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Identification and elimination of bacterial contaminants from yam tissue cultures

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ABSTRACT

Yam tissue cultures grown on yam multiplication medium are often contaminated with endogenous bacteria. These contaminants affect multiplication rates of plant cultures and may provide a source of inoculum in the progeny of microplants. The aim of this study was to detect and control bacterial contamination in yam tissue cultures using antibiotics.

The bacterial isolates were identified using standard microbiological methods. Antibiotic sensitivity test was carried out on the bacterial isolates to know their sensitivity or resistance to some common antibiotics used in plant tissue culture. Streptomycin, Rifampicin and Gentamicin chosen for further investigation were screened for bactericidal activity and phytotoxic effects on yam plantlets in liquid yam multiplication (YM) medium. Single nodes from infected plants were treated by inoculating into liquid YM medium containing antibiotics either singly or in combination. The effect of pH on bactericidal activity of the antibiotic was also determined. The antibiotics were added to yam multiplication medium in the following dosages: Streptomycin-500µg, 750µg, 1000µg and 1250µg/ml, Rifampicin-40µg, 80µg, l60µg, and 320µg/ml and gentamicin- 20µg, 40µg, 80µg and 160µg/ml. Antibiotic sensitivity test was also performed on the bacterial isolates and three antibiotics were identified as being potentially useful in eliminating the bacterial contaminants.

Five bacterial isolates were obtained from contaminated yam cultures and these were classified as Corynebacterium fascians, Corynebacterium michiiganese, Paracoccus denitrificans, Erwinia stewartii and one unidentified species. The best results were obtained with the addition of 320µg/ml of Rifampicin. Combination of Rifampicin (80 µ/ml) and Streptomycin (250 µ/ml) were effective in treatment. Inhibition of growth by antibiotics was best at pH 6.9.

Keywords: Bacterial contaminants, Yam tissue cultures, Antibiotics, *Dioscorea rotundata*, phytotoxicity

INTRODUCTION

Modern way of yam propagation has necessitated the use of tissue culture technique. This technique is used for yam propagation and genotype modification (plant breeding), biomass production of biochemical products, preservation and storage, scientific investigations and others (Hudson *et al*., 1990). Plant tissue culture may be infected by a wide range of bacteria many of which are species specific.

Bacterial contamination can reduce growth rate, retard rooting and even cause plant death (Leifert, 1990; Nakhzari-Moqadam and Sabbagh, 2012). Serious losses have been reported in tissue cultures due to the presence of latent bacteria (Kulkarni *et al*., 2007). These organisms have been found to affect multiplication rates of plant cultures (Leifert, 1990) and because they multiply within the plant tissue, they may provide a source of inoculum in the progeny of microplants. Endophytic bacterial contamination is an important problem in plant tissue culture (Kneifel and Leonhardt, 1992) and cannot be eliminated using any surface sterilization techniques, thus it requires antibiotic therapy (Mathias *et al*., 1987). Antibiotics have being successfully used in controlling contamination in plant cultures (Leifert *et al.*, 1991; Kneifel and Leonhardt, 1992). It has been reported however that, antibiotics may be more effective as bacteriostatic, rather than as bactericidal agent (Fisse *et al.*, 1987; Leifert *et al.*, 1992). Combinations of antibiotics may be advantageous where synergistic action occurs, but some are incompatible and the combination may neutralize any positive effect of the individual drugs (Kneifel and Leonhardt, 1992; Reed and Tanprasert, 1995). Combinations of antibiotics at bactericidal concentrations are likely to be phytotoxic, but repeated use of single antibiotics may lead to bacterial resistance (Kneifel and Leonhardt, 1992; Leifert *et al.*, 1992). Antibiotics effective on isolated organisms may be ineffective in treating contaminated plants due to phytotoxicity or poor penetration into plant tissues (Reed *et al*., 1995, Reed and Tanprasert, 1995). Horsch and King, 1983 reported that certain antibiotic treatments had no effect on contamination. However, many antibiotics have been found to be phytotoxic to plant *in vivo* and *in vitro*, and should therefore be incorporated into plant growth media for limited period of time. Some bacteria, *Lactobaccillus plantarum*, *Staphylococcus saprophyticus*, *Corynebacterium* species, *Pseudomonas pau cimobilis* and *Hyphomicrobum*) have been eliminated from contaminated plant tissue cultures by treatment with combinations of antibiotics involving an aminoglycosides, a penicillin and Cephalosporin Rifampicin or Polymicin into the growth medium. The plant cultures stayed free of the contaminants when assessed for up to two years after antibiotic treatment (Leifert *et al*., 1991). Knowledge of the effect of antibiotics on both bacteria and plants is essential for the elimination of contaminants and recovery of healthy plants (Reed and Tanprasert, 1995). It has been reported by some workers that prophylactic incorporation of antibiotics into the plant growth media only eliminates or suppresses a certain species of bacteria and most antibiotics have been shown to inhibit multiplication and rooting of some plant shoot cultures (Leifert *et al.*, 1992, 1994).

Phytotoxicity and the development of antibiotic-resistant bacteria populations have also restricted the use of antibiotics (Dodds and Roberts, 1981; Phillips *et al.*, 1981; Pollock *et al.*, 1983; Leifert *et al.*, 1992). These side effects of the antibiotics can be taken care of by the use of combination of antibiotics at relatively lower concentrations (Leifert *et al.*, 1992). Streptomycin, Carbenicillin, Rifampicin, Gentamicin, Cephalothin and Polymycin alone or in various combinations with one another are frequently used to eradicate bacterial contaminants from in vitro plant cultures (Kneifel and Leonhardt, 1992).

Bacterial contaminants which have being isolated from yam tissue culture include *Pseudomonas* sp, *Xanthomonas* sp, *Erwinia* sp, *Burkholderia* spp., *Luteibacter rhizovicinus* and *Bacillus cereus* (Ng and Mantell, 1996; Mba and Wakil, 2012).

MATERIALS AND METHODS

Isolation and identification of bacterial strains from *in vitro* cultures

Two genotypes of white yam (TDr 179 and TDr 131) *Dioscorea rotundata* (Poir) plantlets showing bacterial contamination obtained from Tissue culture laboratory, International Institute of Tropical Agriculture, Ibadan were used. The bacteria causing contamination in yam tissue plants were isolated using Tryptone Soya broth and Nutrient Agar (NA) medium for forty eight hours at 37 ^{o}C under 12h photo-period.

Characterization and identification of isolates

The bacterial isolates were identified using cultural, physiological and biochemical tests, some of the tests include Gram staining, spore staining, motility test, catalase production, oxidase test, indole production, citrate utilization, urease activity, hydrogen sulphide production, gelatine hydrolysis and carbohydrate utilization.

Antibiotic sensitivity test

Antibiotic sensitivity test was carried out on the bacterial isolates to know their sensitivity or resistance to some common antibiotics used in plant tissue culture. Antibiotic sensitivity discs were prepared with different concentration of antibiotics. The antibiotics used and their different concentrations were: Rifampicin (10µg, 30µg, 50µg/ml), Gentamicin (10µg, 30µg, 50µg, 80µg/ml), Penicillin (5µg, 8µg, 10µg, 12µg/ml), Streptomycin (500µg, 800µg, 1000µg, 1200µg/ml), Claforan (10µg, 20µg, 30µg, 40µg/ml) and Ampicillin (25µg, 50µg, 75µg, 100µg/ml).

Fresh broth culture (24 hours old) of each isolate was used for the sensitivity test. Broth cultures of each isolate (0.1ml) was incorporated into already prepared sterile NA plates and a glass spreader was used to spread the inoculum on the plates. The sensitivity discs were placed with sterile forceps. Sterile paper disc which was soaked in sterile distilled water served as control. The plates were incubated for 48 hours at 37^{0}C. Data recorded on the reaction of the isolates to each antibiotic was reported as resistant or sensitive. The diameters of inhibition zones around the discs were measured and recorded.

Antibiotic treatment of nodal cultures and the effect of pH on contamination

Three antibiotics were selected on the basis of their effectiveness in antibiotic sensitivity test: The antibiotics (rifampicin, gentamicin and streptomycin) were added to Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) in the following dosages: streptomycin-500µg, 750µg, 1000µg and 1250µg/ml, Rifampicin-40µg, 80µg, l60µg, and 320µg/ml and gentamicin-20µg, 40µg, 80µg and 160µg/ml. The shoots were subjected to different concentrations of streptomycin to determine the maximum concentration for treatment that will not be phytotoxic to the plant. The effect of pH on bactericidal activity of the antibiotic was also determined. Ten replicates of each concentration were prepared. Node cuttings from contaminated cultures were placed separately into tubes containing the different concentrations of streptomycin in stationary liquid medium. For each concentration, antibiotic treatment was monitored for bactericidal activity at pH 5.7 and 6.9 in liquid medium. Antibiotic free liquid MS medium inoculated with node cuttings served as control. The culture tubes were then sealed with strips of paraffin and incubated at 25 ± 2 ^{0}C at 16 hours photoperiod and irradiation of 30µt sec^{-1} m^{-2} supplied by daylight fluorescent light for 4 weeks.

Treatment of contaminated nodal cultures with combinations of antibiotics.

Different concentrations of Rifampicin and Streptomycin were combined together for and these were:

Treatment 1: Antibiotic free liquid MS medium (R0+S0)
Treatment 2: Rifampicin 80µg/ml + Streptomycin 250µg/ml (R80+S250)
Treatment 3: Rifampicin 80µg/ml + Streptomycin 500µg/ml (R80+S500)
Treatment 4: Rifampicin 80µg/ml + Streptomycin 750µg/ml (R80+S750)
Treatment 5: Rifampicin 120µg/ml + Streptomycin 250µg/ml (R120+S250)
Treatment 6: Rifampicin 120µg/ml + Streptomycin 500µg/ml (R120+S500)
Treatment 7: Rifampicin 120µg/ml + Streptomycin 750µg/ml (R120+S750)
Treatment 8: Rifampicin 160µg/ml + Streptomycin 250µg/ml (R160+S250)
Treatment 9: Rifampicin 160µg/ml + Streptomycin 500µg/ml (R160+S500)
Treatment 10 Rifampicin 160µg/ml + Streptomycin 750µg/ml (R160+S750)

The antibiotics were incorporated into liquid MS medium for each treatment. Ten replicates were prepared for each treatment, contaminated node cuttings were immersed in individual tubes of antibiotics incorporated liquid MS medium at pH 6.9. Medium without antibiotics inoculated with node cuttings served as control. The plants were incubated at 25 ± 2 ^{0}C for 1 week. Afterward, the nodes were removed from the tubes, drained on sterile paper towel, trimmed to expose fresh tissue and were transferred to tubes of solidified MS medium without antibiotics. Cultures were monitored for bacterial growth by streaking on NA plates at each transfer. The cultures were monitored weekly for bacterial growth. Growth rate and plant appearance were monitored to determine whether the antibiotics had any phytotoxic effects on multiplication and root formation.

Statistical Analysis

All data were subjected to summary statistics and analysis of variance using the Statistical Analysis System version 6.0 for PC SAS. Pairwise means comparison was carried out using the Tukey`s range test at $p \leq 0.05$.

RESULTS AND DISCUSSION

Five bacterial isolates were obtained from contaminated yam cultures. They were classified as *Corynebacterium fascians*, *Corynebacterium michiiganese, Paracoccus denitrificans*, *Erwinia stewartii* and one unidentified species. About 40% of infections in plant tissue culture systems have being attributed to contaminants introduced into culture on or in the original mother stock plant materials while the remaining may be caused by passive contamination during the practical operation of micropropagation (Leifert *et al*., 1989).

Antibiotic sensitivity test revealed that the antibiotics most effective against the contaminating bacteria were streptomycin, rifampicin and gentamicin (Plate 1).

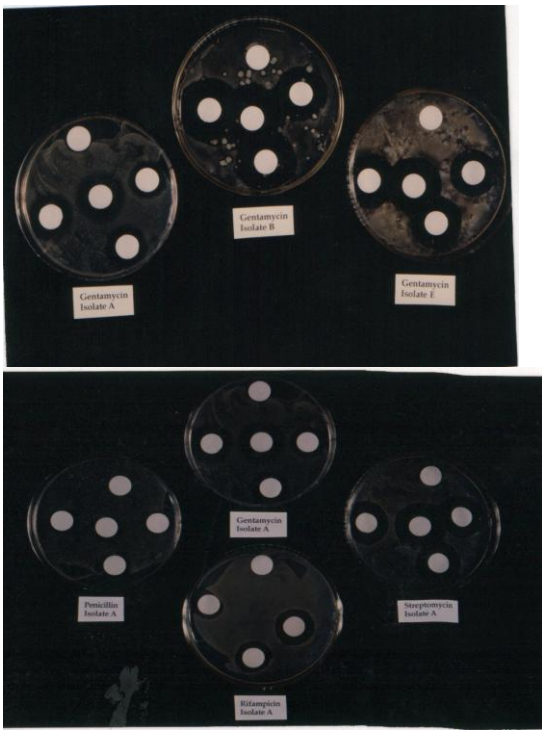

Plate 1: Inhibitory effects of selected antibiotics on bacterial contaminants from yam cultures

C. michiganese was sensitive to four of the antibiotics, streptomycin, rifampicin, gentamicin and ampicillin while it showed resistance to the other two antibiotics: Penicillin and Claforan. *P. denitrificans* was sensitive to three of the antibiotics; streptomycin, rifampicin and gentamicin, while it was resistant to the remaining three antibiotics. *E. stewartii* was sensitive to four antibiotics; streptomycin, rifampicin, gentamicin and claforan and was resistant to the other two antibiotics. The unidentified bacterium was sensitive to three of the antibiotics; streptomycin, rifampicin, and it was resistant to the remaining three (Table 1).

Table 1. Sensitivity of the bacterial contaminants to some antibiotics (µg/ml)

Isolate	Strep 500	Rifam 30	Genta 10	Peni 5	Amp 15	Claf 10
C. fascians	S	S	S	S	S	S
C. michiganese	S	S	S	R	R	S
P. denitrificans	S	S	S	R	S	S
E. stewartii	S	S	S	R	R	S
Unidentified	S	S	S	R	R	R

Strep = Streptomicin; Rifam =Rifampicin; Genta = Gentamicin; Peni = Pencillin; Amp = Ampicillin; Claf = Claforan; R= resistant; S= sensitive

The effect of streptomycin, rifampicin and gentamicin on contamination after four weeks of application is presented in Figures 1, 2 and 3. Streptomycin was used at 500, 750, 1000 and 1250µg/ml and it was most effective at 1000µg/ml. However, it was phytotoxic at different concentrations used on the plantlets (Figure 1). This result

differs from the findings of Reed *et al* (1995) who reported that streptomycin effectively controlled bacterial contamination at 1000µg/ml without showing any phytotoxic effect on mint shoot cultures. Gentamicin and rifampicin reduced contamination by 60% in the cultures at 160 and 320µg/ml respectively, however, gentamicin was found to be phytotoxic while rifampicin was not (Figures 2 and 3).

At higher concentration, the antibiotics were effective in controlling the bacterial contamination. Streptomycin was effective at 1000ug/ml and at pH 6.9, bactericidal activity was enhanced. Rifampicin was most effective at 320ug/ml, enhancing bactericidal activity at pH 6.9. Gentamicin was most effective at 160ug/ml and also enhancing bactericidal activity at pH 6.9.

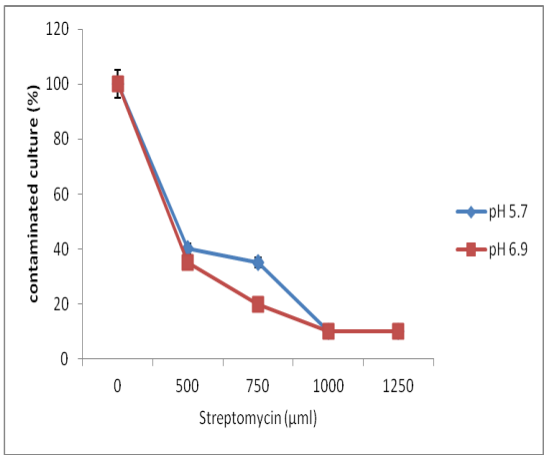

Figure 1. Effect of Streptomycin on the contaminated yam tissue cultures

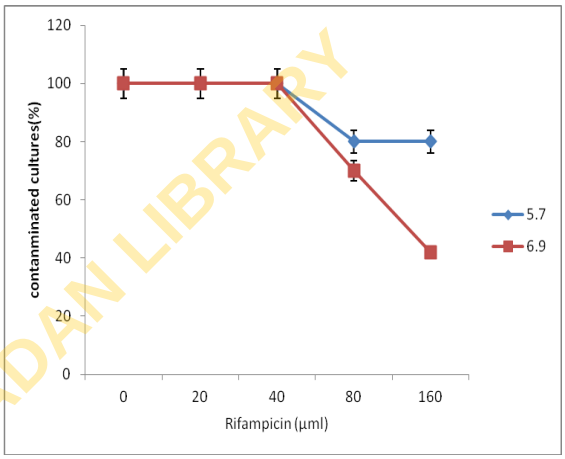

Figure 2. Effect of Rifampicin on the contaminated yam tissue cultures

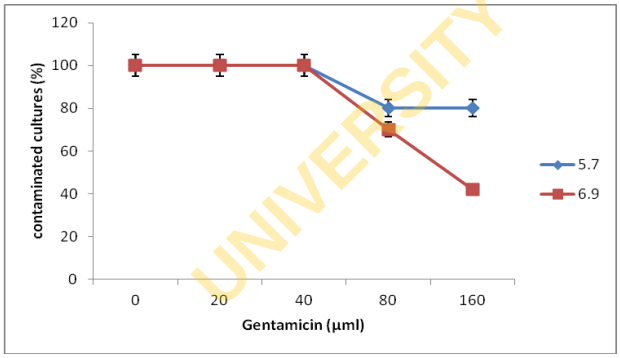

Figure 3. Effect of Gentamicin on the contaminated yam tissue cultures

The phytotoxic effect of the antibiotics on the plantlets is presented in Table 2. Streptomycin was phytotoxic to the growth of the plantlets, at higher concentration resulting in the death of the plantlets. Gentamicin at 160ug/ml was most effective against bacterial contamination; although it was the most phytotoxic to the growth of the plantlets. Rifampicin did not show any phytotoxic effect even at higher concentration. The antibiotics were found to affect the multiplication rate of plantlets (Table 2). For Streptomycin, the multiplication rate could not be determined because of the severe phytotoxicity exhibited by the antibiotics on plantlets. For gentamicin, the multiplication rate was significantly higher in the control treatment

than other treatments (Table 3). Treatment with 20µg/ml and 160ug/ml were not significantly different ($p<0.05$). Treatment with 160µg/ml gave the lowest number of nodes and it was significantly different from the control treatment. The rate of node formation at the two pH were not significantly different. In the formation of roots, control treatment was significantly higher than other treatments followed by treatment with 20µg/ml and in the production of nodes (Table 3).

Table 2. Phytotoxic effect of three antibiotics on yam shoot cultures after 4 weeks of application.

Treatments	No of stunted plants (%)	No of plants showing yellowing (%)	No of dead plants (%)
Streptomycin(µg/ml)			
0	-	-	0
500	100	100	0
750	90	90	0
1000	90	90	100
1250	100	100	100
Gentamicin(µg/ml)			
0	0	0	0
20	0	0	0
40	0	0	0
80	100	100	100
160	100	100	100
Rifampicin(µg/ml)			
0	0	0	0
40	0	0	0
80	0	0	0
160	0	0	0
320	0	0	0

Table 3. Effects of Gentamicin on multiplication rate of the treated yam cultures.

Treatments(µg/ml)	No of nodes	No of roots
0	1.50a	1.72a
20	1.10b	0.73b
40	0.93bc	0.30bc
80	0.81c	0.18c
160	0.72c	0.40c
Ph		
6.9	1.04a	0.63a
5.7	0.98a	0.56a

Means with different letters in the same column are significantly different at $P \leq 0.05$ by Tukey`s Student Range Test.

Table 4. Effects of Rifampicin on multiplication rate of the treated yam cultures.

Treatments(µg/ml)	No of nodes	No of roots
0	1.50a	1.72a
40	1.16b	0.84b
80	1.35ab	1..31ab
160	1.25ab	0.85b
320	1.07b	1.31a
Ph		
6.9	1.32a	1.23a
5.7	1.22a	1.05a

Means with different letters in the same column are significantly different at P≤0.05

On application of rifampicin, control treatment was significantly higher than other treatments in the production and root formation, while other treatments were not significantly different from each other. The pH has no significant effect on the multiplication rate (Table 4).

At four weeks after culturing, the combined effect of rifampicin and streptomycin on contamination, multiplication were most effective at rifampicin 80µg/ml + streptomycin 750µg/ml (R80+S750) (Table 5). With genotype TDr 179, R80+S750 was effective against contamination without, inhibiting the multiplication rate of plants, however, for genotype TDr 131, the combination of R80+S250 was able to inhibit bacterial contamination (Table 6).

Effect of the antibiotics on multiplication rate was determined after 5 weeks (multiplication rate was indexed by the number of roots and nodes produced per plant). For genotype TDr 131, the number of root produced was significantly higher in control than other treatments. There was no significant differences in the number of roots; produced in all the other treatment combination.

Table 5. Percentage of contaminated cultures after treatment with combination of antibiotics.

	TDr179			TDr131		
	No of weeks after treatment			No of weeks after treatment.		
Treatments (µ/ml)	2	4	6	2	4	6
R0+S0	100	100	100	100	100	100
R80+S250	0	40	40	20	20	20
R80+S500	10	40	40	0	10	20
R80+S750	0	20	20	0	0	0
R120+S250	0	40	40	20	20	30
R120+S500	40	40	40	0	0	0
R120+S750	0	0	0	0	20	20
R160+S250	30	30	30	0	10	20
R160+S500	30	30	30	10	20	20

Table 6. Combined effect of rifampicin and streptomycin on multiplication rates in 2 yam genotypes (TDr 131 and TDr 179)

Mean no of root per plant			Mean no of node per plant	
Treatment	TDr 131	TDr 179	TDr 131	TDr 179
R0+S0	1.04a	1.13ab	1.01a	1.09ab
R80+s250	0.15b	1.43ab	0.81ab	01.26a
R80+S500	0.31b	1.56a	0.87a	1.01abc
R80+S750	0.12b	1.35ab	0.74ab	0.71bc
R120+S250	0.15b	1.20ab	0.91a	1.03abc
R120+S500	0.12b	0.30b	0.35b	0.71bc
R120+S750	0.28b	0.60ab	0.87a	0.91abc
R160+S250	0.00b	0.50ab	0.86a	0.84abc
R160+S500	0.00b	0.67ab	0.83a	0.55c
R160+S750				

In genotype TDr 131, number of nodes produced was highest with the control treatment but it was not significantly different from the other treatments except R120+S500 which also has the lowest node number. Node number produced in R80+S250 and R80+S750 were not significantly different from R120+S500 Genotype TDr 179 has the highest number of node produced in R80+S250 The nodes produced in R80+S500,R120+S250, R120+S750 and R160+S250 were not significantly different from one another. R160+S500 has the lowest number of nodes which is significantly different from control treatment and R80+S250 rifampicin.

Streptomycin, and gentamicin were found to inhibit both multiplication and rooting rates of yam shoot cultures while treatment with rifampicin did not affect the multiplication rates, this confirms previous investigation that most antibiotics inhibit both multiplication and rooting rate of some plant species. Combination of rifampicin and streptomycin at 80 and 250 µg/ml was effective in controlling contamination; the combined antibiotics did not affect the multiplication rates of the shoots.

Phytotoxicity tests have been reported to be necessary before using antibiotics in the culture medium. This is because several reports of phytotoxicity have been recorded. Dodds and Roberts (1981) found that gentamicin strongly inhibits the differentiation of tracheary elements in cultured explants of pith parenchyma from heads of romaine lettuce. They also reported that concentration of gentamicin in plant tissue cultures media should not exceed 10µg/ml because of phytotoxicity to Jerusalem artichoke explants and to parenchyma of lettuce, but yam tissue culture was found to tolerate gentamicin up to 40µg/ml. Bastaiens *et al* (1983) also reported that chloroampenicol inhibited organogenesis in various plants. Polymycin B was found to be highly toxic to apple, rhododendron and Douglas fir (Young *et al*., 1984). These results showed that streptomycin and gentamicin were phytotoxic to yam shoot cultures confirming earlier works while rifampicin which show no phytotoxicity differs from these reports. Aminoglycosides (streptomycin and gentamicin) undergo complex interactions with cells such as degradation of polyribomes and disrupting the cytoplasmic membrane leading to loss of permeability control and cell death. Their toxicity to plants may reflect the similarities in

permation and protein synthetic process of bacteria (Pollock *et al*., 1983).

CONCLUSION

Different responses to antibiotic toxicity were observed from the two genotypes evaluated in this study. The application of streptomycin (1000µg/ml) and gentamicin (160 µg/ml) at higher concentrations were phytotoxic to yam shoot cultures resulting into death. Genotype TDr 179 strived better than genotype TDr 131 after treatment with different combinations of antibiotics. The best bacteria inhibition by antibiotics occurred at pH 6.9. Rifampicin was effective at 320ug/ml for most of the isolates. This study concluded that phytotoxicity test should be carried out on yam shoot cultures before application of antibiotics.

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