OPEN ACCESS Freely available online

Journal of Bacteriology & Parasitology

Research Article

Isolation, Characterization and Identification of Contaminant Bacteria from Sugarcane (*Saccharum officinarum L.*) *in vitro* Culture in Tigray Biotechnology Center, Mekelle, Ethiopia

Tsehaye Kidus[1*], Zenebe Teka[2]

[1]Tigray Biotechnology Center, Mekelle, Ethiopia; [2]Aksum University, Aksum, Ethiopia

ABSTRACT

Plant tissue cultures can be contaminated by extensive diversity of bacteria and the contaminations are species specific. The contamination can reduce growth rate of shoot and root, multiplication factor, and even cause plant death. The study was conducted at Mekelle University, College of Veterinary Medicine and Tigray Biotechnology center and contaminated *in vitro* sugarcane culture obtained from laboratory of plant tissue culture. The objective of the study was to isolate, characterize, and identify bacteria from contaminated *in vitro* Sugarcane culture and to test the sensitivity of the isolates to the most commonly used antibiotics. Morphological, Gram stain, Endospore stain and Biochemical test method were used to isolate and identify the contaminates and disk diffusion method was used for the drug susceptibility test. Commonly available antibiotics were used for susceptibility testing. In the present study Escherichia, Bacillus and Micrococcus were isolated and identified as the major contaminant bacteria from *in vitro* sugarcane cultures. In antibacterial susceptibility test the isolates of *Bacillus* and *Micrococcus* were susceptible to Gentamicin, Chloramphenicol, Ciprofloxaciline, Tetracycline, Vancomycine, Streptomycin, Penicillin G and Kanamycine demonstrating the efficacy of these antimicrobials for the treatment of sugarcane *in vitro* culture contamination by incorporating in to the sugarcane media formulation while, the third isolate *Escherichia* were resistance to all antibiotic agents.

Keywords: Antibiotics; Bacterial identification; Drug Sensitivity; *In vitro*; Plant tissue culture

INTRODUCTION

Sugarcane (*Saccharumofficinarum L.*) (Family Poaceae) is a complex aneupolyploidy plant with chromosome number in somatic cells ranging from 2n=8×=80-124 (in cultivated types) to 2n= 10×=48-150 (in wild types). It is perennial herbaceous plant, propagating vegetatively through underground structure. Sugarcane is one of the most efficient species in the plant kingdom in terms of biomass production [1]. It is an economically useful crop in tropical and subtropical countries accounting for nearly 70 to 75% of the world's sugar production [2,3]. Sugarcane is also important source of vinegar, yeast, sprits, rum, antibiotics, paper and particle boards, animal feed, molasses and biofertilizers, and lately bioethanol and other alcohol derived chemicals [2,4,5].

Sugarcane is conventionally propagated through cuttings of mature cane stalk. However, the conventional techniques are faced with critical limitations. First, the use of seed cane cuttings requires considerable volume of cane stalk that would have been sent for processing. It is also quite laborious and time consuming, thus expensive. This technique, with multiplication rate as low as 1:6, does not allow large-scale expansion of sugarcane plantations within few years. Large-scale expansion of sugar estates would require millions of tons of cane as planting material. Second, the techniques do not allow the introduction and expansion of newly developed varieties with good traits quickly. Varieties with good traits can be developed using the conventional and/or micropropagation techniques. But, the newly developed varieties require eight to ten years to bulk up to commercial scale; by the time they start deteriorating due to

Correspondence to: Tsehaye Kidus, Student, Tigray Biotechnology Center, Mekelle, Ethiopia, Tel: +251 9464648185; E-mail: tsehayemkboy@gmail.com

Received: May 15, 2020; **Accepted:** June 05, 2020; **Published:** June 12, 2020

Citation: Kidus T, Teka Z (2020) Isolation, Characterization and Identification of Contaminant Bacteria from Sugarcane (*Saccharum officinarum L.*) *in vitro* Culture in Tigray Biotechnology Center, Mekelle, Ethiopia. J Bacteriol Parasitol. 11:372. DOI: 10.35248/2155-9597.20.11.372.

biological and physical stresses [2,3,6]. Third, vegetative propagation methods are associated with high risk of spreading various parasites and pathogens that reduce the viability and productivity of the crop. Sugarcane plantations are easily affected by bacterial, fungal, viral, and phytoplasmal diseases [5-7].

The development of tissue culture and micropropagation techniques in the 1960s was important break-through solving these problems in sugarcane propagation [8,9]. Micropropagation and tissue culture techniques has several benefits over conventional techniques, including: rapid propagation of new varieties quickly, rapid spreading and bulking up of newly released varieties, production of true-to-type planting materials, production of large number of plants in short time throughout the year, generation of pathogen and pest-free, stress-resistant, healthy and uniform planting materials, elimination of the spread of pathogens and pests, and regaining of vigor and renovation of old and degenerated varieties [6,10].

Plant tissue culture and micropropagation, especially in commercial, large-scale facilities, are affected by microbial (e.g. viral, bacterial, fungal) contaminations [11]. Microbial contamination in tissue culture and micropropagation facilities, oftentimes, affect the tissue culture/micropropagation media and the explants/plantlets. This implies that contamination can occur during explant/plantlet preparation and handling, media preparation, and media inoculation with explants/plantlets at all stages (initiation, propagation and rooting) [12]. Microbial (esp. bacterial) contamination causes growth reduction, poor root health and rooting, and the death of explants/plantlets altogether [13]. The principal causes of microbial contamination in tissue culture and micropropagation facilities are poor aseptic techniques and changing of tissue culture conditions in favor of microbial growth [12,14,15]. Microbial contamination in tissue culture and micropropagation facilities can cause extensive damages and losses due to spoilage of expensive culture, death of plantlets, and waste of money gone for wasteful operational time and inputs as well as production of infected plantlets [16].

The Tigray Biotechnology Center Pvt. Ltd. Co, located in Mekelle, Ethiopia, is probably one of the largest micro propagation facilities in the world with a weekly production capacity of 1.6 to 1.8 million plantlets. The facility rests on 6,700 meter square area with 16 inoculation rooms and 16 growth rooms. It is understandable that the facility can be susceptible to microbial contamination due to technical and cultural limitations both attributable to its massiveness. In fact, the facility is affected by microbial (bacterial and fungal) contaminants coming from the environment because air is source of contamination or Due to may be low personal hygiene and sanitation causing as much as 5% contamination of *in vitro* cultures. Technical personnel of the facility have established that bacterial contamination is more common causing extensive loss of media and plantlets and the wrong design of the premises especially the ceiling part and the growth medium selected for *in vitro* propagation also serves as a good source of nutrients for microbial growth. These microbes further compete adversely with plants for nutrients and environment is also source contamination [17]. Nonetheless, the most common bacterial and fungal contaminants were not yet scientifically identified, thus no remedial measures are put in place to reduce or mitigate contamination. The identification of the culprits and the establishment of their susceptibilities to some common antibiotics are important initiatives towards putting remedial measures to reduce or mitigate contamination. This article reports the findings of a study conducted to isolate, characterize, and identify bacterial contaminants of sugarcane plantlets in the micropropagation facility of the Center and to test the susceptibility of the isolates to different antibiotics.

Statement of the problem

In the Tigray Biotechnology center plant tissue culture laboratory bacterial contamination usually cause great loss of expensive *in vitro* culture of sugarcane, time and production plan and the problem affects the institute and the huge agricultural sectors that depend on Tigray Biotechnology center. Therefore, isolation and characterization and identification of the contaminated *in vitro* culture are necessary and important to initiate and determine control strategies for the critical problem.

Objective

General objective: To isolate, characterize and identify the bacteria from contaminated *in vitro* Sugarcane culture in Tigray Biotechnology Center PLC and to test the susceptibility of the isolates to different antibiotics.

Specific objectives:

- To isolate the contaminating bacteria *in vitro* sugarcane cultures.
- To identify the bacterial isolates to genus level using morphological, biochemical and other parameters.
- To test the sensitivity of the isolates to the most commonly used antibiotics.

Significance of the study

The results obtained from this study were used to devise strategies to control the bacterial contaminants of *in vitro* sugarcane cultures in the company. The Knowledge and skill of the isolation and identification method provides relevant information on the extent of the bacterial contamination, and helps to identify contamination control mechanisms and selection and integrating of appropriate antibiotics for the sugarcane media formulation. The direct beneficiaries of the result of the study were Tigray biotechnology Center and some agricultural sectors.

LITERATURE REVIEW

Definition of plant tissue culture

Plant tissue culture refers to the *in vitro* cultivation of plants, seeds, plant parts (tissues, organs, embryos, single cells, protoplast, etc.) on nutrient media under aseptic conditions. Plant tissue culture is an important tool to propagate the plants in large scale through the eminent way in the short [18]. Culture of plant and various parts in the aseptic condition with the concept of totipotency [19,20]. A special media fortified with

inorganic nutrients, vitamins, carbohydrates and environmental factors are added *in vitro* condition [21]. Cell totipotentiality and cellular plasticity is the major physiological principle behind the plant tissue culture. Cell plasticity responses for the division and differentiation capacity of the culture cells [20]. The ability of the single cell to transform into a whole plant alike as the mother plant [21]. It is possible to distinguish tissue culture in to various types seed culture, embryo culture, organ culture, callus culture, cell culture, protoplast culture, etc [22].

Bacterial contamination in plant tissue culture

Bacterial contaminations are a serious problem in plant *in vitro* cultures, both in commercial plant micro propagation, by making difficult culture initiation, reducing efficiency of multiplication and rooting of shoots, as well as in research laboratories, where contamination can be the causal agent of false results in physiological experiments [23,24]. The diversity and abundance of genera and species of exo and endobiotic bacteria accompanying donor plants is a major challenge in the sterilization of initial explants, a quick detection of bacteria in the first *in vitro* passages and a minimization of their adverse effect on shoot multiplication and rooting efficiency [25]. In most cases, bacteria are introduced to the cultures together with initial explants.

In practice, initial explants are only surface sterilized and thus internally living microorganisms are introduced to *in vitro* cultures. If symptoms of bacteria colonizing plant tissues appeared within a short time, the contaminated explants should be immediately removed. In case when bacterial growth is very slow or temporarily retarded in plant culture conditions, they remain in a cryptic state and may appear only when the culture conditions will drastically change, for example after delayed subculture, increase of temperature, change of medium composition or due to other factors [26].

Sources of contaminants

The sources of contaminated cultures usually are difficult to determine [14]. Bacteria which contaminate plant cultures may originate from explants. Laboratory environments, Operators, mites and thrips or ineffective sterilization techniques Bacteria are associated with plants as epiphytes or entophytes [27,28]. Explants from field grownplants, diseased specimens or from plant parts which are located close to or below the soil may difficult or impossible to disinfect due to both entophytic and epiphytic microbes [14]. Contaminants of greenhouse-grown plants are mostly those associated with soil and may originate from irrigation water [29,30].

Bacteria which infect micropropagated plans can be divided in to three type bacteria which cause disease both during micropropagation and on weaned plants (plant pathogens), bacteria which cause disease only during micropropagation (opportunistic pathogenic contaminants) and bacteria which can not cause disease either during micropropagation or later (contaminants). Most problems occur with opportunistic pathogenic contaminants [31].

Epiphytic bacteria may lodge in plant structures where disinfectants cannot reach [28]. Endophytic bacteria may be localized within the plant at cell junctions and the intercellular spaces of cortical parenchyma [28]. Contaminants found at explants initiation. Present in explants from several collection dates and resistant to surface disinfestations are likely to be endophytic [32].

Effects of bacterial contaminations

Microorganisms at high concentrations increase the risk of Laboratory Acquired Infections (LAI) [33]. *Escherichia coli* provide a good example of the problems of increased virulence. Verotoxin producing *E.coli* 0157:H7 can cause severe human disease [34]. *In vitro* micropropagation of plant cultures with yeast resulted in isolation of thirty one microorganisms including *Salmonella typhi* which causes laboratory acquired fever [14]. Typhoid fever accounted for more fatalities than any other LAI [35]. Microbial contamination has been reported to be higher in preparation rooms than incubating rooms [36]. This is due to the fact that more people frequent the preparation room. Presence of bacterial in the preparation room indicates the presence of people and their levels get higher when the building is heavily populated identified a case of Flexner dysentery spread by *Shigella spp* from quality control specimen. Sources of infections include finger licking, page turning as well as pen chewing among the laboratory personnel [34,37].

Indexing cultures

Detection of bacterial contaminants has traditionally been haphazard Visual inspection of the medium at the base of the plant may provide evidence of some contaminants. But it is not adequate for slow growing bacteria, endophytes or those bacteria which do not grow on plant tissue culture media [38]. Screening methods must be favorable to bacterial or fungal growth, and easily used and interpreted [29]. Screening procedures are available for identifying many contaminants [39,40]. Cultures free of cultivatable contaminants have been established as the result of screening procedures in both commercial and laboratory situations [32,38]. Some bacteria, which are especially difficult to culture, require specialized media [28,41]. But most common contaminants can be detected with screening on two or three commercially available bacteriological media [32,38]. A culture indexing system involving serial stem slices inoculated into liquid and agar solidified yeast extract-glucose, Sabouraud-Glucose and AC media and incubated for three weeks at 30°C detected most contaminants from more than 60 aquatic, marsh, and ornamental woody plant species. In most cases, a contaminant would grow on two of the three media [38]. Initial growth of explants in a liquid culture system at pH 6.9 and later testing on 523 bacterial medium detected most contaminants from over 400 mint explants [32]. Contaminated cultures are sometimes rooted and transferred to the greenhouse instead of being discarded. This is a risky procedure, because contaminants which cause no visible harm to plant cultures may become pathogenic under greenhouse conditions [38].

Identification and characterization

Contaminants can be purified using standard bacteriological methods and characterization with biochemical tests such as Gramstain. Motility, gelatinase, oxidase, and O/F (oxidation/fermentation) [39-42]. Bergey's Manual of Systematic Bacteriology contains descriptions of genera and species which are helpful for identifying bacteria [43]. These traditional tests are labor intensive and time consuming, but may be performed in any laboratory with common chemicals.

The first method of choice for rapid, cost effective identification unknown bacteria is fatty acid profiling. Fatty acid profiling has excellent potential for identification down to species level [31,44]. Identification at infraspecific levels (sub species, biovar, pathovar) where necessary can know be readily obtained by some genetic finger printing techniques. Of these the various repetitive sequence – polymerase chain reaction derived fingerprints are now being used for routine identification by comparison of fingerprints with libraries. These methods also give better absolute proof of identity than fatty acid profiling, but their use complaints rather than replaces such methods [45].

Detection of specific bacteria is now largely done by three methods, traditional isolation, serological methods such as immune fluorescence and ELISA and increasingly by use of chain reaction [46]. This latter method has great potential sensitivity but is often inhibited by plant components. These problems may be less difficult with micropropagated material but hence PCR has great potential for detecting specific pathogens in micro plants.

Identification techniques which provide results in 24-48 h are now available. The Biology system detects carbon source utilization with the reduction of tetrazolium dye in response to cellular respiration. The results are compared with a response data base of Gram negative and positive bacteria, yeasts and lactic acid bacteria [47,48]. The API identification system is also a carbon source utilization test, but it relies on visual detection of the test bacterium [49]. Fatty Acid Analysis Profiles match fatty acid methyl esters with those of known organisms [29,44,50]. DNA probes and 16SrRNA use PCR amplification and probes for known sequences [51]. The reliability of these systems depends upon the number and diversity of bacteria in the databases. Many soil and plant bacteria have not been described or characterized making these procedures less useful for plant biologists suggest the use of more than one test for a more accurate identification [29,49,52].

Antibiotic treatment

Entophytic bacterial contamination is an important problem in plant tissue culture and cannot be eliminated with any surface sterilization techniques, thus require antibiotic therapy [53,54]. Ideal antibiotics should be soluble, stable, unaffected by pH, unaffected by media. Lacking side effects, broadly active, bactericidal, and suitable in combination, non-resistance inducing, inexpensive, and nontoxic to human health [55]. Judicious use of antibiotics is especially important. An analysis of published research concludes that antibiotics are often incorporated as prophylactics in the tissue culture medium or are used to suppressor eliminate bacteria once a contaminant is detected [56]. The continued use of antibiotics in the medium or repeated treatments with a single antibiotic may lead to bacterial resistance [53]. Care must be taken to insure that antibiotics are bactericidal rather than bacteriostatics is often the case, and that the cultures are monitored for recurrence of bacteria [54].

Many antibiotics exist that have not yet been evaluated on plants or their bacteria contaminants [57]. Antibiotics are grouped by mode of action: inhibitors of bacterial cell wall Synthesis, inhibitors of bacterial protein synthesis, and DNA replication blockers [58,59]. Antibiotics can also be grouped by chemical structure: aminoglycosides, quinolones, B Iactams, glycopeptides, polymixins, macrolides and lincosamides [57]. The choice of antibiotic is dependent on the type of bacteria present (i.e. Gram negative or Gram positive), so initial characterization with Gram staining and some simple biochemical tests is essential [29]. Carbenicillin, Cephalothin, Gentamicin, Polymyxin, Pifampicin, Streptomycin, and Timentin have been used to treat plant tissue cultures [29,55,60].

Antibiotics are incorporated into plant tissue culture media or used as a brief treatment for specific contaminants [12]. Authors recommend the use of short antibiotic treatments to prevent the development of antibiotic resistance in bacterial contaminants [12,60]. It is also very important to determine whether antibiotics are bactericidal instead of bacteriostatic to avoid reoccurrence of bacteria [54]. Combinations of antibiotics may be more effective in killing contaminants [55,60]. Cornu and Michel (1987) suggested that it is crucial to know the effect of antibiotic on both the bacteria and explant to be able to eliminate contaminants [61].

Diagnostic techniques

Culture and morphological staining of bacteria: The isolation and identification of colonies in different culture media should perform using standard bacteriological procedures as described by H.C.Gram in 1884 discovered the Gram stain classification remains an important and useful technique until today [62,63]. This technique classifies bacteria as either Gram positive or negative based on their morphology and differential staining properties [64]. The representative bacterial colonies in any clinical materials should be characterized morphologically using Gram's stain described by Merchant and Packer (1967).

Hemolytic activity: To characterize the hemolytic patterns isolated strains should be tested for hemolysis on bovine BA plate by incubating them at 37°C for 24 hours. Hemolytic patterns should categorized as: Alpha (α) hemolysis: a zone of greenish discoloration around the colony manifested by partial hemolysis. (β) Hemolysis; complete clear zone of hemolysis around the colony and Gamma (γ) hemolysis: no detectable hemolysis [65].

Reactions of the organisms in TSI agar slants: Triple sugar iron agar (TSI agar) used to detect the lactose, sucrose and dextrose fermenter and also the bacteria which produce hydrogen sulphide. The organisms seeded over the surface of the slants

and stabbed into the butt where the cases changes after an incubation of 24 hours at 37°C [65].

Sugar fermentation test: The sugar fermentation test used to perform whether the bacteria utilize sugar or not, five basic sugars (e.g., dextrose, sucrose, lactose, maltose, and mannitol) separately according to the procedure described [66].

Catalase test and coagulase test: Slide catalase and tube catalase tests used to perform to differentiate the isolated bacteria whether coagulase positive or negative samples should be recorded according to the procedure described by [66].

Indole test, Methyl red test, and Voges-Proskauer test: These tests used to differentiate the isolated bacteria from various bacteriological samples collected. The test should perform and result should be interpreted according to the standard procedure described by [66].

Methods of microbial preservation: Several methods have been successfully used for the preservation of microorganisms: repeated sub-culturing, preservation on agar beads, oil overlay of slant-grown cultures, use of silica gel and other sterile supports, cryopreservation and lyophilization [67-72].

Hydrogen sulfide (H_2S): In presence of H^+ and a sulfur source (sodium thiosulfate, sulfur-containing amino acids and proteins) many bacteria produce the colorless gas H_2S. For detection of H_2S, a heavy-metal (iron or lead) compound is present that reacts with H_2S to form black colored ferrous sulfide. H_2S producing microbes are *Salmonella*, *Edwardsiella*, *Citrobacter*, and *Proteus sp*. [73].

Citrate utilization: Citrate is utilized by several of the *Enterobacteriaceae*as a single carbon source. To test this ability bacteria are incubated in medium that contains only citrate as a source of carbon [74]. Ammonium phosphate is available as a nitrogen source. *Enterobacteriaceae* that can utilize citrate will extract nitrogen from ammonium phosphate releasing ammonia. Ammonia produces an alkaline pH shift and the indicator bromothymol blue turns blue from its green color at neutral pH. Citrate utilization is a key biochemical property of *Salmonella*, *Citrobacter*, *Klebsiella*, *Enterobacter* and *Serratia sp* [75].

Urease reaction: Urease hydrolyzes urea releasing ammonia which alkalinizes the medium by forming ammonium carbonate, and the pH indicator phenol red becomes red. Proteus, Morganella, and Providencia are strong urease producers, Klebsiella a weak urease producer and *Yersinia enterocolitica* frequently a urease producer [75]. Urease Producing bacteria include *Proteus Morganella*, *Providenciarettgeri*, *Klebsiella pneumonia*, *Klebsiellaoxytoca*, *Enterobacter cloacae* and *Yersinia enterocolitica* [76].

MATERIALS AND METHODS

Description of study area

The experiment of this study was conducted at the Tigray biotechnology center and Mekelle University college of Veterinary Medicine and in Mekelle, Tigray, and Northern Ethiopia. Both centers are located 789 km North of Addis Ababa at an altitude of 1979 meter above sea level, 13030' 0" N latitude and 39028'11"E longitudes.

Study material

Sugarcane tissue culture bottles contaminated with frequently encountered bacterial contaminants were used as the starting material for the study.

Study material collection and transportation

The study was conducted at the research laboratory of the Tigray Biotechnology Center PLC (formerly Mekelle Plant Tissue Culture Laboratory) and the microbiology laboratory of the College of Veterinary Medicine of Mekelle University in Mekelle, Tigray, Ethiopia. Fifty (50) bottles with contaminated *in vitro* sugarcane shooting media were collected from the growth rooms of the commercial micropropagation facility of Tigray Biotechnology Center. The bottles were immediately sealed off using film tape to avoid further contamination of the media as well as to prevent the spread of the contaminants from the bottles to the facility and the surrounding. The bottles were individually labeled, packed in medical primary box, and were shipped to the microbiology laboratory of the College of Veterinary Medicine, Mekelle University. The samples were stored in a refrigerator set at 4°C until needed [62] (**Figure 1**).

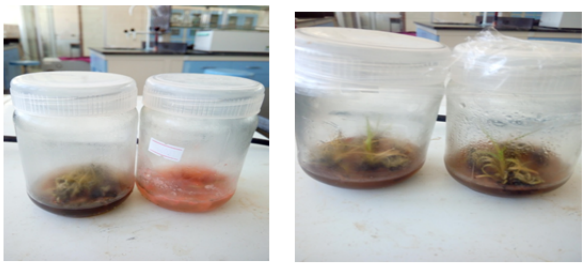

Figure 1: Contaminated *in vitro* sugarcane culture bottle.

Study methodology

Bacterial isolation: A Suspension of 1 g of contaminated sample was taken from each samples of tissue culture bottles and adds in to 9 ml of sterile distilled water. The sample were serially diluted from 10-1 until 10-6 and then each dilutions was inoculated on to pre-prepared Nutrient agar plates by taking 0.1 ml inoculums and then incubated at 37°C for 24 hours. Then plates with countable colonies were selected and the colonies were carefully picked using an inoculating loop and streaked on to freshly prepared nutrient agar separately. All together 50 colonies were isolated for further screening.

Bacterial identification: The isolates were sub cultured to get consistent and pure colonies morphology. In doing so each colony was given a different code (they were numbered from 1-50).Finally, all the colonies merged to only three different colony morphologies and these were coded as WC, PC, and YC. Once consistent colony morphologies were arrived at, they were

transferred in to nutrient agar slants and were kept in a refrigerator at 40°C for further characterization.

Bacterial characterization: Then the isolates will be characterized by applying different microbiological tests including gram staining, spore staining, and different biochemical tests. In the identification process colony morphologies (colony size, margin, elevation, color, smoothness, roughness), biochemical characteristics (Catalase test, Citrate fermentation, MRVP, Indole test, and triple sugar iron tests for sugar fermentation) were carried out. Colonies from primary plates were also cultured in Blood agar, Mannitol salt agar (MSA), *salmonella-shigella* (SS) agar, MacConkey agar, and MacConkeysarbitol agar and incubated at 37°C for 24 to 48 hours for confirmation.

Antimicrobial susceptibility test of isolates: Antimicrobial susceptibility test was conducted using the disk diffusion method as defined by Jorgensen and Ferraro (2009) and the inoculum or isolates were prepared separately by touching with a loop full of 3-5 colonies of similar appearance of the organisms to be tested and transferred to a tube of 0.38% normal saline and the samples were compared with the 0.5 McFarland turbidity standard(approx. cell density 1.5 × 108 CFU/ml) and Streaked the swab all over the surface of freshly prepared medium called Mueller Hinton agar three times by rotating the plate through an angle of 60° after each application and finally the swab were pass round the edge of the agar surface and after 5 minutes the 13 common antibiotics impregnated disc including Ampicilline, Gentamicin, Kanamycin, Norfloxacin, Streptomycin, Chloramphenicol, Ciprofloxacin, Tetracycline, Rifampicine, Vancomycine, Penicillin G, Erythromycin and Amikacine were placed on the superficial of the inoculated plates using a pairs of forceps separately **(Table 1)**.

Table 1: Antibiotics and their contents.

Antibiotics	Content in µg	Antibiotics	Content in µg
Amikacin	30	Norfloxacin	10
Ampicillin	10	Penicillin G	10
Chloramphenicol	30	Rifampicin	5
Ciprofloxacin	5	Streptomycin	10
Erythromycin	15	Tetracycline	3
Gentamicin	10	Vancomycin	30
Kanamycin	30		

The plates were incubated for 24 hours at 35°C according to Disk diffusion or Kirby- Bauer method [77]. The diameters of the inhibition zones around the disks were measured and recorded. The antimicrobial agents were categorized into susceptible, intermediate, and resistant categories according to National Committee for Clinical Laboratory Standards NCCLS (2007) [78].

Data management and analysis

The collected laboratory results were coded and entered in to Microsoft Excel and analyzed. Descriptive analyses and frequency distribution were computed. Data were narrated and interpreted using narration approach of data analysis. Then the results were presented using tables, figures and graphs accordingly.

RESULTS

Cultural and morphological characteristics

In the current study, the major bacterial species isolated from *in vitro* sugarcane culture were coded as PC, YC and WC based on cultural response on different selective media and morphological features and there consistent characteristics. The isolated bacteria coded as PC produced Greyish white colored colonies on Nutrient agar and the growth was displayed by Circular, moist, smooth, raised, the isolated bacteria coded as YC produced yellow colored colonies and the growth was showed similar as the bacterial isolate code PC while the isolated organism WC were produced white colored colonies and the growth was indicated by irregular, Mucoid and flat on the Nutrient agar plate **(Table 2)** **(Figure 2)**.

Table 2: Morphological characterization of the isolates (PC, YC and WC) isolated from the contaminated *in vitro* sugarcane culture after 24 hours in NA.

Culture Media Used	Morphological characteristics of colonies	Isolated bacterial		
		PC	YC	WC
NA	Color	Greyish white	Yellow	White
NA	Shape	Circular	Circular	Irregular
NA	Texture	Moist	Moist	Mucoid
NA	Size	Medium	Medium	Large
NA	Nature	Discrete (separate)	Discrete (separate)	Connected
NA	Degree of growth	Profuse	Moderate	Moderate
NA	Elevation	Raised	Raised	Flat
NA	Margin	Smooth	Smooth	-

Cultural response of the three isolates on the different selective and differential media shows that bacterial isolate PC were response Gamma (γ) hemolysis and WC was response Alpha (α) hemolysis on Blood Agar respectively while in YC the culturing test was not conducted and the cultural test on MacConkey, MacConkeysarbitol agar, Salmonella shegella Agar (SSA) and Mannitol salt Agar (MSA) represted **(Table 3)**.

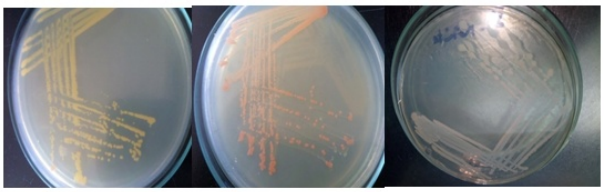

Figure 2: YC growth on NA found in left, PC growth on NA in middle and WC growth on NA in right.

Table 3: Cultural characteristics of representative bacteria isolates on different cultural media for confirmatory test.

Characteristic growth in	Isolated bacterial code		
	PC	YC	WC
Blood Agar (BA)	**Gamma hemolytic**	**N.d**	**Alpha hemolytic**
MacConkey	+	-	-
MacConkey sarbitol agar	+	-	-
Salmonella shegella Agar (SSA)	-	-	-
Mannitol salt Agar (MSA)	-	-	-

Gram stain and biochemical characteristics

The microscopic investigation of Grams stained slurs from agar plates displayed isolates were found Gram-negative, Gram-positive and Gram-positive for the isolated colonies of PC, YC and WC respectively. As confirmed in the experiment PC fermented galactose, glucose, sucrose, xylose, maltose and fructose with the production of acid. WC fermented only glucose, sucrose, and fructose with acid production were as YC fermented none of the tested sugars. The result of Catalase, citrate, TSI Indole, MR and V-P of the isolates is presented **(Tables 4 and 5)**.

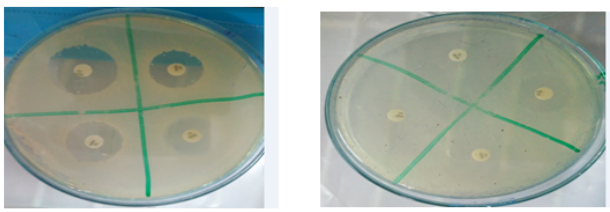

Figure 3: Inhibition of the growth of bacteria isolated from sugarcane culture in the presence of different antibiotics.

Table 4: Gram stain, Endospore and Biochemical test result.

Biochemical Characteristics		Representative bacteria isolates		
		PC	YC	WC
Gram reaction		-	+	+
Endospore		-	-	+
MR		+	-	-
VP		-	-	+
Indole		+	-	-
Catalase		+	+	+
Citrate		-	-	+
TSI	Slant	Yellow	Yellow	Yellow
	Butt	Yellow	Yellow	Red
	Gas	+	-	-
	H2S	-	-	-
Carbohydrate fermentation Test		GA,A		GA,A
		SR,A		GL,A
		GL,A		SU,A
		SU,A	-	XY,A
		XY,A		ML,A
		ML,A		FT,A
		FT,A		

MR:Methyl red; VP :Voges-proskauer; TSI :Triple sugar iron agar; H2S:Hydrogen sulphide production; +:Positive; – :Negative; Galactose:GA; SR:Sorbitol; GL:Glucose; ML:Maltose; SU:Sucrose; A:Acid; AG:Acid and gas.

In the current study the major bacterial species isolated from the contaminated *in vitro* sugarcane cultures were temporarily coded as PC, YC and WC for simplicity. Then their colony morphologies, gram staining, spore staining and biochemical reactions were compared and isolate PC was found to be highly correlated with genus *Escherichia*, YC with genus *Micrococcus* and WC with genus *Bacillus* and thus they were identified to be *Escherichia*, *Micrococcus* and *Bacillus*, respectively.

Table 5: Bacterial Identification based on morphological, Gram stain, Endospore and Biochemical test.

	Identification Keys					
Morphological Characteristics	**Escherichia**	**PC**	**Micrococcus**	**YC**	**Bacillus**	**WC**
Color	Greyish white	Greyish white	Yellow	Yellow	White	White
Shape	Circular	Circular	Circular	Circular	Rod	Rod
Texture	Moist	Moist	Moist	Moist	Mucoid	Mucoid
Size	Medium	Medium	Medium	Medium	Connected	Connected
Nature	Discrete	Discrete	Discrete	Discrete	Large	Large
Degree of growth	Profuse	Profuse	Moderate	Moderate	Moderate	Moderate
Elevation	Raised	Raised	Raised	Raised	Flat	Flat
Margin	Smooth	Smooth	Smooth	Smooth	-	-
2. Gram Reaction	-	-	+	+	+	+
3.Shape of cells	Rod	Rod	Coccus	Coccus	Rod	Rod
3. Endospore	-	-	-	-	+	+
4. Biochemical Characteristics						
MR	+	+	-	-	-	-
VP	-	-	-	-	+	+
Indole	+	+	-	-	-	-
Catalase	+	+	+	+	+	+
Citrate	-	-	-	-	+	+
TSI	Slant	Yellow	Yellow	Yellow	Yellow	Yellow
	Butt	Yellow	Yellow	Yellow	Yellow	Red
	Gas	+	-	-	-	-
	H2S	-	-	-	-	-
Carbohydrate Fermentation Test		GA,A SR,A GL,A SU,A XY,A ML,A FT,A		-		GA,A GL,A SU,A XY,A ML,A FT,A
Interpreting	Escherichia		Micrococcus		Bacillus	

MR:Methyl red; VP:Voges-proskauer; TSI:Triple sugar iron agar; H2S:Hydrogen sulphide production; +:Positive; –:Negative;GA:Galactose; SR:Sorbitol; GL:Glucose; ML:Maltose; SU:Sucrose; A:Acid

Antibiotic sensitivity of bacterial isolates test

Antimicrobial sensitivity study of the isolates using 13 different antimicrobials showed that bacterial isolates identified as Genus *Micrococcus* was highly susceptible to Gentamicin,Chloramphenicol,ciprofloxaciline,Tetracycline,Amikacin,Vancomycine,Streptomycin,PenicillinG- and Kanamycine and bacterial isolate identified as *Bacillus* was highly susceptible to Gentamicin, Ciprofloxaciline, Chloramphenicol, Vancomycine and Kanamycine while bacterial isolate identified as *Escherichia* was found to be resistance to Gentamicin, Amikacine, Ciprofloxacin, Norfloxacin, Chloramphenicol, Streptomycin, Tetracycline, Rifampicine, Vancomycine, Ampicilline, PenicillinG, Erythromycn and Kanamycine Bacillus was found to be resistant to Ampicilline and Rifampicine and micrococcus to Rifampicine in Tigary Biotechnology Center **(Table 6) (Figure 4)**.

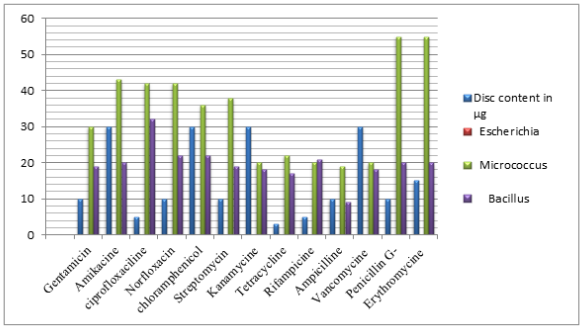

Figure 4: Inhibition zone (mm) of the bacterium of *Escherichia* , *Micrococcus* and *Bacillus*.

Table 6: Antimicrobial Susceptibility pattern the isolated bacteria involved in *in vitro* sugarcane culture.

SN	Antibiotic	Disc Content (in µg)	Inhibition Zone (in mm)			Their Degrees of Susceptibility*		
			Escherichia	Bacillus	Micrococcus	Escherichia	Bacillus	Micrococcus
1	Amikacin	30	0	20	43	R	S	HS
2	Ampicillin	10	0	9	19	R	R	R
3	Chloramphenicol	30	0	22	36	R	S	HS
4	Ciprofloxacin	5	0	32	42	R	HS	HS
5	Erythromycin	15	0	20	55	R	S	HS
6	Gentamicin	10	0	19	30	R	I	HS
7	Kanamycin	30	0	18	20	R	S	S
8	Norfloxacin	10	0	22	42	R	S	HS
9	Penicillin G–	10	0	20	55	R	I	HS
10	Rifampicin	5	0	21	20	R	R	R
11	Streptomycin	10	0	19	38	R	I	HS
12	Tetracycline	3	0	17	22	R	I	S
13	Vancomycin	30	0	18	20	R	S	S

*:HS:Highly susceptible; S:Susceptible; I:Intermediate; R:Resistance

DISCUSSION

Bacterial contaminations are a serious problem in plant *in vitro* cultures, Both in commercial and research laboratories plant micro propagation, by making difficult culture initiation, reducing efficiency of multiplication and rooting of shoots [23,24]. In this current investigation the predominantly isolated were bacteria species include, *Escherichia*, *Bacillus* and *Micrococos* isolated from contaminated *in vitro* sugarcane culture. The

current result is in agreement with a report *Bacillus*, *Corynebacterium*, *Escherichia*, *Streptococcus*, *Staphylococcus*, *Klebsiella*, *Actinobactor*, *Citrobactor*, *Lactobacillus* and *Salmonella* isolated from plant cell laboratories in Nigeria [79]. And the gram negative bacteria which are pathogenic are majorly associated with faecal contamination [80]. The gram positive isolates, *Bacillus sp*, *Corynebacteria sp* and *Actinomycete* were identified as contaminants in the laboratory laminar air flow cabinets and Bacteria species like *Staphylococcus* and *Micrococcus* can be found on human skin scales and during sub-culturing, man can also act as sources of contamination [81,82].

The occurrence of these bacteria contaminants in these plant cultures is supported by others finding this indicates that their presence was not just accidental. Hennerty (1994) identified *Bacillus sp*., a *Corynebacterium sp*. and an *Actinomycete* as contaminants in the M29 rootstocks. Although most of these contaminants might be endogenously embedded in the plant tissues, some of the contaminant might have emanated from contaminated tools.

Remediation of bacterial contamination of plant tissue cultures using exogenous antibiotics is gaining recent acceptance [53,60]. The highest susceptibility of bacterial isolates of Micrococcus and Bacillus attained from the contaminated sugarcane *in vitro* culture to antimicrobials such as Gentamicin, Chloramphenicol, Amikacine and Kanamycine in *in vitro* drug sensitivity test were in agreement with the previous reports and that of Tetracycline, ampiciline and Erythromycin according to the report [83-90]. The resistance of Escherichia isolates to Gentamicin, Amikacine, Ciprofloxacin, Norfloxacin, Chloramphenicol, Streptomycin, Tetracycline, Rifampicine,Vancomycine, Ampicilline, PenicillinG, Erythromycin and Kanamycine, and that of Micrococcus to Rifampicine, tetracycline, ciprofloxacin, penicillin, Amoxicillin, and Norfloxacin. Bacillus to Ampicilline, penicillin and Tetracycline supports the reports of previous studies [90-103].

CONCLUSION AND RECOMMENDATIONS

The outcome of the current study show that *Escherichia*, *Micrococcus* and *Bacillus* as the principal bacteria species isolated from *in vitro* sugarcane contaminated culture bottle indicating that these bacteria are the major cause of contamination. The gram positive bacterial isolates were found to be susceptible to Gentamicin, Chloramphenicol, Ciprofloxaciline, Tetracycline, Vancomycine, Streptomycin, Penicillin G- and Kanamycin. The third gram negative bacterial isolate *Escherichia* were found resistance to all antimicrobial agents, making them ineffective in the fight against *Escherichia*. The multi-drug resistant *Escherichia* isolated in the current study may be a threat because it may spread to other cultures found in the laboratory and even can infect the personnel working in the laboratory. Therefore, based on the results of the present study the following recommendations are given:

Stringent aseptic working conditions be practiced by operators, supervisors and attending to the maintenance and use of autoclaves, laminar air flow, transfer rooms and growth rooms are the first important steps toward evading environmental contaminants and other source of contamination and screening cultures at the initiation stage and again throughout the culture cycle is a second step which can relentlessly reduce the number of contaminants.

Well-disciplined approaches should be established for the prevention and control of bacterial contamination through the coherent using and incorporating the effective antimicrobials originated on the study in to the *in vitro* sugarcane media formulation.

Research should be conducted on the extent of the problem of the multi-drug resistant strain of *Escherichia* by using the different combination antibiotics and this approach can have synergistic effect in controlling the bacterium.

Research should be conduct to characterized bacteria isolates for further identification of species level from tissue culture using molecular tools.

REFERENCES

1. Brumbley SM, Purnell MP, Petrasouits LA, Nielson LK, Twine PH. Developing the sugarcane biofactory for high value biomaterials. Int sugar J. 2007;109(1):5-15.
2. Jalaja NC, Neelamathi D, Sreenivasan TV. Micropropagation for Quality Seed Production in Sugarcane in Asia and the Pacific. Food and Agriculture Organization of the United Nations, Rome, Italy 2008 p:1-46.
3. Pandey RN, Rastogi J, Sharma ML, Singh RK. Technologies for cost reduction in sugarcane micropropagation. Afr J Biotechnol. 2011;10(40):7814-7819.
4. De Oliveira MED, Vaughan BE, Rykiel EJ. Ethanol asfuel: energy, carbondiox balance and ecological foot print. Bioscience. 2005;55(7):593-602.
5. Warakagoda PS, Subasinghe S, Kumar DLC, Neththikumara TS. Micropropagation of sugarcane (*Saccharumofficinarum L.*) through auxiliary buds. Proceedings of the Fourth Academic Sessions. 2007.
6. Singar RS, Sengar K, Garg SK. Biotechnological approaches for high sugarcane yield. Plant Sci Feed. 2011;1(7):101-111.
7. Shannon GJ, Pace R, Di Bella LP. Experience with micropropagated plants of sugarcane in the Hebert. Aust. Soc. Sugar Cane Technologists. 2008;30(1):303-308.
8. Lal N, Singh HN. Rapid clonal multiplication of sugarcane through tissue culture. Plant Tiss Cult. 1994;4(2):1-7.
9. Lee TSG. Micropropagation of sugarcane (*Saccharum spp.*) Plant Cell Tiss Org Cult. 1989;10(2):47-55.
10. Sugarcane Breeding Institute (SBI). Micropropagation for Quality Seed Production in Sugarcane. 2010.
11. Felise HB, Nguyen HV, Pfuetzner RA, Barry KC, Jackson SR, Blanc MP, et al. An inhibitor of gram-negative bacteria virulence protein secretion. Cell Host & Microbe. 2008;4(4):325-336.
12. Cooke DL,Waites WM, Leifert C. Effects of *Agrobacterium tumefaciens*, *Erwiniacarotovora*, *Pseudomonas siringae* and *Xanthomonascampestris* on plant tissue cultures of Aster, Cheiranthus, Delphinium, Iris and Rosa; disease development in vivo as a results of latent infection *in vitro*. J Plant Dis Protec. 1992;99(2):469-481.
13. Leifert C, Camotta H, Waites WM. Effect of combinations of antibiotics on micropropagated Clematis, Delphinium, Hosta, Iris, and Photinia. Plant Cell Tiss Org Cult. 1992;29(1):153-160.
14. Leggat IV, Waites M, Leifert C, Nicholas J. Characterization of micro-organisms isolated from plants during micro-propagation in Nigeria. ISHS ActaHorticulturae. 1994;225(1):240.

15. Leifert C. Quality Assurance systems for plant cell and tissue culture: The problem of latent persistence of bacterial pathogens And Agrobacterium – based transformation vector systems. Act Hort. 2000;530(1):87-91.
16. Sattigeri VJ, Soni A, Singhal S, Khan S, Pandya M, Bhateja P, et al. Synthesis and antimicrobial activity of novelthiazolidinones. Arkivoc. 2005;2(2):46-59.
17. Odutayo OI, Amusa NA, Okutade OO, Ogunsanwo YR. Determination of the sources of microbial contaminants of cultured plant tissues. Plant Pathol J. 2007;6(2):77-81.
18. Alkhateeb AA. A review the problems facing the use of tissue culture technique in date palm (*Phoenix dactylifera L.*). Sci J King Faisal Univ. 2008;9(2):1429.
19. Assareh MH; Sardabi H. Macropropagation and micropropagation of Ziziphusspina-christi. Pesqagropec bras. 2005;40(5):459-465.
20. Bhojwani SS, Razdan MK. Plant Tissue Culture: Theory and Practice: Developments in Crop Science (1st Edn) Elsevier, Amsterdam 1996 Pp:766.
21. Boxus PH, Terzi JM. Control of accidental contaminations during mass propagation. ActaHortic. 1988;225(2):189-192.
22. Chawla HS. Plant biotechnology. Laboratory manual for plant biotechnology Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi, India 2005.
23. Orlikowska T, Zawadzka MB. Invitro Bacteria in plant tissue culture. Biotechnologia. 2006;4(75):64-77.
24. Orlikowska T, Sobiczewski P, Zawadzka M, Zenkteler E. *In vitro*/ The control and eradication of bacterial infections and contaminations in plant tissue culture. Biotechnologia. 2010;2(89):57-71.
25. Leifert C, Ritchie JY, Waites WM. Contaminants of plant-tissue and cell cultures. World J MicrobiolBiotechnol. 1991;71(2): 452-469.
26. Thomas P. *In vitro* decline in plant cultures: detection of a legion of covert bacteria as the cause for degeneration of long term micropropagated triploid water melon cultures. Plant Cell Tiss Org Cult. 2004;7(1):173179.
27. Sigee DC. Bacteria as plant pathogens. In: Sigee DC (eds). Bacterial Plant Pathology: Cell and Molecular Aspects. Cambridge University Press, Cambridge, 1993 pp:1-12.
28. Gunson HE, Spencer-Phillips PTN. Latent bacterial infections: Epiphytes and endophytes as contaminants of micropropagated plants. Physiology, Growth and Development of Plants in Culture. 1994;2(1):379-396.
29. Buckley PM, Dewilde TN, Reed BM. Characterization and identification bacteria isolated microproped mint plants. *In vitro* cell Div Biol. 1995;31(1):58-64.
30. Seabrook JM, Farrell G. City water can contaminate tissue culture stock plants. Bort Science. 1993;28(2):628-629.
31. Stead DE. Identification of bacteria by computer assisted fatty acid profiling. Actahortic. 1988;225(3):39-46.
32. Reed M, Buckley PM, DeWilde TN. Detection and eradication of endphytic bacteria from micro propagated mint plants. *In Vitro* Cell Dev Biol. 1995;3(2):53-57.
33. Flaning B, Morey PR. Control of moisture affecting biological indoor air quality: International society of indoor air quality and climate, Ottawa, Canada. International Society of Indoor Air quality and climate. 2009.
34. Kimura K, Suzuki S, Wachino J, Kurokawa H, Yamane K, Shibata N, Nagano, et al. First molecular characterization of group B *Streptococci* with reduced penicillin susceptibility. Ann Rev Microbiol. 2008;52(8):2890-2897.
35. Pike RM. Laboratory-associated infections: incidence, fatalities, causes and prevention. Ann Rev Microbiol. 2009;33(1):41-66.
36. Odutayo OI, Amusa NA, Okutade OO, Ogunsanwo YR. Sources of microbial contamination in tissue culture laboratories in South western Nigeria. Afr J Agric Res. 2004;2(3):67-72.
37. Homles MB, Johnson DL, Vesley D. Acquisition of typhoid fever from proficiency testing specimens. N Engl J Med. 1980;9(1): 519-521.
38. Kane ME. Indexing explants and cultures to maintain clean stock. *In Vitro*. 1995;31(1):25.
39. Debergh PC, Vanderschaeghe AM. Some symptoms indicating the presence of bacterial contaminants in plant tissue culture. ActaBort. 1988;255(1):77-81.
40. Viss PR, Brooks EM, Driver JA. A simplified method for the control of bacterial contamination in woody plant tissue culture. *In Vitro* Cell/Div-Biol. 1991;279(2):42.
41. George KL, Falkinham JO. Selective medium for the isolation and enumeration of *Mycobacterium avium*-intracellulare and M. *scrofulaceum*. Can J Microbiology. 1986;32(1):1014.
42. Klement Z, Rudoph K, Sands DC. Methods in Phytobacteriology. AkademiaiKiado, Budapest 1990.
43. Krieg NR, Holt JG. Bergey's Manual of Systematic Bacteriology (1st Edn) Lippincott Williams & Wilkins, Philadelphia, United States 1984.
44. Stead DE, Sell wood JE,Wilson J, Viney I. Evaluation of a commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. J ApplBacteriol. 1992;72(3):315-321.
45. Stead DE, Henssey J, Wilson J. Modern methods for identifying bacteria cell. Tissue Org Cult. 1997;52(1):17-25.
46. Elphinstone JG, Stanford HM, Stead DE. Detection of *Ralstoniasolanacearum* in potato tubers *Solanumdulcamara* and associated irrigation water. Bacterial Wilt Disease. 1998;5(1)133-139.
47. Bouzar H, Jones JB, Hodge NC. Differential Characterization of Agrobacterium species using carbon source utilization patterns and fatty acid profiles. Phytopathology. 1993;83(7):733-739.
48. Hildebrand DC, Hendson M, Schroth MN. Usefulness of nutritional screening for the identification of *Xanthomonascampestris* DNA homology groups and pathovars. J Appl Bact. 1993;75(2):447-455.
49. Verniere C, Provost O, Civerolo EL, Garnbin O, Jacquemoud Collet JP, Luisctti J. Evaluation of the biology substrate utilization system to identify and assess metabolic variation among strains of *Xanthomonascampestis*pv.Citri. Appl Environ Microbiol. 1993;59(1):243-249.
50. Chase AR, Stall RE, Hodge NC, Jones JB. Characterization of *Xanthomonascampestris* strains from aroids using physiological, pathological, and fatty acid analysis. Phytopath. 1992;82(1): 754-759.
51. Kjijn N, Weerkamp AH, Davos WM. Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16srRNA and specific DNA probes. Appl Environ Microbiol. 1991;57(1):3390-3393.
52. Jones JB, Chase AR, Harris GK. Evaluation of the Biolog GN Micro Plate system for identifications of some plant-pathogenic bacteria. Plant Dis. 1993;77(1): 553-558.
53. Fellner M, Kneifel W, Gregorits D, Leonhardtm W. Identification and antibiotic sensitivity of microbial contaminants from callus cultures of garlic Allium sativum L. and Allium longicuspis Regel. Plant Science. 1996;113(1):193-201.
54. Mathias PJ, Alderson PG, Leakey RRB. Bacterial contamination in tropical hardwood cultures. Acta Hort. 1987;212(2):43-48.

55. Falkiner FR. Strategy for the selection of antibiotics for use against common bacterial pathogens and endophytes of plants. ActaBort. 1988;225(2):53-56.
56. Leifert C, Waites WM. Bacterial growth in plant tissue culture media. J Appl Bacterial. 1992;72(1):460-466.
57. Falkiner FR. The criteria for choosing an antibiotic for control of bacteria in plant tissue culture. Int Ass Plant Tissue Culture. 1990;60(3):13-23.
58. Pollock K, Badield DG, Shields R. The toxicity of antibiotics to plant cell cultures. Plant Cell Repts. 1982;2(2):36-39.
59. Quesnel LB, Russell AD. Antibiotics: Assessment of Antimicrobial Activity and Resistance. Academic Press, New York. 1983 pp:1-17.
60. Kneifel W, Leonhardr W. Testing of different antibiotics against gram positive and gram negative. Bacteria isolated from plant tissue cultures. Plant Cell Tissue Org Cult. 1992;29(1):139-144.
61. Cornu D, Michel MF. Bacterial contamination in shoot cultures of *PrunusauiumL.*choice and phytotoxicity of antibiotics. Acta Hort. 1987;212(1):83-86.
62. Quinn PJ, Carter ME, Markey BK, Carter GR. Veterinary Microbiology Microbial Diseases, Bacterial Causes of *Bovine Mastitis* (8th Edn). Mosby International Limited, London. 2002 pp:465-475.
63. Swayne DE, Glisson JR, Jack wood MW, Pearson JE, Reed WM. A laboratory manual for the isolation and identification of avian pathogens (4th Edn). Pennsylvania, USA. American Association of Avian Pathologists, University of Pennsylvania, United States 1998 pp:4-16.
64. Frank L. Bacterial classification, structure and function. Pathophys. 2009;1(1):1-2.
65. Cheesbrough M. District Laboratory Practice in Tropical Countries (2nd Edn) Cambridge University Press, Cambridge, United Kingdom 2006 P: 100-194.
66. Ryan KJ, Ray CG. Sherris Medical Microbiology (4th edn). McGraw Hill, New York, United States 2004 pp:232-390.
67. Winters RD, Winn WC. A simple effective method for bacterial culture storage: a brief technical report. J BacteriolVirol. 2010;40(3):99-101.
68. Nakasone KK, Peterson SW, Jong SC. Preservation and distribution of fungal cultures. In: Muller BG, Foster MS (eds). Biodiversity of Fungi: Inventory and Monitoring Method. Elsevier Academic Press, Amsterdam, 2004 pp:37–47.
69. Smith D, Ryan J, Stackebrandt E. The ex situ conservation of microorganism: aiming at a certified quality management. EOLSS Publisher, Oxford, UK. 2008.
70. Gorman R, Adley CCN. An evolution of five preservation techniques and conventional freezing temperatures of -20 C and -85 C for long term preservation of *Campylobacter jejuni*. LettApplMicrobiol. 2004;38(3):306-310.
71. Berner D, Viernstein H. Effects of protective agents on the viability of *Lactococcuslatis* subjected to freeze-thawing and freezing drying. Sci Pharm. 2006;74(3):137-149.
72. Morgan CA, Herman N, White PA, Vesey G. Preservation of micro-organisms by drying; a review. J Microbiol Methods. 2006;66(3):183-193.
73. Abdurakhmonov IY, Buriev ZT, Saha S, Pepper AE, Musaev JA, Almatov A, et al. Microsatellite markers associated with lint percentage trait in cotton, *Gossypiumhirsutum*. Euphytica. 2007;156(1):141-156.
74. McCrea K, Xie J, LaCross N, Patel M, Mukundan D, Murphy T, et al. Relationships of non-type able Haemophilus influenza Strains to Hemolytic and Nonhemolytic*Haemophilushaemolyticus* strains. J ClinMicrobiol. 2007;46(2):406-416.
75. Del L, Jaramillo M, Talledo M, Pons M, and Flores L, Quispe RL, et al. Development of a 16S rRNA PCR-RFLP assay for Bartonella identification. Applicability in the identification of species involved in human infections. Universal Journal of Microbiology Research. 2014;2(1):15-22.
76. Mukundan D, Ecevit Z, Patel M, Marrs C, Gilsdorf J. Pharyngeal Colonization Dynamics of *Haemophilus influenza* and *Haemophilushaemolyticus* in Healthy Adult Carriers. J ClinMicrobiol. 2007;46(4):1575.
77. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J CliPathol. 1966;45(4):493-496.
78. Performance standards for antimicrobial susceptibility testing; Seventeenth informational supplement, 27 M100-S17, NCCLS, Wayne, PA. National Committee for Clinical Laboratory Standards. 2007.
79. Okuniola A, Adewoyin A, Oluwatoyin A. Evaluation of pharmaceutical and microbial qualities of some herbal medicinal products in SouthWestern Nigeria. Trop J Pharm Res. 2007;6(1): 661-670.
80. Mendoza J, Caso W, Valdez C, Pons M, Valle L, Ore V, et al. Diagnosis of carrion's disease by direct blood PCR in thin blood smear negative samples. J ClinMicrobiol. 2014;9(1):92-183.
81. Liberto MC, Lamberti AG, Marascio N, Matera G, QuirinoA. Molecular identification of *Bartonellaquintana* infection using species specific real time PCR targeting transcriptional regulatory protein (bqtR) gene. Journal of Molecular Cell Probes. 2011;25(1): 238–242.
82. Trudeau WL, Fernández-Caldas E. Identifying and measuring indoor biologic agents. J Allergy ClinImmunol. 1994;2(1):393-400.
83. Fazlani SA, Khan SA, Faraz S, Awan MS. Antimicrobial susceptibility of bacterial species identified from mastitic milk samples of camel. Afr J Biotechnol. 2010;10(15):2961-2962.
84. Lee YJ, Kim AR, Jung SC, Song SW, Kim JH. Antibiotic resistance pattern of *E. coli* and *Salmonella spp*. Isolated from chicken feces. Korean J Vet Res. 2005;45(1):75-83.
85. Nasrin S, Islam MA, Khatun ML, Akhter S. Characterization of bacteria associated with omphalitis in chicks. Bangl J Vet Med. 2012;29(2):63-68.
86. Amare A, Amin AM, Shiferaw A, Nazir S, Negussie H. Yolk Sac Infection (Omphalitis) in Kombolcha Poultry Farm, Ethiopia. Am-Euras J Sci Res. 2013;8(1):10–14.
87. Abass MH. Microbial contaminants of date palm (Phoenix dactylifera L.) in Iraqi tissue culture laboratories. Emir J Food Agric. 2008;25(11):875-882.
88. Ahmad I, Hussain T, Ashraf I, Nafees M, Maryam, Rafay M, et al. Lethal Effects of Secondary Metabolites on Plant Tissue Culture. Am-Euras J Agric& Environ Sci. 2013;13(4):539–547.
89. Bailey RA, Bechet GR. A comparison of seedcane derived from tissue culture with conventional seedcane. Proc S Afr Tech Assoc. 1989;20(1):125-129.
90. Bhoite HA, Palshikar GS. Plant tissue culture: A review. World J Pharm Sci. 2014;2(1):565-572.
91. Boxus PH, Terzi JM. Big losses due to bacterial contamination can be avoided in mass propagation schemes. Acta Hort. 1987;212(1): 91-93.
92. Bunn E, Tan B. Microbial contaminants in plant tissue culture propagation. Microorganisms in plant conservation and biodiversity. 2004;12(1):307-335.
93. Cassels AC, Tahmatsidou V. The influence of local plant growth conditions on non-fastidious bacterial contamination of meristem-tips of Hydrangea cultured *in vitro*. Plant Cell Tiss Organ Cul. 1996;47(2):15-26.

94. Debergh PC. A few academic approaches to problems in tissue culture propagation. Arxius de l'Escola Superior d'Agricultura de Barcelona. 2003;12(1):8-9.

95. Hendre RR, Iyer RS, Kotwal M, Khuppe SS, Mascarenhas AF. Rapid multiplication of sugarcane by tissue culture. Sugarcane. 1983;1(1):4-9.

96. Jorgensen JH. Antimicrobial susceptibility testing: A review of general principles and contemporary practices. Clin Infect Dis. 2009;49(2):1749-1755.

97. Leifert C, Waites B, Keetley JW, Wright SM, Nicholas JR, Waites WM. Effect of medium acidification on filamentous fungi, yeasts and bacterial contaminants in Delphinium tissue cultures. Plant Cell Tiss Org Cult. 1994;36(2):149-155.

98. Progress report on Metrological data. Mekelle Agricultural Research Center. 2012.

99. Misra P, Gupta N, Toppo DD, Pandey V, Mishra MK, Tuli R. Establishment of long-term proliferating shoot cultures of elite *Jatrophacurcas L.* by controlling endophytic bacterial contamination. Plant Cell Tiss Org Cult. 2010;100(2):189-197.

100. Nand L, Singh HN. Rapid clonal multiplication of sugarcane through tissue culture. Plant Tiss Cult. 1994;4(1):1-7.

101. Reed BM, Mentzer J, Tanprasert P, Yu X. Internal bacterial contamination of micropropogated hazelnut: identification and antibiotic treatment. Plant Cell Tiss Org Cult. 1998;52(1):67-70.

102. Williams SD, Boehm MJ, Hand FP. Bacterial Diseases of Plants. Ohio State University. 2008.

103. Seckinger G. The use of antibiotics in plant tissue culture. *In Vitro*. 1995;31(1):25.