ELIMINATION OF BACTERIA FROM *IN VITRO* YAM TISSUE CULTURES USING ANTIBIOTICS

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SUMMARY

In vitro germplasm collections are always under the threat of air-borne microbial contaminants following poor laboratory practices and endogenous contaminants. Visibly clean cultures of aseptically micropropagated shoot cultures of plants of the genus *Dioscorea* (yam) grown on yam multiplication media are often contaminated with covert bacteria. The bacteria may survive endophytically within plantlets thereby making them unsuitable for *in vitro* maintenance of germplasm. The aim of this study was to evaluate and determine the efficacy of bactericidal doses of antibiotics on contaminated *in vitro* cultures of *Dioscorea rotundata*. Both single antibiotics [rifampicin (Rn) at 125 µg/ml], combination of two antibiotics [tetracycline plus rifampicin (TR), streptomycin plus gentamycin (SG) and vancomycin plus streptomycin (VS) each at 125 µg/ml] and combination of five antibiotics [tetracycline + vancomycin + streptomycin + gentamycin + rifampicin (TVSGR) at a final concentration of 100 µg/ml] were tested on contaminated cultures by growing non-disinfected nodal cuttings on a semi-solid yam multiplication media supplemented with the antibiotics for 3 to 4 weeks. During the period of antibiotic exposure, Rn, TR and TVSGR significantly ($P>0.001$) inhibited bacterial growth on non-disinfected cultures though without complete elimination. Further tests carried out with these three promising antibiotic treatments on both disinfected and non-disinfected *in vitro* yam cultures showed that only 33% of the yam genotype TDr 95/19177 treated with TVSGR were completely free from contaminating bacteria. Phytotoxicity (necrosis) was not observed between the first two weeks of antibiotic (Rn, TR and TVSGR) treatment but only after prolonged exposure.

Key words: antibiotics, bacterial contamination, *Dioscorea* species, yam multiplication media, phytotoxicity.

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INTRODUCTION

In vitro germplasm collection of yam is maintained in tissue culture, a technique that is not deprived of risks because of endophytic bacterial contamination that can occur at any stage of the process (Leifert, 2000).

Media in which plant tissues are cultivated are good sources of nutrients for bacterial growth (Odutayo *et al*., 2007) and so are the plant tissue exudates that serve as additional growth factors (Leifert and Waites, 1992; Leifert *et al.*, 1994). Bacteria can reduce growth rate, retard rooting, increase culture mortality, result in variable growth, cause tissue necrosis, and even plant death (Lefert and Waites, 1992; Kane, 2003). Bacteria are usually difficult to control (Agrios *et al*., 1997) and the most difficult are the endogenous ones which do not cause any visible symptoms in contaminated cultures (Wojtania *et al*., 2005). Most of these bacteria escape the initial surface sterilization (Van Dan Houwe *et al*., 2000) and remain latent during growth on plant multiplication media but will appear after subsequent sub culturing (Cassells *et al*., 1991, 1992, 2001). Endophytic bacterial contamination cannot be eliminated with any surface sterilization techniques, thus requires antibiotic therapy (Mathias *et al*., 1987).

Antibiotics are grouped by their mode of action, i.e. inhibitors of bacterial cell wall synthesis, inhibitors of bacterial protein synthesis and DNA replication blockers, or by their chemical structure, i.e. β-lactans, amino glycosides, quinolones, glycopeptides, polymixins, macrolides and lincosamides (Quesnel and Russell, 1983; Falkiner, 1990). Ideally, antibiotics used in plant tissue culture should be soluble, stable, unaffected by the components or pH of the medium, lack side effects, broadly active, non-resistance inducing, inexpensive and non-toxic to humans (Falkiner, 1990). Many attempts have been made to suppress/eliminate endogenous bacteria from plant cultures with antibiotics with varying degrees of success (Falkiner, 1990). Often, bacterial growth is only suppressed (bacteriostatic effect) by antimicrobial treatments and when chemicals are removed, the bacteria resume growth (Falkiner, 1990, 1997; Barret and Cassells, 1994). In other instances, antibiotics effective on isolated organisms cannot be used

for treating contaminated plants, due to phytotoxicity or poor penetration into tissues (Reed *et al*., 1995). Although phytotoxicity and development of antibiotic-resistant bacterial populations have restricted the use of antibiotics, these side effects can be taken care of by the use of combination of antibiotics at relatively lower concentrations (Leifert *et al*., 1992).

In view of the above problems, this study was aimed at evaluating the bactericidal doses of antibiotics on endogenous bacteria of contaminated *in vitro* cultures of *Dioscorea rotundata*.

MATERIALS AND METHODS

Plant materials. Different genotypes (TDr 95/19177 and TDr 95/00929) of *D. rotundata* which were naturally contaminated with *Burkholderia* spp., *Luteibacter rhizovicinus* and *Bacillus cereus* as identified by CABI (Commonwealth Agricultural Bureax) were used for this study. *Burkholderia* bacteria are Gram-negative, motile, rod shaped and obligately aerobic the same as *L. rhizovicinus.* By contrast*, B. cereus* is Gram-positive, sporulating, rod-shaped and aerobic. These bacterial species usually escape surface sterilization of the *in vitro* yam cultures, remain latent during growth on explant multiplication media and appears after subsequent sub culturing. Roots of contaminated yam cultures appear flocculent/cloudy on yam multiplication medium (Fig. 1b), which consists of MS-basal medium (4.43 g/l), myo-inositol (100 mg/l), sugar (30 g/l), kinetin (0.5 mg/l), L-cysteine (20 mg/l) and agar (7.5 g/l).

Antibiotics. Tests encompassed the use of: (i) single antibiotics, rifampicin (Rn) at 125 µg/ml; (ii) combination of two antibiotics, TR (tetracycline+rifampicin), SG (streptomycin+ gentamycin), VS (vancomycin + streptomycin) each at 125 µg/ml; (iii) combinations of five antibiotics TVSGR (tetracycline + vancomycin + streptomycin + gentamycin + rifampicin) at a final concentration of 100 µg/ml were tested.

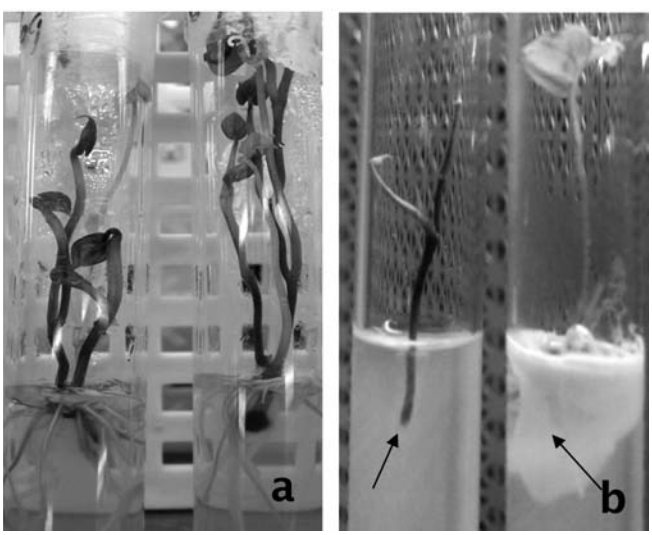

Fig. 1. Healthy (a) and contaminated (b) plantlets of *Dioscorea rotundata.*

Sterilization of yam cultures. Nodal cuttings 0.5-1.0 cm in length were disinfected by immersion into 10% La Croix (2.6% NaOCl) for 20 min after rinsing with 70% ethanol for 2 min. The explants were rinsed in 3 successive changes of sterile distilled water. Non-disinfected cuttings served as controls.

Antibiotics treatments of contaminated plants. Elimination of bacteria by the use of antibiotics alone (trial 1) or by antibiotics with or without surface disinfection (trial 2) were conducted on two different genotypes of contaminated *D. rotundata* (i.e. TDr 95/19177 and TDr 95/00929). The above specified bactericidal doses of the selected fresh filter-sterilized antibiotics (Rn, TR, VS, SG) and TVSGR were incorporated into a sterile semi-solid yam multiplication medium.

Trial 1 was carried out by growing for a period of 3 weeks contaminated, non-disinfected *D. rotundata* cuttings in a semi-solid yam multiplication media at pH 5.7, supplemented with the five antibiotic (Rn, TR, VS, SG and TVSGR) used separately. Trial 2 consisted of growing for a period of 4 weeks contaminated disinfected and non-disinfected *D. rotundata* cuttings in a semi-solid yam multiplication medium at pH 5.7 supplemented with the three promising antibiotics (Rn, TR and TVSGR) selected from the previous trial. After 3-4 weeks of growth, plantlets without visible signs of contamination were subcultured (i.e. first subculture) onto semi-solid yam multiplication medium without antibiotics and grown for additional 3-4 weeks, followed by a second subculture for 3-4 weeks so as to ascertain the effectiveness of the antibiotics used in the two treatments.

At every subculture, reoccurrence of bacterial contamination was monitored and the presence of clean shoots was assessed. The bacterium-free status of clean shoots was ascertained after every second subculture by streaking nodal cuttings on Mueller-Hinton medium (i.e. incubating streaked plates for 18-24 h at 35-37°C) prior to transfer to yam multiplication medium. All cultures were kept in a culture room at 25°C under 12 h photoperiod under cool-white fluorescent lamps with light intensity of 4,000 lux.

For statistical analysis, antibiotic treatments of the two trials were arranged in a completely randomized design with ten replications and the data subjected to analysis of variance (ANOVA) using the statistical analysis system (SAS) software.

Phytotoxicity. The two genotypes of *D. rotundata* (TDr 95/19177 and TDr 95/00929) treated with single

antibiotics (Rn), combination of two antibiotics (VS, TR and SG) and combinations of five antibiotic (TVSGR) were monitored to evaluate the level of antibiotic toxicity during their period of application and during the first and second subculture on to a non-antibiotic media. Phytotoxicity was determined visually checking for necrosis (death of the plantlets) and chlorosis (bleaching of the leaves).

RESULTS

Antibiotic treatments of contaminated yam tissue cultures. The result of the evaluation of the effect of the different antibiotics (Rn, TR, VS, SG and TVSGR) tested on the two contaminated *D. rotundata* genotypes to determine their ability to eliminate bacterial species without adversely affecting the *in vitro* yam cultures, are shown in Fig. 2, 3 and 4.

Trial 1 (Fig. 2) showed that when non-sterilized contaminated nodal cuttings of both genotypes were treated with the five antibiotics for three weeks, bacterial growth was totally inhibited by TR, Rn and TVSGR with no significant difference (P>0.001), but not by SG and VS. However, in the first subculture of the Rn-, TR- and TVSGR-treated cultures onto a non-antibiotic medium, 100% re-occurrence of the contaminating bacterial species was observed in both genotypes. Since three (TR, Rn and TVSGR) out of the five (TR, Rn VS, SG and TVSGR) antibiotics treatment on non-disinfected cultures of both *D. rotundata* genotypes inhibited bacterial growth, the three antibiotics were selected for trial 2, which involved the determination of the susceptibility of the disinfected contaminated cultures to these antibiotics.

Results of this trial showed that during the 4 weeks of treatment, bacteria were inhibited in both disinfected and non-disinfected explants. However, the percentage of clean cultures of disinfected explants (60-90%) was lower than that of non-disinfected counterparts (100%) (Fig. 3 and 4a). This contrast in result may be attributable to the toxicity induced by the synergistic effect of the disinfectants and the antibiotics. At the first subculture of the Rn-, TR- and TVSGR-treated cultures onto a non-antibiotic medium, only TVSGR treatment on disinfected cultures of genotype TDr 95/19177 yielded 33% of sanitized cultures (Fig. 3b), which became 100% at the second subculture (Fig. 3c), as confirmed also on Mueller–Hinton medium (not shown). All bacteria-free, non-disinfected cultures obtained after antibiotic treatment had 100% reoccurrence of the contaminating bacteria at the first subculture.

TVSGR-treated cultures that appeared to be sanitized at the second subculture onto a non-antibiotic media, remained bacteria-free after 6 to 8 months of subculturing.

In conclusion, the experiments carried out on *in vitro*-grown contaminated *D. rotundata* genotypes showed that disinfected explants were more susceptible to contaminating bacterial species than the non-disinfected explants (Fig. 2-4), although depending on the plant genotype. Disinfected contaminated nodal cuttings grown on a semi-solid yam multiplication medium

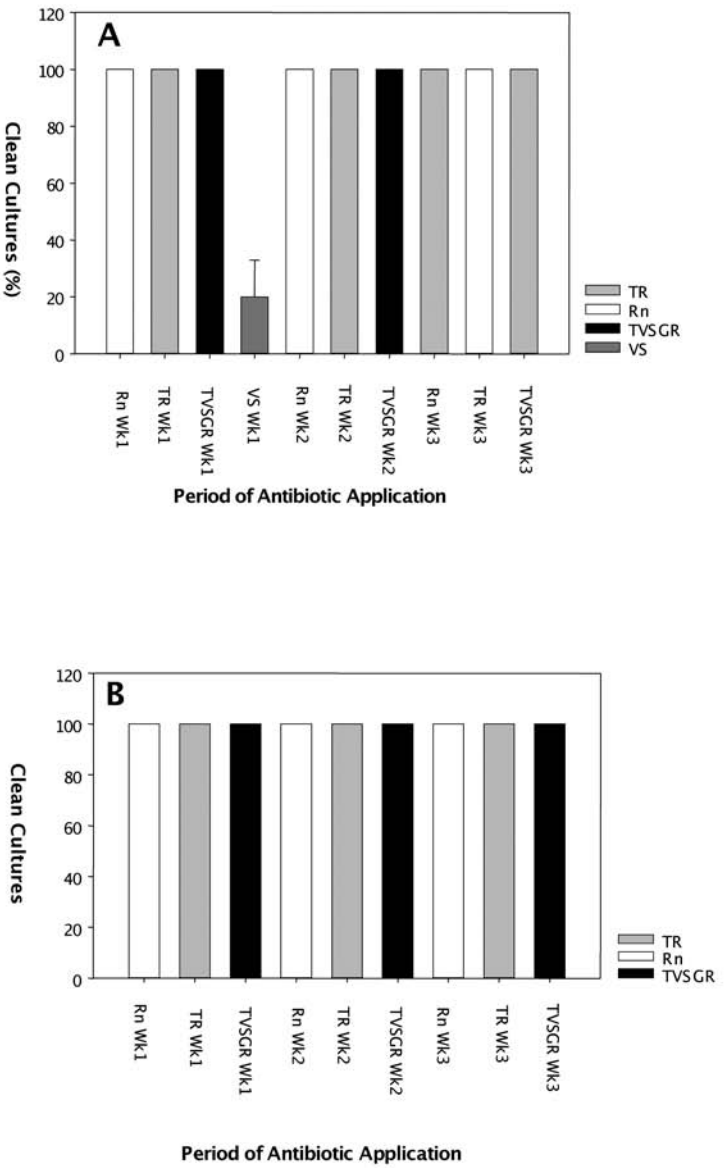

Fig. 2. Effect of semi-solid yam multiplication media at pH 5.7 amended with different antibiotics (Rn, VS and TR at 125 µg/ml and TVSGR at 100 µg/ml) for the elimination of bacteria from non-sterilized *in vitro* cultures of *Dioscorea rotundata* (i.e. genotype: TDr 95/19177 and TDr 95/00929). Vertical bars on the histograms represent the standard errors of the mean of clean (bacteria-free) plants to non-sterilized TDr 95/19177 (A) and TDr 95/00929 (B). Rn, VS, TR and TVSGR refer to non-sterilized plantlets treated with rifampicin, vancomycin+streptomycin, tetracycline+rifampicin and tetracycline+vancomycin+streptomycin+gentamycin+rifampicin, respectively, while WK refers to the various weeks at which the antibiotics applied to the contaminated cultures were monitored for effectiveness.

at pH 5.7 amended with TVSGR antibiotics for 4 weeks were effective in sanitizing 33% of the explants.

Phytotoxicity tests. Results revealed that none of the antibiotic treatments (Rn, TR, SG, VS at 125µg/ml and TVSGR at 100 µg/ml) showed toxicity (i.e. necrosis or leaf chlorosis) to the non-disinfected cultures of *D. rotundata* genotypes (Fig. 2). Phytotoxicity was only observed on the disinfected cultures (Fig. 5) and was significant during the period of antibiotic application (third to fourth week). Toxicity levels at the fourth week were higher than those at the third week but no significant (P>0.001) difference was observed. At first subculture onto a non-antibiotic medium of the Rn-, TR- and TVSGR-treated cultures, necrosis were only observed on 17% of the TVSGR- treated cultures.

DISCUSSION

Serious losses have been reported in tissue cultures due to the presence of endogenous bacteria that multiply within the explants thereby affecting their growth (Leifert *et al*., 1992). Antibiotics are not commonly employed in the treatment of *in vitro* plant tissue cultures contaminated by bacteria unless contamination proves difficult to eliminate by other means. Many attempts have been made to suppress or eliminate endogenous bacteria from cultures with antibiotics with varying degree of success (Falkiner, 1990), although at times faced with the problems of phytotoxicity (Cornu and Michael, 1987). Our data clearly shows that antibiotic treatments of non-disinfected *in vitro* cultures of *D. rotundata* are bacteriostatic rather than bactericidal (Fig. 3 and 4). All doses of the antibiotics applied to the non-sterilized contaminated cultures were only inhibitory and could not ultimately control the contamination. Hence, all the antibiotic-treated plantlets without initial cleaning (disinfection) showed 100% bacterial contamination after one week of growth. This supports the findings of Falkiner *et al.* (1990) that bacteria can lose their cell walls following antibiotic treatment but remain viable as sphaeroplasts persisting as cryptic contaminants. The cell wall can be regenerated and the organisms can become active when the antibiotics are removed, i.e. when transferred to a non-antibiotic medium.

When antibiotics were applied to disinfected *in vitro*-grown yam cultures, only TVSGR treatment afforded about 33% sanitation of *D. rotundata* genotype TDr 95/19177. According to Keskitalo *et al.* (1996), single antibiotic treatments including rifampicin could not control the bacterial contamination in shoot cultures of tansy (*Tanacetum vulgare*). From our study, a treatment with rifampicin alone was unable to eliminate the bacteria and so was also the combination of two antibiotics (TR and SG) but not when five combined antibiotics (TVSGR) were used. Earlier report by Leifert *et al.* (1991) showed that a range of different bacteria were eliminated from contaminated plant tissues of *Hemero-*

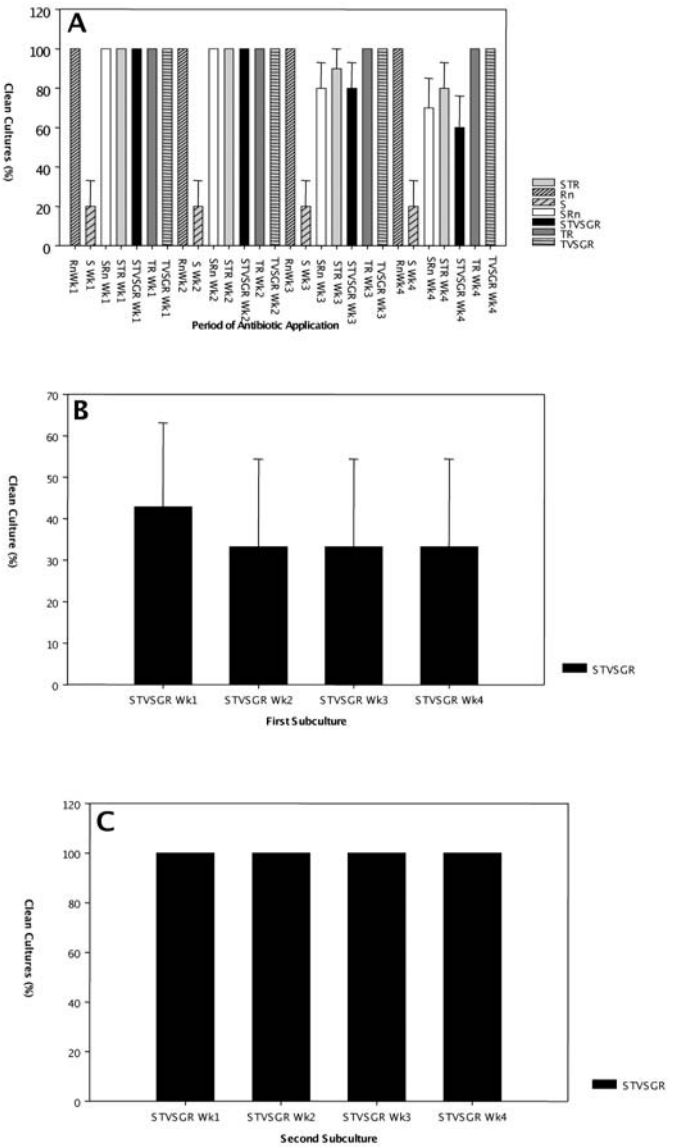

Fig. 3. Effect of semi-solid yam multiplication media at pH 5.7 amended with different antibiotics (Rn, TR at 125 µg/ml and TVSGR at 100 µg/ ml) for the elimination of bacteria from both sterilized and non-sterilized *in vitro* cultures of *Dioscorea rotundata* (i.e. genotype: TDr 95/19177). Vertical barson the histograms represent the standard error of the mean of clean (bacteria-free) plants to sterilized or non-sterilized *in vitro* cultures. Rn, TR and TVSGR refers to non-sterilized plantlets treated with rifampicin (Rn), tetracycline+rifampicin (TR) and tetracycline+vancomycin+streptomycin+gentamycin+rifampicin (TVSGR), respectively, while SRn, STR and STVSGR refer to sterilized plantlets treated with rifampicin (Rn), tetracycline+rifampicin (TR) and tetracycline+vancomycin+streptomycin+gentamycin+rifampicin (TVSGR), respectively. First and second subculture refer to the first and second transfer of clean/treated or bacteria-free plantlets on to an antibiotic-free media, while WK refer to the various weeks at which the antibiotics applied on the first day of treatment to contaminated cultures were monitored for effectiveness**.**

callis, *Choisya* and *Delphinium* using combinations of gentamycin, streptomycin, rifampicin, carbenicillin and cephalotaxim. However, the TVSGR combination we have successfully used has apparently never benn used before. According to Leifert *et al.* (1991), treated plants had to be separated (to avoid cross-contamination by plants that remained infected during sub culturing) and had to be tested for the presence of contaminants for at least 3 months after antibiotic treatment to ensure the absence of latent contaminants. Treated cultures from this study after several subcultures (i.e. 3-6 months) remained bacteria-free.

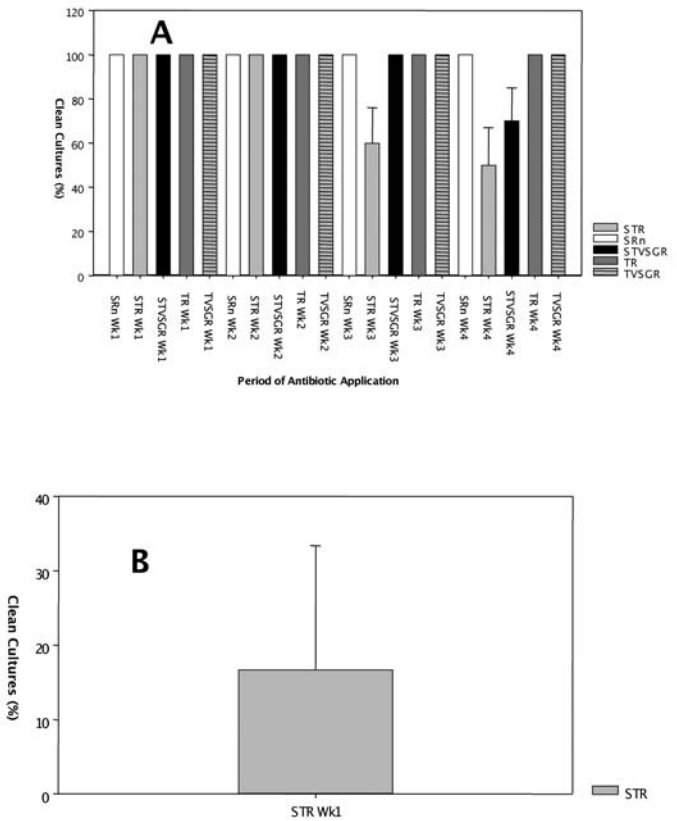

Fig. 4. Effect of semi-solid yam multiplication media at pH 5.7 amended with different antibiotics (Rn, TR at 125 µg/ml and TVSGR at 100 µg/ml) for the elimination of bacteria from both sterilized and non-sterilized *in vitro* cultures of *Dioscorea rotundata* (genotype: TDr 95/00929). Vertical bars on histograms represent the standard error of the mean of clean (bacteria-free) plants to sterilized or non-sterilized *in vitro* cultures. Rn, TR and TVSGR refers to non-sterilized plantlets treated with rifampicin (Rn), tetracycline+rifampicin (TR) and tetracycline+vancomycin+streptomycin+gentamycin+ rifampicin (TVSGR), respectively, while SRn, STR and STVSGR refer to sterilized plantlets treated with rifampicin (Rn), tetracycline+rifampicin (TR) and tetracycline+vancomycin+streptomycin+gentamycin+rifampicin (TVSGR), respectively. First and second subculture refer to the first and second transfer of clean/treated or bacteria-free plantlets on to an antibiotic-free media, while WK refer to the various weeks at which the antibiotics applied on the first day of treatment to the contaminated cultures were monitored for effectiveness**.**

Buckley *et al.* (1995) found that antibiotics cause stunting, yellowing, curling, bleaching of the leaves, or death of mint plants, depending on the antibiotic used and its concentration. From our study, analysis of the phytotoxic effect of antibiotics on *D. rotundata* tissue cul-

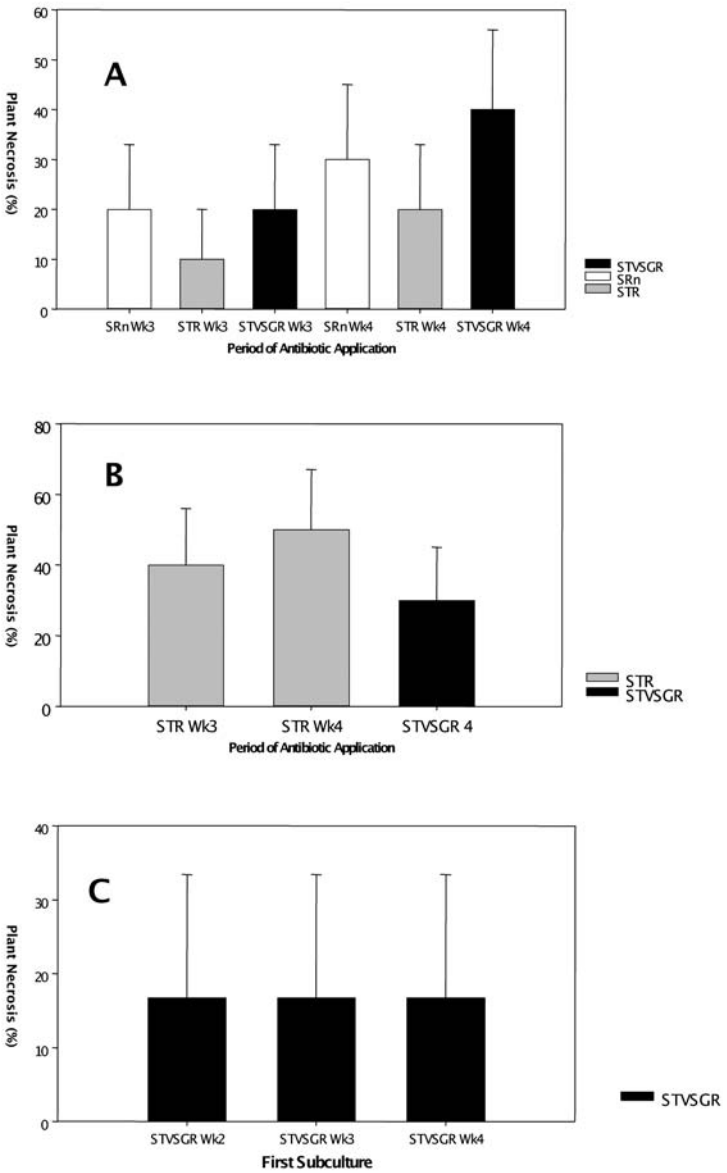

Fig. 5. Phytotoxic effects of Rn, TR (125µg/ml) and TVSGR (100 µg/ ml) on *in vitro* sterilized cultures of *Dioscorea rotundata* genotype: TDr 95/19177 (A) and TDr 95/00929 (B) treated for 4 weeks in a semi-solid yam multiplication media at pH 5.7 amended with the antibiotics (Rn, TR and TVSGR), followed by the first subculture of clean cultures of TDr 95/19177 (C) on to an antibiotic-free semi-solid yam multiplication medium. SRn, STR and STVSGR refer to sterilized plantlets treated with rifampicin (Rn), tetracycline+rifampicin (TR) and tetracycline+vancomycin+streptomycin+gentamycin+rifampicin (TVSGR) respectively, while WK refer to the various weeks at which the antibiotics applied on the first day of treatment to the contaminated cultures were monitored for phytotoxic effects. For each antibiotics type at different week of monitoring, vertical bars represent standard errors of mean necrosis.

tures showed that antibiotic treatments (Rn, TR, SG, VS at 125 µg/ml and TVSGR at 100 µg/ml) to non-disinfected cultures did not induce any apparent toxicity but were significantly toxic (necrosis and leaf chlorosis) to the disinfected cultures during the period of antibiotic application (third to fourth week). Thus, to avoid phytoxicity, antibiotic treatments should be applied only for one to two weeks, followed by two subcultures on a non-antibiotic containing medium. Necrosis of cultures (17%) observed during the first subculture of TVSGR-treated plantlets (Fig. 5) were not observed in other antibiotic-treated cultures at the first subcultures. This might be the result of antibiotic (TVSGR) carryover.

In conclusion, elimination of *D. rotundata* endogenous bacteria is not successful with exposure to single antibiotic or a combination of two. The combination of five antibiotics (tetracycline+vancomycin+streptomycin +gentamycin+rifampicin i.e. TVSGR), which yielded 33% sanitation in one of the yam genotypes (TDr 95/19177), is highly recommended, keeping however in mind that the efficacy of TVSGR varies with the plant genotype. Since antibiotic treatment or disinfection of plantlets alone were ineffective (i.e. neither inhibited nor eliminated bacteria), the synergistic effect between antibiotics and disinfection may be responsible for the 33% bacterial elimination observed in this study. Thus, subjecting nodal cuttings to disinfection before antibiotic treatment is recommended since it controls a higher percentage of contaminating bacteria.

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