# Screening Antibiotics for the Elimination of Bacteria from *in vitro* Yam Plantlets

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## *Abstract*

*Plant tissue culture technology has been successfully used for the commercial production of pathogen-free plants and for germplasm conservation of rare and endangered species. Clean cultures of aseptically micropropagated shoot cultures of the genus* Dioscorea *(yam) grown on yam multiplication media are often contaminated with covert bacteria. The bacteria may survive endophytically within these plantlets thereby making the plantlets unusable for* in vitro *maintenance of germplasm. This study aimed at screening some antibiotics with a view of identifying the best one that can eradicate these endophytic bacteria from yam germplasm maintained* in vitro*. Eleven antibiotics, at different concentration range, were evaluated on the identified isolates to determine their potential to inhibit these contaminants, using the disk diffusion technique. Single or combined antibiotic treatments effective against the contaminants were also screened for their stability and minimum bactericidal concentration (MBC). The results shows that tetracycline combined with rifampicin (TR), streptomycin plus gentamycin (SG), rifampicin (Rn), vancomycin plus streptomycin (VS) at ≥ 125 µg/ml and TVSGR (tetracycline + vancomycin + streptomycin + gentamycin + rifampicin) at ≥ 100 µg/ml were highly bactericidal. These potential bactericidal doses can be further tested* in vivo *to determine their efficacy in the eradication of bacterial contaminants from the* in vitro *yam tissue cultured.*

***Keywords:*** *Endophytic bacteria, Discorea species,* in vitro *susceptibility test, yam germplasm.*

## 1. Introduction

Plant tissue culture refers to the aseptic growth, multiplication and maintenance of cells, tissues or organs of plants isolated from the mother plant, on a defined solid or liquid media under controlled environment. Basically, the technique consists of taking a piece of a plant referred to as the explants and placing it in a sterile nutrient where it multiplies (Odutayo *et al*. 2007). The formulation of the growth medium depends upon whether it is intended to produce undifferentiated callus tissue, multiply the number of plantlets, grow roots or multiply embryo for artificial seed (George 1993). The main advantage of tissue culture technology lies in the production of high quality and uniform planting material that can be multiplied on a year-round basis under disease-free conditions, irrespective of the season and weather. This goal can be attained, if tissues cultured are essentially free from all infecting microorganisms. Aseptic conditions are usually implied but many plant cultures do not stay aseptic *in vitro* and contamination by micro-organisms, especially bacteria, is a continuing problem for commercial and research plant micropropagators (Cassells 2000; Duhem *et al.* 1988; Debergh and Vanderschaeghe 1991).

Bacterial contamination is a major threat in plant tissue culture. Plant tissue cultures could harbour bacteria in a totally unsuspecting manner, either externally in the medium/plant or endophytically (Pious 2004). Epiphytic bacteria may lodge in plant structures where disinfectants cannot reach (Gunson and Spencer-Phillips 1994; Leifert and Waites 1992) while endophytic bacteria may be localized within the plant at cell junctions and

the intercellular spaces of cortical parenchyma (Gunson and Spencer-Phillips 1994). According to Reed *et al.* (1995), bacterial contaminants found at explants initiation, present in explants from collection dates and resistant to surface disinfection are likely to be endophytic. Both surface sterilization-resistant micro-organisms and endophytic micro-organisms (Cassells 2001) may survive in the plant material for several subculture cycles and over extended periods of time without expressing symptoms in the tissue or visible signs in the medium (Van den Houwe and Swennen 2000). Presence of these bacteria in the cultures is highly undesirable due to adverse effects on growth (Leifert and Waites 1992; Thomas 2004), lack of reproducibility of tissue-culture protocols (Thomas 2004) ramifications in cell cultures (Horsch and King 1983), possibility of carrying pathogens (Cooke *et al.* 1992), potential risk to *in vitro* gene banks (Van den Houwe and Swennen 2000) and a barrier for safe exchange of germplasm (Salih *et al.* 2001). These factors reduce the potential reliability of plant cell/tissue-culture systems (Cassells 2001; Thomas 2004).

The major challenges that yam production is facing using tissue culture techniques are that of endophytic bacterial contamination. *Burkholderia* spp., *Luteibacter rhizovicinus and Bacillus cereus* are the major bacteria contaminants implicated in the Yam Tissue Culture at the Institute of Tropical Agriculture Ibadan (IITA), Nigeria. These bacteria have been found to be plant-associated bacteria (Kotiranta *et al.* 2000; Coenye and Vandamme 2003; Johansen *et al*. 2005; Janssen 2006; and Compant *et al.* 2008) which infect cultures when explants materials from the field are not properly disinfected/ decontaminated. In addition, *Bacillus cereus* has also been reported as opportunistic human pathogen (Hoffmaster *et al*. 2006) which is always derived from the plant propagator.

Several antibiotics are frequently used in plant biotechnology to eliminate endogenous bacteria in plant tissue culture although aaccording to Cornu and Michel (1987), knowledge of the effect of antibiotics, on both bacteria and plants is important for the elimination of contaminants and the recovery of healthy plants. Bonev *et al.* (2008) reported that the efficacy of antibiotics can be assessed by their ability to suppress bacterial growth, described by the MIC, or by their ability to kill bacteria, characterized by the minimal bactericidal concentration (MBC). Hence, antibiotic screening remains the primary requisite for tackling the covert contamination problem. Therefore, the objectives of this research was to screen a selected range of antibiotics against the three identified yam tissue culture bacterial contaminants (*Burkholderia* spp., *Luteibacter rhizovicinus* and *Bacillus cereus)* and to determine the bactericidal activities of the most effective antibiotics.

## 2. Materials and Methods

### 2.1 Test Organisms

Three bacterial isolates obtained from contaminated *in vitro* yam plantlets by the pathology team and identified as *Burkholderia* spp. (IMI No 395525), *Luteibacter rhizovicinus* (IMI No 395527), and *Bacillus cereus (*IMI No 395528) by the Commonwealth Agricultural Bureaux (CABI) were used for the study. All isolates were cultivated by streaking onto Muller-Hinton agar medium (MHA) and incubating at 35-37$^{o}$C for 18-24 hours.

### 2.2 Antimicrobial Agents

A total of eleven antibiotics, obtained from Sigma-Aldrich, Corporation (Poole, UK), were used for the preliminary screening (ampicillin, penicillin G, tetracycline, vancomycin, streptomycin, rifampicin, gentamycin, bacitracin, cefotaxime, trimethoprim and carbenicillin). All stock solutions were prepared from reagent-grade powders to produce 1-mg/ml solutions by using the solvents and diluents suggested in Clinical Laboratory Standards Institute document M100-S17 (NCCLS 2001). Stock solutions were filter-sterilized (pore 0.22 µm, millipore), stored at -20$^{o}$C, and used within the recommended period.

Antibiotic paper discs of different known concentrations were prepared by soaking sterile paper discs with appropriate antibiotic concentrations derived from a two-fold dilution of the stock solutions. The method employed for impregnating antibiotics to discs was the immersion method. It was assumed by the World Health Organization (WHO) and NCCLS that a paper disc could absorb 0.02 ml of the solutions (NCCLS 1984). The wet inoculated antibiotic paper discs plate were sealed with Para films and dried inside an incubator at 35-37$^{o}$C.

**2.3 Antibiotic Susceptibility Testing**

Prior to the antibiotic susceptibility test, each of the three isolates (*Burkholderia* spp., *Luteibacter rhizovicinus* and *Bacillus cereus*) grown on a Muller-Hinton agar plate for 18-24 hours at 35-37$^{o}$C was standardized. Bacterial standardization was done by suspending well isolated colonies of the same morphology in a test tube containing sterile normal saline and mixed until it attained the turbidity of 0.5 McFarland standard (i.e. each suspension containing 1-2×10$^{8}$ cfu /ml of bacteria).

The antibiotic minimum inhibitory concentration (MIC) was determined according to the procedures detailed by the NCCLS (NCCLS 2001). The antibiotic susceptibility testing of the three isolates (i.e. *Burkholderia* spp., *Luteibacter rhizovicinus* and *Bacillus cereus*) was carried out using a final antibiotic concentrations ranging from 3.9-1,000 mg/L following the procedure of the disc diffusion method (Saeed *et al.* 2007). In this method, sterile cotton swabs dipped into appropriate adjusted bacterial suspensions of 0.1 optical densities, squeezed against the side of the suspension tubes to remove excess fluid were streaked across the sterile Mueller-Hinton agar plates. The streaking was done thrice for each plate, with the plate rotated approximately 60$^{o}$ between each streaking. After approximately 10 to 15 min, to allow absorption of excess moisture into the agar, three antimicrobial discs of the same concentrations were placed aseptically and at opposing directions on each plate. In the control plates, sterile disc containing no antibiotics were inoculated on seeded plates. All samples were tested in triplicates. The inoculated plates were sealed and incubated at 35-37$^{o}$C for 18-24 hours in an inverted position. The diameters of the inhibition zones around the discs were measured and recorded. MIC was defined as the lowest concentration of antibiotic to inhibit bacterial growth.

Five antibiotics (gentamycin, vancomycin, tetracycline, rifampicin and streptomycin) selected based on their effectiveness in the susceptibility test conducted were combined together as TVSGR (tetracycline + vancomycin + streptomycin + gentamycin + rifampicin) and also in the combinations of two forming ten antibiotic combination treatments: tetracycline + vancomycin (TV), tetracycline + streptomycin (TS), tetracycline + gentamycin (TG), vancomycin + streptomycin (VS), vancomycin + gentamycin (VG), vancomycin + rifampicin (VR), streptomycin + gentamycin (SG), streptomycin + rifampicin (SR) and gentamycin + rifampicin (GR). All the antibiotics combination treatments at a concentration range of 15.6-250 µg/ml for the two-combination treatments and 3-200 µg/ml for the five-combination treatments were also subjected to a preliminary screening following the procedures detailed in the single antibiotic treatment. The MIC's were recorded after 18-24 hours of incubation. Antibiotics screening for all the obtained records (i.e. from the single, combination of two and five antibiotic treatments) at their different concentration levels on the three isolates were repeated once and each isolate treatment was replicated three times in a single run.

**2.4 Antibiotic Stability Determination**

The clear zones of inhibition in the various inoculated plates were monitored in the incubator for a period of 5 days to check for the stability of the antibiotic treatments. Plates showing absence of resistant colonies on the clear zones of inhibition were considered stable and used for the minimum bactericidal concentration (MBC) determination while presence of mutants proved the antibiotics unstable and were not used for the MBC determination.

### 2.5 Minimum Bactericidal Concentration Determination (MBC)

The various antibiotic concentrations selected from the antibiotic stability determination were used for this experiment. MBC determination was effectively done, using the broth dilution method. Antibiotic-containing broth of appropriate concentrations was inoculated with 1 ml of the corresponding bacterial suspensions and incubated for 18-24 hours at 35-37$^{o}$C. Antibiotic-free broth inoculated with 1 ml of bacterial suspension served as a control for each of the isolates. After incubation, turbid broth in tubes indicated visible growth of microorganisms while non turbid broth showed no visible growth. The quantity, 0.1 ml of the non-turbid broth were inoculated onto a sterile Mueller-Hinton agar medium using a pour plate method and incubated for 18-24 hours to ascertain the bactericidal effectiveness of the antibiotics.

### 2.6 Statistical Analysis

The final data are reported as the mean of three replications for each antibiotic treatment. Statistical analysis of the data recorded from the experiment was performed using the analysis of variance (ANOVA) and $P < 0{\cdot}0001$ value was taken to indicate significance. All analyses were performed in SAS (version 9.1, SAS Institute (2003), Cary, NC, USA) using the PROC GLM procedure.

## 3. Results

### 3.1 Evaluation of Antibiotics Screening on the Bacterial Isolates

Susceptibility test results for the antibiotics were obtained from the data generated by the disc diffusion method. The disc diffusion method for antibacterial activity showed significant reduction in bacterial growth in terms of zones of inhibition around the discs. The mean of the growth inhibition zones of each of the bacteria at their different antibiotics (single and in combinations of two and five) concentration levels are shown in Tables 1 and 3. Results of the activities of the single, combinations of two and five-antibiotics on each of the bacteria revealed that the zones of inhibition increased with increase in the antibiotic concentrations, thus, exhibiting concentration dependent activity.

### 3.2 Single Antibiotics Treatments

The antibacterial activities and their corresponding MIC of the 11 selected antibiotics screened against *Burkholderia* spp., *Luteibacter rhizovicinus* and *Bacillus cereus* are shown in Table 1. The antibiotic sensitivity tests revealed that all isolates were more sensitive to tetracycline, vancomycin, streptomycin, gentamycin and rifampicin (i.e. wider zones of inhibition and sensitive at lower antibiotics concentration (Table 3)). Among the five most efficient antibiotics (tetracycline, vancomycin, streptomycin, gentamycin and rifampicin), gentamycin showed the largest zones of inhibition (18.5-25.2 mm) against the three bacteria at higher concentrations (250-1,000 µg/ml) with no observed significant differences ($P > 0.0001$) between the Bacteria strains. Results also showed that carbenicillin and trimetoprim showed no inhibitory effect against the three bacteria studied at all the different levels of concentrations (3.0-1,000 µg/ml). On the contrary, rifampicin presented growth inhibition zones at all the different concentration levels ranging from 9.7-22 mm while bacitracin, penicillin G, ampicillin and cefortaxime had poor activity with very small zones of inhibition (0-12 mm) which occurred only at the higher concentration levels (250-1,000 µg/ml). In general, the least inhibitory effect of the five effective antibiotics was found on *Luteibacter rhizovicinus* while *Bacillus cereus* and *Burkholderia* spp. were detected to be the most sensitive bacteria. No growth inhibition was observed in the control group.The variation in size of zones of inhibition with different antibiotic concentrations was found to be statistically significant at most concentration.

Table 1. Inhibitory zones (mm) around discs containing different concentrations of various antibiotics placed on the surface of Mueller-Hinton agar (MHA) medium inoculated with the standardized yam tissue culture bacterial contaminants.

| Bacteria | Antibiotics | Concentration (µg/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 3.9 | 7.8 | 15.6 | 31.3 | 63 | 125 | 250 | 500 | 1,000 |
| *Burkholderia* spp. | Rifampicin | 10.2 | 11 | 12 | 12.7 | 14.2 | 16.3 | 17.8 | 18.7 | 20 |
| | Sterptomycin | 0 | 5.2 | 7.2 | 10.2 | 14.7 | 15.5 | 18.8 | 19.3 | 21.2 |
| | Vancomycin | 0 | 0 | 1.5 | 1.3 | 4.3 | 11.5 | 13 | 15.2 | 17.7 |
| | Gentamycin | 0 | 0 | 3 | 11.7 | 14.3 | 18.7 | 20 | 21.3 | 25.2 |
| | Tetracycline | 0 | 0 | 0 | 0 | 4 | 8.7 | 9 | 11 | 13 |
| | Ampicillin | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 1.3 | 8.5 |
| | Penicillin G | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7.8 | 9.7 |
| | Cefortaxime | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4.8 | 10.8 |
| | Bacitracin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4.8 |
| | Trimetoprim | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Carbenicillin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LSD | 0.3 | 2 | 2.8 | 2.2 | 2.5 | 1.2 | 1.2 | 2.3 | 2.1 |
| *Luteibacter rhizovinus* | Rifampicin | 9.7 | 9.8 | 11 | 12 | 13 | 14.3 | 15.3 | 17 | 17.5 |
| | Sterptomycin | 0 | 6.5 | 12 | 11.5 | 16.7 | 17.7 | 19 | 20.2 | 21.7 |
| | Vancomycin | 0 | 0 | 2.7 | 4.2 | 8.2 | 13.2 | 13.2 | 16 | 16.7 |
| | Gentamycin | 0 | 0 | 3 | 11 | 14.7 | 17.5 | 18.5 | 22 | 22.8 |
| | Tetracycline | 0 | 0 | 0 | 1.7 | 10.2 | 12.7 | 12.7 | 15 | 17.8 |
| | Ampicillin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 11 |
| | Penicillin G | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.8 | 6.3 |
| | Cefortaxime | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.5 | 12 |
| | Bacitracin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.5 | 8 |
| | Trimetoprim | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Carbenicillin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LSD | 0.4 | 1.8 | 2.3 | 2.2 | 1.7 | 1.2 | 1.2 | 3.1 | 2.3 |
| *Bacillus cereus* | Rifampicin | 10.3 | 11 | 13 | 13.8 | 15.5 | 17.2 | 16.3 | 21.5 | 22 |
| | Sterptomycin | 0 | 0 | 4 | 8.7 | 13 | 13.8 | 15.8 | 17.2 | 20.2 |
| | Vancomycin | 0 | 0 | 0 | 1.5 | 4 | 11.7 | 13 | 16 | 18.3 |
| | Gentamycin | 0 | 0 | 4 | 11.8 | 16.7 | 19.3 | 21.5 | 23.8 | 25 |
| | Tetracycline | 0 | 0 | 0 | 0 | 3.8 | 4.2 | 9.3 | 10.8 | 12.7 |
| | Ampicillin | 0 | 0 | 0 | 0 | 0 | 0 | 4.2 | 2.8 | 9.5 |
| | Penicillin G | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.5 | 9 |
| | Cefortaxime | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10.5 |
| | Bacitracin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| | Trimetoprim | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Carbenicillin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LSD | 0.4 | 0.7 | 3 | 2.3 | 2.5 | 2.1 | 1.8 | 2.8 | 3.7 |

### 3.3 Combination of Two Antibiotics

From the initial 11 antibiotics screened by the disc diffusion assays, the 5 antibiotics that showed effectiveness were subjected to further analysis and 10 combination treatments were obtained from the combination of antibiotics in two's. Representative results of the disc diffusion assays, with varying concentrations of the combination of two antibiotics treatments are shown in Tables 2 and 4.

Table 2. Inhibitory zones (mm) around discs containing different concentrations of various antibiotics in combinations of two placed on the surface of Mueller-Hinton agar (MHA) medium inoculated with the standardized yam tissue culture bacterial contaminants.

| Bacteria | Antibiotic combination | Concentration(µm/ml) | | | | |
|---|---|---|---|---|---|---|
| | | 15.6 | 31.3 | 62.5 | 125 | 250 |
| *Burkholderia* spp. | SG | 11 | 14.3 | 17 | 20 | 23.7 |
| | TR | 10 | 12 | 15 | 17.3 | 20 |
| | VR | 9 | 6.7 | 14.3 | 16.3 | 18 |
| | SR | 0 | 0 | 16 | 17.3 | 21 |
| | GR | 8.3 | 11.7 | 18 | 18 | 21.7 |
| | VG | 0 | 0 | 13.7 | 18.7 | 21.7 |
| | TS | 6.3 | 12.7 | 16.7 | 19.7 | 21.7 |
| | TG | 3.3 | 14 | 16.7 | 20.7 | 24 |
| | VS | 3.3 | 10.7 | 13.3 | 18 | 19.7 |
| | TV | 0 | 0 | 10 | 10 | 13.3 |
| | Control | 0 | 0 | 0 | 0 | 0 |
| | LSD (p<0.0001) | 3 | 2.4 | 1.4 | 1.3 | 1.2 |
| *Luteibacter rhizovicinus* | SG | 11.5 | 15.7 | 18.2 | 22.2 | 25.2 |
| | VG | 9.8 | 13.8 | 17 | 19.5 | 22.8 |
| | VS | 9.5 | 10.8 | 14.7 | 17 | 19.8 |
| | SR | 0 | 0 | 12.8 | 19.2 | 21.5 |
| | VR | 9 | 9.5 | 13.3 | 15 | 17 |
| | GR | 8.7 | 11.7 | 14.2 | 18 | 20.5 |
| | TR | 8.7 | 10.8 | 12.2 | 13.5 | 15.3 |
| | TV | 7.3 | 9.8 | 11.5 | 16.3 | 16.5 |
| | TS | 5.2 | 14.8 | 16.7 | 19.8 | 20.8 |
| | TG | 3.3 | 10.8 | 14.5 | 20.7 | 23.8 |
| | Control | 0 | 0 | 0 | 0 | 0 |
| | LSD (p<0.0001) | 3.2 | 1.7 | 1.4 | 1.5 | 1.5 |
| *Bacillus cereus* | SR | 0 | 0 | 15.5 | 17.8 | 19.5 |
| | TR | 11.2 | 11.7 | 14.7 | 16 | 18.3 |
| | VR | 9.7 | 9.3 | 15.7 | 17.2 | 19.8 |
| | VG | 9.2 | 12.2 | 16.2 | 20.2 | 21.3 |
| | GR | 8.7 | 12.3 | 16 | 17.2 | 20.8 |
| | TV | 2.7 | 5.7 | 9 | 12.3 | 13.3 |
| | VS | 0 | 10.3 | 14.5 | 16.7 | 18.5 |
| | TS | 0 | 9 | 14.7 | 17 | 18.3 |
| | TG | 0 | 7.8 | 14 | 18.5 | 22.6 |
| | SG | 0 | 5 | 16.2 | 19.5 | 22.1 |
| | Control | 0 | 0 | 0 | 0 | 0 |
| | LSD (p<0.0001) | 1.8 | 3.8 | 1.7 | 1.6 | 1.8 |

LSD = Least significant difference,
tetracycline + vancomycin (TV),
tetracycline + streptomycin (TS),
tetracycline + gentamycin (TG),
tetracycline + rifampicin (TR),
vancomycin + streptomycin (VS),
vancomycin + gentamycin (VG),
vancomycin + rifampicin (VR),
streptomycin + gentamycin (SG),
streptomycin + rifampicin (SR),
gentamycin + rifampicin (GR),
control = no antibiotics.

Among the three bacteria tested with the 10 antibiotic combinations treatments (TV, TS, TG, TR, VS, VG, VR, SG, SR, GR) at a concentration range of 15-250 µg/ml, *Luteibacter rhizovicinus* and *Burkholderia* spp. were found to be more sensitive to the combination of the two antibiotics, streptomycin + gentamycin (SG) with no significant differences ($P > 0.0001$) observed in their mean zones of inhibition.

Table 3. MIC of different concentration of various antibiotics on the bacterial contaminants.

| Antibiotics | Minimal inhibitory concentration (µg/ml)/ organisms | | |
|---|---|---|---|
| | *Burk-holderia* spp. | *Lutei-bacter rhizo-vicinus* | *Bacillus cereus* |
| Rifampicin | 3.9 | 3.9 | 3.9 |
| Sterptomycin | 7.8 | 7.8 | 15.6 |
| Vancomycin | 15.6 | 15.6 | 31.3 |
| Gentamycin | 15.6 | 15.6 | 15.6 |
| Tetracycline | 62.5 | 31.3 | 62.5 |
| Ampicillin | 250 | 500 | 500 |
| Penicillin G | 500 | 500 | 1,000 |
| Cefortaxime | 500 | 500 | 500 |
| Bacitracin | 1,000 | 500 | 1,000 |
| Trimetoprim | 0 | 0 | 0 |
| Carbenicillin | 0 | 0 | 0 |
| Control | 0 | 0 | 0 |

Table 4. MIC for different concentration of various antibiotics in combination of two bacterial contaminants.

| Anti-biotics | Minimal Inhibitory concentration (µm/ml) | | |
|---|---|---|---|
| | *Burk-holderia* spp. | *Luteibacter rhizoviccinus* | *Bacillus cereus* |
| SG | 15.6 | 15.6 | 31.3 |
| TR | 15.6 | 15.6 | 15.6 |
| GR | 15.6 | 15.6 | 15.6 |
| TS | 15.6 | 15.6 | 31.3 |
| VS | 15.6 | 15.6 | 31.3 |
| TG | 15.6 | 15.6 | 31.3 |
| VR | 15.6 | 15.6 | 15.6 |
| SR | 62.5 | 62.5 | 62.5 |
| VG | 62.5 | 15.6 | 15.6 |
| TV | 62.5 | 0 | 15.6 |
| Control | 0 | 0 | 0 |

LSD = Least significant difference,
tetracycline + vancomycin (TV),
tetracycline + streptomycin (TS),
tetracycline + gentamycin (TG),
tetracycline + rifampicin (TR),
vancomycin + streptomycin (VS),
vancomycin + gentamycin (VG),
vancomycin + rifampicin (VR),
streptomycin + gentamycin (SG),
streptomycin + rifampicin (SR),
gentamycin + rifampicin (GR),
control = no antibiotics.

For *Bacillus cereus*, streptomycin + gentamycin (SG) showed higher zones of inhibition at a minimal inhibitory concentration (MIC) range of ≥ 62.5 µg/ml. Also, results revealed that the combination of the two antibiotics: streptomycin + rifampicin. (SR) showed no inhibitory effect against the three bacteria studied at 15.6-31.3 µg/ml concentrations.

### 3.4 Combination of Five Antibiotics

Results obtained from the antibiotics combination sensitivity tests revealed that *Burkholderia* spp., *Luteibacter rhizovicinus* and *Bacillus cereus* were significantly sensitive to the cocktail of the 5 effective antibiotics (tetracycline + vancomycin + streptomycin + gentamycin + rifampicin, i.e. TVSGR) at their different concentration levels (i.e. 3-200 µg/ml). For the combination of the five-antibiotics, TVSGR at the concentration of 200 µg/ml was found to present the highest mean zones of inhibition (23-24 mm) while the 3 µg/ml presented the lowest mean zones of inhibition (5-6 mm) on the three isolates. Significant differences ($P < 0.0001$) were observed in the susceptibility of *Luteibacter rhizovicinus* to the various TVSGR concentration levels (i.e. 3-200 µg/ml) while for *Burkholderia* spp. and *Bacillus cereus*, there was no significant difference ($P > 0.0001$) observed in the activity of the TVSGR concentration at 100-200 µg/ml and 12-25 µg/ml, respectively.

### 3.5 Evaluation of the Antibiotic Stability

Resistant colonies were observed in the zones of inhibition of most of the single and antibiotic combination treatments derived from the antibiotic susceptibility tests. Antibiotic stability was achieved more on the antibiotic combination treatments than on the single treatments as observed after 5 days of monitoring the clear zones of inhibition on the isolates (Table 5). The results also shows that for both assays (single antibiotics and antibiotic combinations), clear zones of inhibition in the inoculated plates assessed 5 days after the antibiotic susceptibility tests revealed that two

antibiotics (rifampicin and streptomycin) out of the 11 single antibiotic treatments were stable while in the combination of two-antibiotics, 3 out of 10 antibiotic combination treatments:
vancomycin + streptomycin **(**VS),
tetracycline + rifampicin **(**TR), and
streptomycin + gentamycin **(**SG),
showed stability. The combination of five-antibiotics (TVSGR) was also observed to be stable after 5 days. When antibiotic stability was assessed beyond five days in the incubator at 35-37$^{o}$C after the antibiotic susceptibility tests, the MHA medium at which the standardized inoculums were seeded on the sterile plates became exhausted (dried up). Isolates exhibited marked similarities in their response to antibiotics stability at their various concentration levels. For all unstable antibiotics on the isolates, resistant colonies (mutants) were observed on the clear zones of inhibition.

### 3.6 Evaluation of the minimal bactericidal concentrations (MBCs)

All stable antibiotics against the contaminating bacteria were evaluated at their variable concentrations by the broth dilution method to determine their bactericidal doses (Table 6). From the minimal bactericidal concentrations analysis conducted on the 6 stable antibiotics:
rifampicin (Rn),
streptomycin (S),
vancomycin + streptomycin **(**VS),
streptomycin + gentamycin (SG),
tetracycline + rifampicin **(**TR), and
tetracycline + vancomycin + streptomycin + gentamycin + rifampicin (i.e. TVSGR),
5 antibiotics (Rn, VS, SG, TR and TVSGR) out of the 6 had bactericidal effects on the three contaminating bacteria (Table 6). The data revealed that rifampicin had the lowest MBC (7·8 µg/ml) on *Burkholderia* spp. and *Bacillus cereus* amongst all the antibiotics tested. The result also revealed that 4 antibiotics (Rn, VS, SG, and TR) had a common bactericidal dose of ≥ 125 µg/ml, and TVSGR, a bactericidal dose of ≥ 100 µg/ml. Hence, this MBC (≥ 125 and 100 µg/ml) can be used for the elimination of *Burkholderia* spp., *Luteibacter rhizovicinus* and *Bacillus cereus* in an *in vitro* environment. For the unstable antibiotics which had resistant colonies (mutants) on their clear zones of inhibition, their bactericidal doses were not determined because it is a clear indication that they are bacteristatic to the yam tissue culture contaminants: *Burkholderia* spp., *Luteibacter rhizovicinus* and *Bacillus cereus*.

Table 5. Concentrations of the various antibiotics that had stable activity on the *in vitro* yam bacterial contaminants, determined after 5 days of the antibiotics susceptibility result.

| Antibiotics | Antibiotics concentration(µg/ml) | | |
|---|---|---|---|
| | *Burk-holderia* spp. | *Lutei-bacter rhizo-viccinus* | *Bacillus cereus* |
| Rifampicin | ≥7.8 | ≥7.8 | ≥7.8 |
| Streptomycin | ≥7.8 | ≥7.8 | ≥7.8 |
| VS | ≥15.6 | ≥15.6 | ≥15.6 |
| TR | ≥15.6 | ≥15.6 | ≥15.6 |
| SG | ≥15.6 | ≥15.6 | ≥15.6 |
| TVSGR | ≥25 | ≥25 | ≥25 |
| Control | 0 | 0 | 0 |

Vancomycin + streptomycin(VS),
tetracycline + rifampicin(TR),
streptomycin + gentamycin(SG),
tetracycline + vancomycin + streptomycin + gentamycin + rifampicin (TVSGR),
control = no antibiotics.

Table 6. Minimal bactericidal concentrations (MBC) of the effective antibiotics determined after ascertaining their stability on the *in vitro* yam bacterial contaminants.

| Antibiotics | Antibiotics concentration µg/ml) | | |
|---|---|---|---|
| | *Burk-holderia* spp. | *Lutei-bacter rhizo-viccinus* | *Bacillus cereus* |
| Rifampicin | ≥7.8 | ≥62.5 | ≥7.8 |
| Streptomycin | ≥250 | ≥125 | ≥250 |
| VS | ≥125 | ≥125 | ≥125 |
| TR | ≥62.5 | ≥62.5 | ≥62.5 |
| SG | ≥62.5 | ≥62.5 | ≥62.5 |
| TVSGR | ≥50 | ≥25 | ≥100 |
| Control | 0 | 0 | 0 |

## 4. Discussion

Bacterial contamination is one of the major challenges that yam production is facing using tissue culture techniques. These bacteria which may originate from explants, laboratory environments, operators, mites, thrips or ineffective sterilization techniques normally escape the initial surface sterilization (Van den Houwe and Swennen 2000) and remain latent during growth on plant multiplication media but will appear after subsequent sub culturing (Cassells 1991, 2000, 2001).They hinder the international exchange of germplasm and also become nuisance when contaminated yam cultures are used as explants material for cryopreservation. Antibiotics may be needed to eliminate these endogenous bacterial infection but the type, the efficiency, the level and duration of exposure for different *in vitro* plant varies and needs to be determined before use. Thus, availability of a reliable antibiotics screening method remains the primary requisite for tackling the covert contamination problem.

Our goal in this research was to screen a selected range of antibiotics against the three identified yam tissue culture bacterial contaminants (*Burkholderia* spp., *Luteibacter rhizovicinus* and *Bacillus cereus)* and to determine the bactericidal activities of the most effective antibiotics. According to Reed and Tanprasert (1995), knowledge of the effect of the antibiotics on both bacteria and the plants is essential for the recovery of healthy plants. Thus, knowledge of the effect of the selected antibiotics against the three isolates was achieved by determining their minimal inhibitory concentration (MIC) and subsequently the minimal bactericidal concentration (MBC) of all stable antibiotics after 5 days of incubation succeeding the antibiotic susceptibility test. MIC indicates the inhibitory potential, while MBC shows the cidal potential of the antibiotics on the isolates. According to Bonev *et al*. (2008), the effectiveness of antibiotics can be assessed by their ability to suppress bacterial growth, described by the MIC, or by their ability to kill bacteria, characterized by the minimal bactericidal concentration (MBC).

Previous studies from some researchers revealed that single antibiotic treatments were ineffective against bacteria isolated from plant tissue cultures (Keskitalo *et al.* 1996; Mentzer *et al.* 1996; Reed *et al*. 1995) while Van den Houwe and Swennen (2000) revealed Rifampicin as the only single antibiotics that showed effectiveness against banana tissue cultures. From this study, the results of the preliminary antibiotic susceptibility screening of the single antibiotics treatments showed that all isolates were very susceptible to tetracycline, vancomycin, streptomycin, gentamycin and rifampicin, and statistical analysis showed that the susceptibility is highly significant. The results also showed slight susceptibility to ampicillin, cefortaxime, penicillin G and bacitracin only at higher concentrations (500-1,000 µg/ml). Also, carbenicillin at 500 µg/ml was known to eliminate 40% of bacterial contaminants (*Paenibacillus glycanilyticus* and *Lactobacillus paracasei*) in *Pelargonium* Tissue (Wojtania *et al.* 2005) but on the contrary, our studies clearly showed that the isolates were significantly resistant to carbenicillin and trimethoprim at all tested concentrations.

According to Leifert *et al*. (1991, 1992) and Young *et al*. (1984), combinations of antibiotics are used against bacteria from plant tissue cultures and they may be more effective than single antibiotics in killing contaminants and reducing the risk of antibiotic resistance developing in the microbial population (Falkiner 1988; Leifert *et al.* 1991; Kneifel and Leonhardt 1992). Thus, in our study, in addition to examining the effects of the various antibiotics on the isolates, we also considered combining the 5 effective antibiotics in a two- and five- combination treatments. The two-antibiotic combinations of the 5 effective antibiotics which produced a 10 combination treatments (TV, TS, TG, TR, VS, VG, VR, SG, SR, and GR) and a cocktail of the 5 effective antibiotics (TVSGR) revealed from the antibiotic susceptibility test conducted that all isolates were sensitive to all the antibiotics combination treatments. Most of the combinations of two-antibiotics were bacteristatic on all the isolates except VS, TR and SG with a common MBC of ≥ 125 µg/ml.

Also, for the cocktail of the five-antibiotics, TVSGR at an MBC of ≥ 100 µg/ml, all the isolates were completely inhibited.

In conclusion, rifampicin was the only single antibiotics that was bactericidal to *Burkholderia* spp. and *Bacillus cereus* at ≥ 7.8 µg/ml and *Luteibacter rhizovicinus at* ≥ 62.5 µg/ml. Combinations of antibiotics were most effective for growth inhibition of the bacterial isolates (TR and SG at ≥ 62.5 µg/ml, VS at ≥ 125 µg/ml for the three isolates and TVSGR at ≥ 50 µg/ml for *Burkholderia* spp., ≥ 25 µg/ml for *Luteibacte*r *rhizovicinus*, and ≥ 100 µg/ml for *Bacillus cereus).* The major limitation involved in the use of antibiotics bactericidal doses to eliminate bacteria in plant tissue culture is that sensitivity may be reduced in complex plant tissue culture media (Barrett and Cassells 1994). Thus, the sensitivity of the bactericidal doses needs to be determined when incorporated into the plant tissue culture growth media.

## 5. Recommendations

Further studies are required on the antibiotic bactericidal doses to test their effectiveness in eliminating bacterial contamination from *in vitro* plantlets of *Dioscorea rotundata*. Also, phytotoxicity studies should be done to determine the effect of the antibiotic on the *in vitro* plantlets growth. A promising study in this direction was conducted by Mbah and Wakil (2012).

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## 7. References

Barrett, C.; and Cassells, A.C. 1994. An evaluation of antibiotics for the elimination of *Xanthomonas campestris* pv. *pelargonii* (Brown) from *Pelargonium* x *domesticum* cv. 'Grand Slam' explants *in vitro*. Plant Cell, Tissue and Organ Culture 36(2): 169-75.

Bonev, B.; Hooper, J.; and Parisot, J. 2008. Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method. Journal of Antimicrobial Chemotherapy 61(6): 1,295-301.

Cassells, A.C. 1991. Problems in tissue culture: culture contamination. *In:* Debergh, P.C. and Zimmerman, R.H. (eds.). Micropropagation. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 31-44.

Cassells, A.C. 2000. Contamination detection and elimination in plant cell culture. *In*: Spier, R.E. (ed.). Encyclopedia of Cell Technology. John Wiley & Sons, Inc., New York, NY, USA. Vol 2, pp. 577-86.

Cassells, A.C. 2001. Contamination and its impact in tissue culture. Acta Horticulturae 560: 353-59.

Coenye, T.; and Vandamme, P. 2003. Diversity and significance of *Burkholderia species* occupying diverse ecological niches. Environ. Microbiol. 5(9): 719-29.

Compant, S.; Nowak, J.; Coenye, T.; Clément, C.; and Ait Barka, E. 2008. Diversity and occurrence of *Burkholderia* spp. in the natural environment. Federation of European Microbiological Societies (FEMS) Microbiology Reviews 32(4): 607-26.

Cooke, D.L.; Waites, W.M.; and Leifert, C. 1992. Effects of *Agrobacterium tumefaciens*, *Erwina carotovora*, *Pseudomonas syringae* and *Xanthomonas campestris* on plant tissue cultures of *Aster*, *Cheiranthus*, *Delphinium*, *Iris* and *Rosa*; disease development *in vivo* as a result of latent infection *in vitro*. Journal of Plant Diseases and Protection 99(5): 469-81.

Cornu, D.; and Michel, M.F. 1987. Bacteria contaminants in shoot cultures of *Prunus avium* L. choice and phytotoxicity of antibiotics. Acta Horticulturae 212: 83-6.

Debergh, P.C.; and Vanderschaeghe, A.M. 1991. Some symptoms indicating the presence of bacterial contaminants in plant tissue cultures. Acta Horticulturae 225: 77-81.

Duhem, K; Le Mercier, N.; and Boxus, P. 1988. Difficulties in the establishment of

axenic *in vitro* cultures of field collected coffee and cacao germplasm. Acta Horticulturae 225: 67-75.

Falkiner, F.R. 1988. Strategy for the selection of antibiotics for use against common bacterial pathogens and endophytes of plants. Acta Horticulturae 225: 53-6.

George, E.F. 1993. Plant Tissue Culture Techniques. *In:* Plant Propagation by Tissue Culture. Part 1: The Technology. Exegetics Ltd., Edington, Wiltshire, England, UK.

Gunson, H.E.; and Spencer-Phillips, P.T.N. 1994. Latent bacterial infections: Epiphytes and endophytes as contaminants of micropropagated plants. *In:* Physiology, Growth and Development of Plants in Culture. Nicholas, J.R. (ed.). Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 379-96.

Hoffmaster, A.R.; Hill, K.K.; Gee, J.E.; Marston, C.K.; De, B.K.; Popovic, T.; Sue, D.; Wilkins, P.P.; Avashia, S.B.; Drumgoole, R.; Helma, C.H.; Ticknor, L.O.; Okinaka, R.T.; and Jackson, P.J. 2006. Characterization of *Bacillus cereus* isolates associated with fatal pneumonias: strains are closely related to *Bacillus anthracis* and harbor *B. anthracis* virulence genes. Journal of Clinical Microbiology 44(9): 3,352-60.

Horsch, R.B.; and King, J. 1983. A covert contaminant of cultured plant cells: elimination of a *Hyphomicrobium* sp. from cultures of *Datura innoxia* (Mill.). Plant Cell Tissue and Organ Culture 2(1): 21-8.

Janssen, P.H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Applied and Environmental Microbiology 72(3): 1,719-28.

Johansen, J.E.; Binnerup, S.J.; Kroer, N.; and Mølbak, L. 2005. *Luteibacter rhizovicinus* gen. nov., sp. nov., a yellow-pigmented gammaproteobacterium isolated from the rhizosphere of barley (*Hordeum vulgare* L.). International Journal of Systematic and Evolutionary Microbiology 55(6): 2,285-91.

Keskitalo, M.; Pohto, A.; Savela, M.L.; Valkonen, J.P.T.; Pehu, E.; and Simon, J. 1996. Control of bacteria and alteration of plant growth in tissue cultures of tansy (*Tanacetum vulgare* L.) treated with antibiotics. HortScience 31(4): 631.

Kneifel, W.; and Leonhardt, W. 1992. Testing of different antibiotics against Gram-positive and Gram-negative bacteria isolated from plant tissue culture. Plant Cell, Tissue and Organ Culture 29(2): 139-44.

Kotiranta, A.; Lounatmaa, K.; and Haapasalo M. 2000. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes and Infection 2(2): 189-8.

Leifert, C.; and Waites, W.M. 1992. Bacterial growth in plant tissue culture media. Journal of Applied Microbiology 72(6): 460-6.

Leifert, C.; Camotta, H; and Waites, W. M. 1992. Effect of combination of antibiotics on micropropagated *Clematis, Delphinium, Hosta, Iris* and *Photinia*. Plant Cell, Tissue and Organ Culture 29(2): 153-60.

Leifert, C.; Camotta, H.; Wright, S.M., Waites, B.; Cheyne, V.A.; and Waites, W.M. 1991. Elimination of *Lactobacillus plantarum, Corynebacterium* spp., *Staphylococcus saprophyticus* and *Pseudomonas paucimobilis* from micropropagated *Hemerocallis, Choisya* and *Delphinium* cultures using antibiotics. Journal of Applied Microbiology 71(4): 307-30.

Mbah, E.I.; and Wakil, S.M. 2012. Elimination of bacteria from *in vitro* yam tissue cultures using antibiotics. Journal of Plant Pathology 94(1): 53-8.

NCCLS. 1984. Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard M2-A. National Committee for Clinical Laboratory Standards (NCCLS), Villanova, PA, USA.

NCCLS. 2001. Performance standards for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals; approved standard. NCCLS document M31-A. National Committee for Clinical Laboratory Standards (NCCLS), Wayne, PA, USA.

Odutayo, O.I; Amusa, N.A; Okutade, O.O.; and Ogunsanwo, Y.R. 2007. Sources of microbial contamination in tissue culture laboratories in south western Nigeria. African Journal of Agricultural Research 2(3): 67-72.

Pious, T. 2004. A three-step screening procedure for detection of covert and endophytic bacteria in plant tissue cultures. Current Science 87(1): 67-72.

Reed, B.M.; and Tanprasert, P. 1995. Detection and control of bacterial contaminants of plants tissue in cultures. A review of recent literature. Plant Tissue Culture and Biotechnology 1(3): 137-42.

Reed, B.M; Buckley, P.M.; and DeWilde, T.N. 1995. Detection and eradication of endophytic bacteria from micropropagated mint plants. *In Vitro* Cellular and Developmental Biology - Plant 31(1): 53-7.

Salih, S.; Waterworth, H.; and Thompson, D. A. 2001. Role of plant tissue culture in international exchange and quarantine of germplasm in the United States and Canada. HortScience 36(6): 1,015-21.

SAS. 2003. Statistical Analysis System. User's Guide. SAS Institute Inc., Cary, NC, USA.

Thomas, P. 2004. *In vitro* decline in plant cultures: detection of a legion of covert bacteria as the cause for degeneration of long-term micropropagated triploid watermelon cultures. Plant Cell, Tissue and Organ Culture 77(2): 173-9.

Van den Houwe, I.; and Swennen, R. 2000. Characterization and control of bacterial contaminants *in vitro* cultures of banana (*Musa* spp.). Acta Horticulturae 532: 69-79.

Wojtania, A.; Puławska, J.; and Gabryszewska, E. 2005. Identification and elimination of bacterial contamination from *Pelargonium* tissue cultures. Journal of Fruit and Ornamental Plant Research 13: 101-8.

Young, P.M.; Hutchins, A.S.; and Canfield, M.L. 1984. Use of antibiotics to control bacteria in shoot cultures of woody plants. Plant Science Letters 34(1-2): 203-9.