGGGGAGTACGGCCG
CAAGGTTAAAACTCAAATGAATTGAC

Fig.8. Reverse sequence of bacteria B, >B_R_10768-4_P1634, Trimmed Sequence (877 bp).

CTTGCGGTTAGACTAGCTACTTCTGGAGCAACCCA
CTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCC
GGGAACGTATTCACCGTGACATTCTGATTCACGAT
TACTAGCGATTCCGACTTCACGCAGTCGAGTTGCA
GACTGCGATCCGGACTACGATCGGTTTTATGGGAT
TAGCTCCACCTCGCGGCTTGGCAACCCTCTGTACCG
ACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGG
GCCATGATGACTTGACGTCATCCCCACCTTCCTCCG
GTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCAT
GACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCG
TTACGGGACTTAACCCAACATCTCACGACACGAGC
TGACGACAGCCATGCAGCACCTGTGTTCCGATTCC
CGAAGGCACTCTCGCATCTCTGCAAGATTCCGGAC
ATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTC
GAATTAAACCACATGCTCCACCGCTTGTGCGGGCC
CCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGT
ACTCCCCAGGCGGTCGACTTATCGCGTTAGCTGCG
CCACTAAGATCTCAAGGATCCCAACGGCTAGTCGA
CATCGTTTACGGCGTGGACTACCAGGGTATCTAAT
CCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCA
GTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTT
CCTTCCTATATCTACGCATTTCACCGCTACACAGGA
AATTCCACCACCCTCTACCGTACTCTAGTCAGGCA
GTTATGGATGCAGTTCCCAGGTTGAGCCCGGGGAT
TTCACATCCATCTTACCAAACCACCTACGC

The genome of bacteria C cannot be amplified using universal primer by PCR. The sequence of bacteria A and bacteria B were subjected to BLAST analyses (Fig. 5, 6, 7 and 8). The output was compared with nucleotide data bank of NCBI.

4. Discussion

On the day after inoculation of explant, media was about 50% of culture tube were found to be contaminated with microbes. According to earlier studies [8] contamination rate of banana culture was 45%, hence microbial contaminants severely affect commercial propagation of different varieties of banana. Hence eradication of microbial contaminants plays a key role in the commercial production of banana. In the present investigation we could identify the fungus and bacteria in the contaminated tubes even if better sterilization method adopted.

To identify the microbial contaminants in the culture tube we isolated the individual microbes. It was found that the fungus which was frequently found in the culture tube was *Pencillium*. The *Pencillium* could be easily identified under compound light microscope after staining with Methylene blue because they are with branched conidiophores and conidia (Fig.1). Cassells [4] reported that the fungi like *Fusariaum sp, Penicillinum sp.* and *Aspergillus sp* were usually found in tissue culture media. Ketoconazole at a concentration of 200 mg/L was most effective against the fungal contaminants. It suppressed fungal contaminants in animal cell culture, especially *Aspergillus fumigatus, Candida albicans* and *Penicillium sp.* [9]. Ketoconazole was systemic antifungal agent that interferes with the synthesis of fungal cell membranes as well as certain enzyme activities [10] although reports on phytotoxic effect of ketoconazole are scanty, the antifungal agent has been reported to suppress larval development in mussel *in vitro* culture [11]. Application of systemic fungicides like Benomyl before the collection of plants materials was found to reduce the microbial contaminants in plant *in vitro* cultures [12].

It was also observed that, bacterial colonies were also found in the culture medium. Earlier reports [6], [8] revealed that bacteria like *Proteus sp, Erwinia sp, Klebsiella sp, Staphylococcus sp, Pseudomonas sp* etc. were found in banana culture media, which spoil the explant and it will affect the clonal propagation. However there were certain bacteria which were endophytic and are beneficial to the culture media [13]. In the present study the bacterial colonies invaded in the media and almost damaged the explant. Bacterial samples were collected and serially diluted and plated on nutrient agar. We could not identify the bacteria by noting colony characters a (Fig.1). Hence the isolates were subjected to Gram staining [6]. Based on gram staining we could isolate three bacterial types (Fig. 2). Among these two bacteria were gram positive and one was gram negative, and named as Bacteria A, Bacteria B and Bacteria C respectively. Bacteria A were spherical in shape; Bacteria B and Bacteria C was rod shaped. Gram staining itself was not sufficient to identify the genus/species correctly. Hence the isolated bacteria were subjected to 16S DNA analysis. 16S RNA analysis is a new technique to identify genus/species/strains of bacteria [14]. 16s ribosomal RNA genes of approximately 1500 bp in length. rRNA genes contain region of variable DNA sequence that are unique to the species carrying the gene. It forms the part of the 30s subunit of prokaryotic ribosome that binds to the shine-dalgarno sequence. The genes coding for it is referred to as rRNA gene and are used in reconstructing phylogenies, due to the slow rate of evolution of this region. Among the three bacteria viz. bacteria A, bacteria B and bacteria C, only bacteria C 16S DNA could not be amplified using universal primers. The 16S DNA sequence of bacteria A composed of 888bp in forward sequence (Fig.5) and 866 bp in reverse sequence (Fig.6). The 16S DNA sequence of bacteria B composed of 862 bp in forward sequence (Fig.7) and 877 bp in reverse sequence (Fig.8). The sequences were subjected to BLAST analyses. The output was compared with nucleotide data bank of NCBI. On comparison it was found that, the bacteria A identified was *Staphylococcus warneri* (Sequence ID: MH014968.1) and the bacteria B identified as *Pseudomonas nitroreducens* (Sequence ID: MF627712.1).

The isolated bacteria were subjected for antibiotic sensitivity study [6]. Antibiotics such as Taxim-O, Tetracycline (tet) and Ampicillin (amp) were used to study the sensitivity. For the study isolated bacteria were plated on nutrient agar. Concentrations used were 0.0833 gm/ 100ml, 0.1666gm / 100ml and 0.332gm / 100ml. The method adopted was disc diffusion method. The antibiotic sensitivity tests revealed that all bacteria were more sensitive to Tetracycline than Ampicillin (Fig. 2, 3, 4). Results also showed that Taxim-o had inhibitory effect on all bacteria studied at higher concentration (0.3332 gm/100ml). Bacteria C were more sensitive to Tetracycline at a lower concentration of 0.1666gm/100ml.

The antibiotic Taxim-O was commonly used in banana tissue culture due to its low cost and broad spectrum nature [15]. However in our study the Taxim was less effective towards bacteria A, bacteria B and bacteria C. The bacteria very susceptible to Tetracycline, Vancomycine and Streptomycine showed slight susceptibility to Ampicillin, Cefotaxime and Penicillin G only at high concentrations [16].

Previous studies revealed that single antibiotic treatments were ineffective against bacteria isolated from plant tissue cultures [17]. Hence other antibiotics and combination may be tried latter. In the present study Tetracycline was found to be more effective.

In the present study it was found that *Pencillium* was the fungus which was frequently found in culture media, can be eradicated by using Sodium hypochlorite solution at 0.3 to1.0% 5 to 30 minutes or aqueous Mercuric chloride at 0.1 to 1.0% for 8 minutes [18]. Generally, this surface sterilization eliminates most epiphytic contaminants expect endophytic ones [19]. Procedure including Bavistin treatment at 0.5% for 2 hours [18] might eliminate the *Penicillium*.

It was reported that bacteria like *Proteus sp, Erwinia sp, Klebsiella sp, Staphylococcus sp, Pseudomonas sp* etc. were common in banana culture media [6] [8]. In present investigation the species of *Staphylococcus* and *Pseudomonas* were identified as *Staphylococcus warneri* and *Pseudomonas nitroreducens*. Tetracycline was found to be more effective and a composition of tetracycline and rifampicin antibiotics could be used to control both these genera [16]. Further investigation with more antibiotics are required to suggest new combination of antibiotics to eradicate these two species of bacteria in banana tissue culture. Also, phytotoxicity studies to be carried to determine the effect of the antibiotic on the *in vitro* plantlet growth.

5. Conclusion

Microbial contaminants are very common in the mass production of banana under *in vitro* condition. We conducted a study on the microbial contaminants of banana tissue culture media and found that both fungi and bacteria were present. On identification it was found that *Penicillium* was the fungus present in the medium. Three types of bacteria could be isolated from the media and it could not be identified by conventional methods hence subjected for 16S RNA assay. It revealed that the bacteria present in the medium was *Staphylococcus warneri* (Sequence ID: MH014968.1) and the bacteria B identified as *Pseudomonas nitroreducens* (Sequence ID: MF627712.1). We could not identify one of the isolates using universal primers. An antibiotic sensitivity study was conducted and found that the antibiotic Tetracycline was more effective for all the bacteria isolated. However more combinations and phytotoxic studies should be conducted to ascertain its suitability.

6. Acknowledgement

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7. References

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