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Common Bacterial Contaminants of *invitro* Sugarcane Culture in the Micro propagation Facility of Tigrai Biotechnology Center, Mekelle, Ethiopia

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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.

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Keywords

Antibiotics, Bacterial identification, Drug sensitivity, *in vitro*, Plant tissue culture

Introduction

Sugarcane (*Saccharum officinarum* 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 (Brumbley *et al.*, 2007). It is an economically useful crop in tropical and subtropical countries accounting for nearly 70 to 75% of the world's sugar production (Jalaja *et al.*, 2008; Pandey *et al.*, 2011). 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 (De Olivier *et al.*, 2005; Jalaja *et al.*, 2008; Warakagoda *et al.*, 2007).

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 sugar-cane 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 biological and physical stresses (Jalaja *et al.*, 2008; Pandey *et al.*, 2011; Singar *et al.*, 2011). 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 (Shannon *et al.*, 2008; Singar *et al.*, 2011; Warakagoda *et al.*, 2007).

The development of tissue culture and micropropagation techniques in the 1960s was important break-through solving these problems in sugarcane propagation (Lal & Singh, 1994; Lee, 1989). 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 (Singar *et al.*, 2011; Sugarcane Breeding Institute [SBI], 2010).

Plant tissue culture and micropropagation, especially in commercial, large-scale facilities, are affected by microbial (e.g. viral, bacterial, fungal) contaminations (e.g. Felise *et al.*, 2008). 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) (Leifert, 1992). Microbial (esp. bacterial) contamination causes growth reduction, poor root health and rooting, and the death of explants/plantlets altogether (Leifert and Waites, 1992). The principal causes of microbial contamination in tissue culture and micropropagation facilities are poor asceptic techniques (e.g. Leifert *et al.*, 1994) and changing of tissue culture conditions in favor of microbial growth (e.g. Cooke *et al.*, 1992; Leifert, 2000). 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 (e.g. Sattigeri *et al.*, 2005).

The Tigrai 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 (Odutayo, 2007) and environment is also source contamination. 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 usualy cause great loss of expensive *inviro* 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.

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 (Alkhateeeb,2008).Culture of plant and various parts in the aseptic condition with the concept of totipotency (Assareh and sardabi,2005;Bhozwani and Razdan,1996).A special media fortified with inorganic nutrients, vitamins, carbohydrates and environmental factors are added *in vitro* condition (Boxus and Terzi,1988).

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 Bhozwani and Razdan, 1996). The ability of the single cell to transform into a whole plant alike as the mother plant (Boxus and Terzi, 1988). It is possible to distinguish tissue culture in to various types seed culture, embryo culture, organ culture, callus culture, cell culture, protoplast culture,etc (Chawla,2005).

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 (Orlikowska *et al.*, 2006; Orlikowska *et al.*, 2010).

The diversity and abundance of genera and species of *exo*- and *endobiotic* bacteria accompanying donor plants (Leifert *et al.*, 1991) 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. 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 (Thomas, 2004).

Sources of contaminants

The sources of contaminated cultures usually are difficult to determine (Leifert and Waites,1994).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 (Sigee,1993,Gunson and Spencer Phillips,1994). Explants from field grown plants, 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 (Leifert *et al*.,1994). Contaminants of greenhouse-grown plants are mostly those associated with soil (Buckley *et al*., 1995) and may originate from irrigation water (Seabrook and Farrell, 1993).

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 cannot cause disease either during micropropagation or later (contaminants). Most problems occur with opportunistic pathogenic contaminants (Stead, 1988).

Epiphytic bacteria may lodge in plant structures where disinfectants cannot reach (Gunson and Spencer-Phillips,1994).Endophytic bacteria may be localized within the plant at cell junctions and the intercellular spaces of cortical parenchyma (Gunson and Spencer-Phillips, 1994). Contaminants found at explants initiation. Present in explants from several collection dates and resistant to surface disinfestations are likely to be endophytic (Reed *et al*., 1995).

Effects of bacterial contaminations

Microorganisms at high concentrations increase the risk of Laboratory Acquired Infections (LAI) (Flaning and Morey,1996). *Escherichia coli* provide a good example of the problems of increased virulence. Verotoxin producing *E.coli* 0157: H7can cause severe human disease (Kimura *et al*., 2008). *In vitro* micropropagation of plant cultures with yeast resulted in isolation of thirty one microorganisms including *Salmonella typhi* which causes laboratory acquired fever (Leggat *et al*., 1994). Typhoid fever accounted for more fatalities than any other LAI (Pike, 2009). Microbial contamination has been reported to be higher in preparation rooms than incubating rooms (Odutayo *et al*., 2004).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 (Kimura *et al*., 2008). Homles *et al*., (1980) 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.

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 is not adequate for slow growing bacteria, endophytes or those bacteria which do not grow on plant tissue culture media (Kane, 1995). Screening methods must be favorable to bacterial or fungal growth, and easily used and interpreted (Reed *et al*., 1995)

Screening procedures are available for identifying many contaminants (Debergh and Vander schaeghe, 1988; Viss *et al*.,1991).Cultures free of cultivatable contaminants have been established as the result of screening procedures in both commercial and laboratory situations (Kane, 1995; Reed *et al*., 1995). Some bacteria, which are especially difficult to culture, require specialized media (George and Falkinham, 1986; Gunson and Spencer-Phillips, 1994), but most common contaminants can be detected with screening on two or three commercially available bacteriological media (Kane, 1995; Reed *et al*., 1995).

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 (Kane. 1995). 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 (Reed *et al*., 1995). 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 (Kane, 1995).

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) (Buckley *et al*., 1995; Klement *et al*., 1990). Bergey's Manual of Systematic Bacteriology contains descriptions of genera and species which are helpful for identifying bacteria (Krieg and Holt, 1984). 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 (Stead, 1988; Stead *et al*., 1992).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. This methods also give better absolute proof of identity than fatty acid profiling, but their use complaints rather than replaces such methods (Stead *et al*., 1997).

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 (Elphinstone *et al*., 1998). 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 (Bouzar *et al*., 1995; Hildebrand *et al*., 1993; Jones *et al*., 1993). The API identification system is also a carbon source utilization test, but it relies on visual detection of the test bacterium (Verniere *et al*., 1993). Fatty Acid Analysis Profiles match fatty acid methyl esters with those of known organisms (Buckley *et al*., 1995; Chase *et al*., 1992; Stead *et al*., 1992). DNA probes and 16SrRNA use PCR amplification and probes for known sequences (Klijn *et al*., 1991). 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 (Buckley *et al*., 1995, Jones *et al*., 1993 and Verniere *et al*., 1993) suggest the use of more than one test for a more accurate identification.

Antibiotic treatment

Entophytic bacterial contamination is an important problem in plant tissue culture (Kneifel and Leonhardt, 1992) and cannot be eliminated with any surface sterilization techniques, thus require antibiotic therapy (Mathias *et al*., 1987). 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 (Falkiner, 1990; 1988). 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 (Leifert *et al*.,1992). The continued use of antibiotics in the medium or repeated treatments with a single antibiotic may lead to bacterial resistance (Kneifel and Leonhardt, 1992).Care must be taken to insure that antibiotics are bactericidal rather than bacteriostaticas is often the case, and that the cultures are monitored for recurrence of bacteria (Mathias *et al*., 1987).

Many antibiotics exist that have not yet been evaluated on plants or their bacteria contaminants (Falkiner, 1990; Seckinger, 1995). Antibiotics are grouped by mode of action: inhibitors of bacterial cell wall Synthesis, inhibitors of bacterial protein synthesis, and DNA replication blockers (Pollock *et al*., 1983; Quesnel and Russell,1983). Antibiotics can also be grouped by chemical structure: aminoglycosides, quinolones,B Iactams, glycopeptides, polymixins, macrolides, and lincosamides (Falkiner, 1990). The choice of antibiotic is dependent on the type of bacteria present (i.e.Gram negative or Gram positive), soinitial characterization with Gram staining and some simple biochemical tests is essential (Buckley *et al*., 1995). Carbenicillin, Cephalothin, Gentamicin, Polymyxin, Pifampicin, Streptomycin, and Timentin have been used to treat plant tissue cultures (Buckley *et al*., 1995; Falkiner, 1988; Kneifel and Leonhardt, 1992).

Antibiotics are incorporated into plant tissue culture media or used as a brief treatment for specific contaminants (Leifert *et al*., 1992). Kneifel and Leonhardt (1992), and Leifert *et al*., (1992) recommend the use of short antibiotic treatments to prevent the development of antibiotic resistance in bacterial contaminants. It is also very important to determine whether antibiotics are bactericidal instead of bacteriostatic to avoid reoccurrence of bacteria (Mathias, 1987). Combinations of antibiotics may be more effective in killing contaminants (Falkiner, 1988; Kneifel and Leonhardt, 1992).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.

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 (Quinn *et al*., 2002; Swayne *et al*., 1998). Gram in 1884 discovered the Gram stain classification remains an important and useful technique until today. This technique classifies bacteria as either Gram positive or negative based on their morphology and differential staining properties (Frank, 2009).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 (Cheesbrough, 2006).

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 (Cheesbrough, 2006).

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 by (Ryan and Ray, 2004).

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 tothe procedure described by (Cheesbrough, 2006).

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 (Cheesbrough, 2006).

Methods of microbial preservation

Several methods have been successfully used for the preservation of microorganisms: repeated sub-culturing, preservation on agar beads (Winters and Winn, 2010), oil overlay of slant-grown cultures (Nakasone *et al.*, 2004), use of silica gel and other sterile supports (Liao and Shollenberger, 2003; Pérez-García *et al.*, 2006; Smith *et al.*, 2008), cryopreservation (Gorman and Adley, 2004; Smith *et al.*, 2008) and lyophilization (Berner and Viernstein, 2006; Morgan *et al.*, 2006).

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. (Abdurakhmonov *et al*., 2007).

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 (McCrea *et al*., 2007). 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 (Del *et al*., 2014).

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 (Del *et al*., 2014). Urease Producing bacteria include *Proteus Morganella*, *Providencia rettgeri*, *Klebsiella pneumonia*, *Klebsiellaoxytoca*, *Enterobacter cloacae* and *Yersinia enterocolitica* (Mukundan *et al*., 2007).

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, $13^0$30' 0" N latitude and $39^0$28'11"E longitudes (MARC, 2012).

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 Tigrai Biotechnology Center PLC (formerly Mekelle Plant Tissue Culture Laboratory) and the microbiology laboratory of the College of Veterinary Medicine of Mekelle University in Mekelle, Tigrai, Ethiopia. Fifty (50) bottles with contaminated *in vitro* sugarcane shooting media were collected from the growth rooms of the commercial micropropagation facility of Tigrai 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 (Quinn *et al.*, 2002).

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 37^0C 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 4^0C 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 MacConkey sarbitol 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 x10^8 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.

The plates were incubated for 24 hours at 35^{o}C according to Disk diffusion or Kirby- Bauer method (Bauer *et al.*, 1966). 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).

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 and Discussions

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).

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, MacConkey sarbitol agar, Salmonella shegella Agar (SSA) and Mannitol Salt Agar (MSA) represented in (Table 3).

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 in (Table 4).

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.

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).

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		

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	Rod
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	-

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)	-	-	-

+ = Positive, – = Negative,N.d. = not determined

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	+	-	-
	H_2S	-	-	-
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

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

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

	Identification Keys					
1.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	Yellow
TSI Butt	Yellow	Yellow	Yellow	Yellow	Red	Red
TSI Gas	+	-	-	-	-	-
TSI H_2S	-	-	-	-	-	-
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 and + = Positive, – = Negative, Galactose=GA, SR=Sorbitol, GL: Glucose, , ML = Maltose, SU = Sucrose, , A = Acid

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

SN	Antibiotics	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

Figure.1 Contaminated *in vitro* sugarcane culture bottle

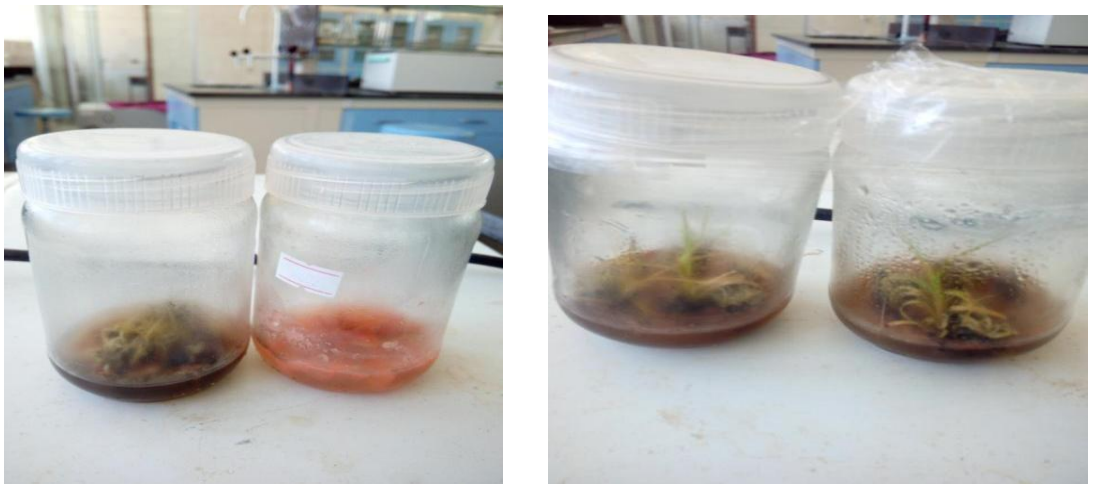


Figure.2 YC growth on NA found in left, PC growth on NA in middle and WC growth on NA in right

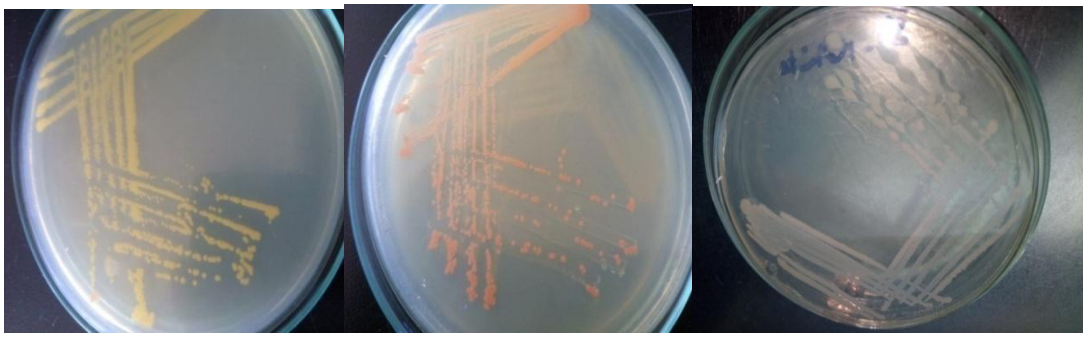


Figure.3 Inhibition of the growth of bacteria isolated from sugarcane culture in the presence of different antibiotics

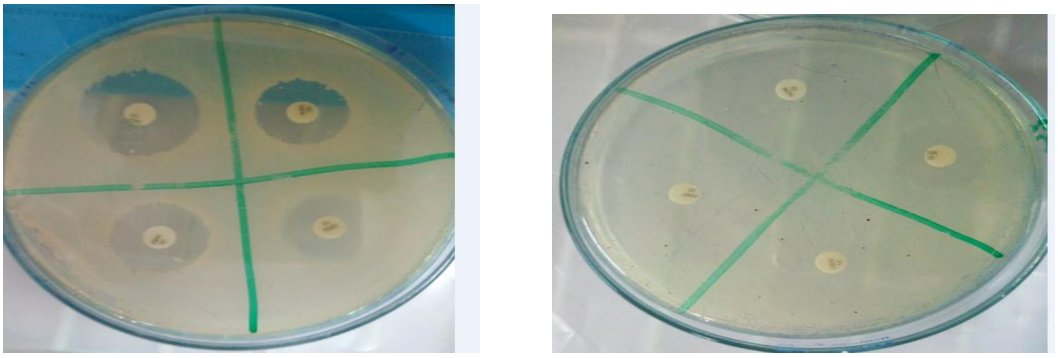
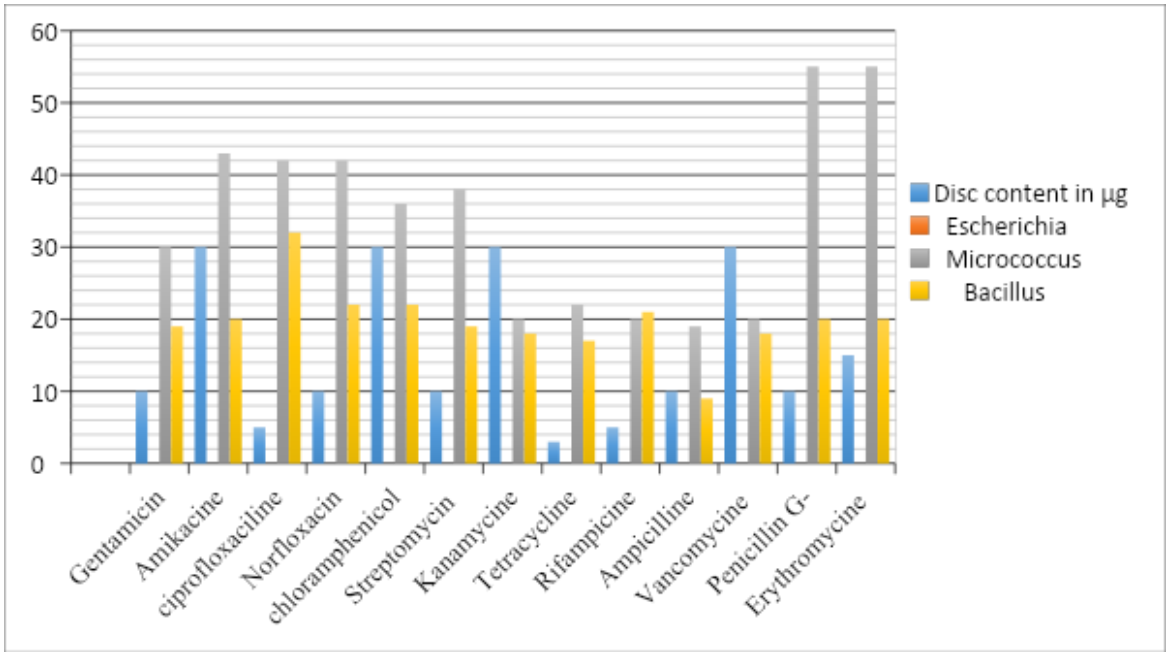


Figure.5 Inhibition zone (mm) of the bacterium of *Escherichia, Micrococcus* and *Bacillus*



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 (Holl and Polacco, 1994; Orlikowska and Zawadzka, 2006; Orlikowsk *et al*., 2010). 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 Okuniola *et al*., (2007) who reported *Bacillus, Corynebacterium, Escherichia, Streptococcus, Staphylococcus, Klebsiella, Actinobactor, Citrobactor, Lactobacillus* and *Salmonella* isolated from plant cell laboratories in Nigeria. And the gram negative bacteria which are pathogenic are majorly associated with faecal contamination (Mendoza *et al*., 2014). The gram positive isolates according to Liberto *et al*., (2011), *Bacillus sp*, *Corynebacteria sp* and *Actinomycete* were identified as contaminants in the laboratory laminar air flow cabinets and according to Trudeau, Fernández-Caldas, (1994) 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.

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 (Prierik, 1988), 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 (Fellner *et al*.,1996; Kneifel &Leonhardt, 1992). 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 (Salehi *et al.*, 2006; Sharada *et al*., 2010) and that of Tetracycline, ampiciline and Erythromycin according to the report of Fazlani (2010). 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 (Khan *et al.,* 2002; Lee *et al.,* 2005; Nasrin *et al.,* 2012; Abadi *et al.,* 2013).

Conclusion and recommendations are as follows:

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.

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