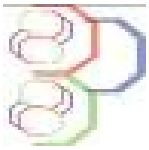

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# Identification and management of microbial contaminants of banana *in vitro* cultures

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## ABSTRACT
Microbial contamination is one of the major challenges hampering the application of *in vitro* micropropagation technique for mass production of pest-free banana planting materials at the Sokoine University of Agriculture in Tanzania.
*Objectives*: The objectives of this study were to identify bacterial and fungal contaminants of banana *in vitro* cultures and to test the efficacy of selected antibiotics and antifungal agents in the elimination of such contaminants.
*Methodology and results*: Purified bacterial isolates were identified based on vegetative cell shape, gram reaction, fluorescent pigment and standard biochemical tests. On the other hand, pure fungal isolates were microscopically identified based on structural and morphological characters. Four antibiotics, namely rifampicin, gentamicin, chloramphenicol and vancomycin each at 100, 150 and 200mg /litre and three antifungal agents, namely ketoconazole, fluconazole and nystatin each at 100, 150 and 200 mg/litre were used in the culture susceptibility tests of the identified bacteria and fungi, respectively. The bacterial contaminants of banana *in vitro* cultures were *Proteus* spp., *Erwinia* spp., *Klebsiella* spp. and *Staphylococcus* spp. while the fungal contaminants were *Aspergillus* spp., *Fusarium* spp,. *Penicillium* spp. and *Candida* spp. Culture susceptibility tests revealed that gentamicin, rifampicin and chloramphenicol each at 150mg/litre effectively suppressed the growth of all the identified bacteria while only ketoconazole at 200mg/litre inhibited the growth of all the identified fungal contaminants.
*Conclusion and application of results*: *Proteus*, *Erwinia*, *Klebsiella* and *Staphylococcus* are the major bacterial contaminants while *Aspergillus, Fusarium, Penicillium* and *Candida* are the main fungal contaminants of banana *in vitro* cultures. These contaminants can effectively be eliminated by incorporation in the growth media of gentamicin, rifampicin and chloramphenicol each at 150mg/litre and ketoconazole at 200mg/litre. Further studies are required to investigate the negative side-effects of these antibiotics and antifungal agents on the growth and genetic stability of banana *in vitro* cultures.
**Key words:** Antibiotic treatment, Antifungal treatment, Microbial contamination, *in vitro* micropropagation, banana

## INTRODUCTION
Plant *in vitro* micropropagation is an aseptic technique for rapid multiplication of pest-fee plant materials from organs, tissues and cells of desirable plants (Vuylsteke and De Langhe, 1985). The growth media in which the plant tissue is cultivated is also a good source of nutrients for microbial growth. These microbes compete with plant tissue cultures for nutrients and some of

them produce phytotoxins which result in culture mortality, tissue necrosis, and reduced shoot proliferation and rooting (Kane, 2003). For instance, fungi *Aspergillus niger* and *Aspergillus flavus* have been reported to produce oxalate and aflatoxin poisons, respectively, that kill plant cultures (Obuekwe and Osagie, 1989). Microbial contamination is one of the major challenges facing plant *in vitro* propagation during different stages of culture processes such as culture initiation and sub-culturing. Sub-culture process is a major source of contamination with about 5-15% of contaminants being introduced for every sub-culture (Leifert, 1990). The major cause of the microbial contamination is insufficient sterilization of explants, growth media, working tools and operators' hands (Omamor *et al*., 2007). The principal microbial contaminants frequently reported in plant *in vitro* cultures are bacteria and fungi (Cassels, 1996). *Pseudomonas syringae*, *Bacillus licheniformis, Bacillus subtilis, Cornebacterium* sp. and *Erwinia* spp. have been reported to be the major bacterial contaminants in plant tissue cultures (Odutayo *et al*., 2004) while the main fungal contaminants frequently observed in plant tissue cultures are *Alterneria tenius, Aspergillus niger, Aspergillus fumigatus* and *Fusarium culmorum* (Odutayo *et al*., 2004; Odutayo *et al*., 2007). Plant materials for *in vitro* propagation are surface-sterilized using either sodium hypochlorite solution at 0.3-1.0% (m/v) for 15-30 minutes or aqueous mercuric chloride at 0.1-1.0%(m/v) for 8 minutes (Meghwal *et al*., 2000; Maina *et al*., 2010). Generally, this surface sterilization eliminates most epiphytic contaminants expect endophytic ones (Habiba *et al*., 2007). An application of systemic fungicides such as benomyl (benlate®) before the collection of plant materials also suppresses microbial contaminants in plant *in vitro* cultures (Mng'omba *et al*., 2007). Alternatively, an incorporation of antibiotics and antifungal agents into the growth media of plant cultures has been reported to eliminate microbial contaminants (Reed *et al*., 1995; Habiba *et al*., 2002). Daily observation has shown that the plant tissue culture laboratory at Sokoine University of Agriculture (SUA) faces serious microbial contamination with about 40 - 60% of the banana *in vitro* cultures being lost. The main aseptic procedures in this laboratory involve growth media sterilization at 121°C for 15 minutes and explant treatment with 4.5% (m/v) laundry sodium hypochloride for 15 minutes, dry heat sterilization of working tools at 180˚C for 120 minutes, flaming of tools during working in 99% methylated spirit and disinfection of lamina flow bench and operators' hands with 70% methylated spirit (Maerere *et al*., 2003). Despite following these aseptic procedures, microbial contamination still remains a major problem affecting banana *in vitro* propagation in this laboratory. The objectives of this study were (i) to identify bacterial and fungal contaminants of banana *in vitro* cultures and (ii) to evaluate the efficacy of antibiotics and antifungal agents on the suppression of the identified microbial contaminants.

## MATERIALS AND METHODS

**Characterization and identification of bacterial and fungal contaminants:** Microbial contaminants were isolated from banana cultures at SUA Plant tissue culture laboratory. Bacterial isolates were aseptically streaked onto sterile nutrient agar (NA) medium and the cultures were incubated at 28˚C for 24 hours. Pure bacterial isolates were obtained by repeated sub-culturing using a serial dilution technique (Collins and Lyne, 1984). The purified isolates were stained for morphological characterization based on vegetative cell shape, gram reaction and presence or absence of spores. Furthermore, standard biochemical tests were conducted, namely methyl red, arginine hydrolase, starch hydrolysis, casein hydrolysis, fluorescent pigment, lactose, citrate and catalase production (Collins and Lyne 1984, Krieg and Holt, 1984; Sneath *et al*., 1986). On ther other hand, fungal isolates were aseptically transferred onto Petri dishes containing potato dextrose agar (PDA) growth medium and the cultures were incubated at 24˚C for 5 to 15 days. The fungal isolates were purified by repeated subcultures onto fresh PDA growth medium. Wet mount slides of pure fungal isolates were prepared and stained with lactophenol cotton blue for identification of the isolates based on microscopic morphological appearance of conidiophores and conidia (Barnett and Hunter, 1972).

**Culture susceptibility tests of identified bacterial and fungal contaminants:** The susceptibility of bacterial cultures to antibiotics was tested using Kirby-Bauer method (Claus, 1995). Briefly, the bacterial growth medium solidified by Mueller-Hinton agar was inoculated with the bacterial isolates. Disks singly impregnated with gentamicin, rifampicin, chloramphenicol and vancomycin each at 100, 150 and 200mg/litre were placed onto the growth medium in 10cm diameter plate after the bacterial inoculation (Figure 1). A treatment consisted of four disks of an antibiotic in a plate replicated four times. The inoculated plates were incubated at 28˚C for 24 hours and the susceptibility of the bacterial isolates to the antibiotics was estimated based on the diameter of the inhibition zone measured using a ruler (Kneifel and Leonhardt, 1992). Inhibition zone diameters of 9 - 14mm, 15 – 19mm and > 20mm meant the bacterial isolate was resistant, intermediate resistant and susceptible to the antibiotic, respectively. On the other hand, ketoconazole, nystatin and fluconazole were tested for anti-fungal activities using agar well diffusion method (Trease and Evans, 1983; Ajaiyeoba *et al*., 1996). Briefly, each test fungal isolate was individually spread using a sterile bent glass rod onto the PDA medium in a 10cm diameter plate and a well was made on each plate using a sterile 6mm diameter cork-borer (Figure 2) . Ketoconazole, nystatin and fluconazole each at concentrations of 100, 150 and 200 mg/litre and sterile water as a negative control were singly filled into the wells with the aid of a pipette. A treatment consisted of 20 plates replicated three times. The plates were incubated at 24˚C for 5 – 7 day and the susceptibility of fungal isolate to the antifungal agents was estimated based on the diameter of the inhibition zone measured using a ruler (Collins and Lyne, 1984). Inhibition zone diameters of 9 - 14mm, 15 – 19mm and > 20mm meant the fungus was resistant, intermediate resistant and susceptible to the antifungal agents, respectively. Data analysis involved computing mean diameters and comparing them with the inhibition zone diameter range.

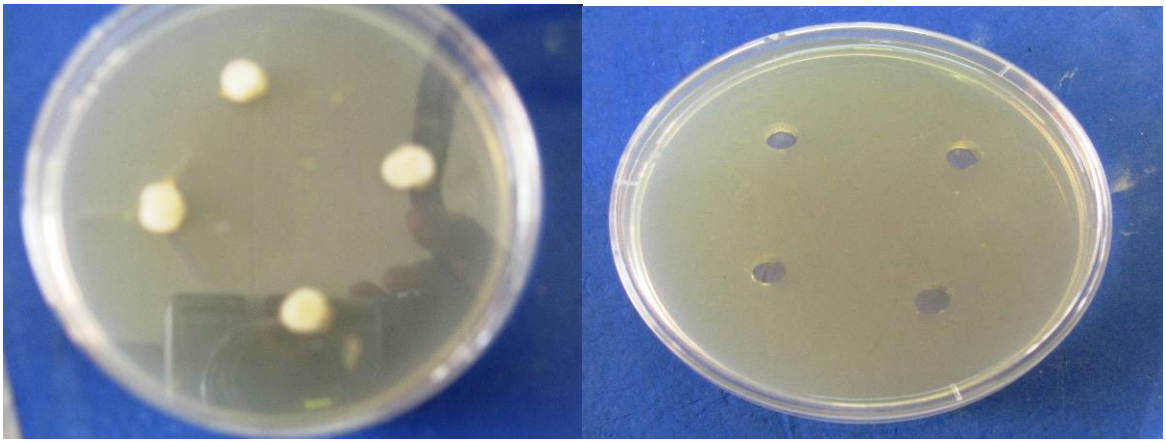

**Figure 1:** Plate of fungal and bacterial growth medium: Left - disks impreginated with antibiotis and Right - wells drilled into the media for injection of anti-fungal agents.

## RESULTS AND DISCUSSION

**Identification of microbial contaminants in banana tissue cultures*:*** The bacterial contaminants of banana *in vitro* cultures at SUA were *Proteus* spp., *Erwinia* spp., *Klebsiella* spp. and *Staphylococcus* spp. (Table 1). The isolated bacterial contaminants in this study have earlier been frequently reported in plant tissue cultures (Kneifel and Leonhardt, 1992; Odutayo *et al*., 2007). For example, *Klebsiella* has endophytically been isolated in internal tissues of banana, maize and wheat (Martinez *et al*., 2003). Endophytic bacteria are beneficial to host plants as they enhance plant defence against diseases (Guan *et al*., 2005) but become problematic in tissue cultres where total asepsis is required. The elimination of endophytic bacteria through surface sterilization is usually ineffective except when stronger and systemic sterilants are used such as mercuric chloride and systemic fungicides like benomyl (Danso *et al*., 2011).

**Table 1:** Characterization and identification of bacterial contaminants of banana *in vitro* cultures

| Vegetative cells | Spore formation | Gram reaction | Methyl red test | Arginine hydrolase test | Starch hydrolysis test | Casein hydrolysis test | Fluorescent pigment test | Lactose utilization test | Citrate test | Catalase test | Name of isolate |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rods | None | _ | + | - | - | - | - | - | - | - | *Proteus* spp. |
| Rods | None | _ | + | + | - | - | - | + | + | - | *Erwinia* spp. |
| Rods | None | - | - | - | - | + | - | + | + | - | *Klebsiella* spp. |
| Cocci | None | + | - | - | + | + | - | + | + | + | *Staphylococccus* spp. |

+: Positive result and - : Negative result

Conversely, *Proteus* spp., *Erwinia* spp. and *Staphylococcus* spp. are exogenous bacteria that are found in soils, water and plant surfaces. The occurrence of exogenous bacteria in plant tissue culture in this study was probably due to an insufficient surface sterilization of explants, tools and culture vessels. Being on plant surfaces, exogenous bacteria are generally easy to eliminate using normal surface sterilization techniques (Mathias *et al*., 1987; Meghwal *et al*., 2000; Kane, 2003). The fungal contaminants in banana *in vitro* cultures in this study were *Aspergillus spp., Fusarium spp.*, *Penicillium spp.* and *Candica* spp. (Table 2).

**Table 2:** Characterization and identification of fungal contaminants of banana *in vitro* cultures

| Isolate description | Name of isolate |
|---|---|
| Colonies flat, filamentous, velvety, woolly, or cottony in texture. Colonies initially white but later becoming blue green or grey green at centre surrounded by white. Isolates appear simple or branched with conidiophores, metulae, phialides and conidia. Metulae carry flask-shaped phialides which form brush-like clusters. | *Penicillium* spp. |
| Immature heads white while mature heads are in shades ranging from yellowish cream to green or black. Conidiophores bear heads, long and hyaline that terminates in bulbous heads while conidia are globose to subglobose and usually rough yellowish green and dark brown. | *Aspergillus* spp. |
| Isolates white to off-white growth, pionnotes wet and full of microconidia with oval, elliptical or kidney-shapes and held together in false-head on monophialides and polyphialides, and falcate macroconidia with 3-5 sepates. | *Fusarium* spp. |
| Colonies generally flat, smooth, moist, glistening or dull, and cream to tannish cream in colour and sometimes peach coloured. Microscopically, blastoconidia unicellular, globose and ellipsoid to elongate in shape. Multipolar budding is typical, pseudohyphae, if present, are rudimentary and hyphae are absent. | *Candida* spp. |

*Fusarium* spp., *Penicillium* spp. and *Aspergillus* spp. are exogenously found in soils, water and plant surfaces (Cassels, 1990) but are also endophytes in some plant species (Suryanarayanan *et al*., 2000). For instance, *Fusarium* has been reported as an endophytic fungus in banana and pumpkin plants while *Penicillium* spp. and *Aspergillus* spp. were found in internal tissues of mallow plants *(*Suryanarayanan *et al*., 2000; Odutayo*etal.*, 2007*)*. The occurrence of exogenous fungal contaminants in banana *in vitro* cultures in this study was possibly due to an inadequate surface sterilization. Several studies have also associated the incidence of exogenous fungal contaminants in plant *in vitro* cultures with an insufficient sterilization (Cassells, 1991; Kane, 2003). *Candida* is a genus of yeasts that only occurs in animals and humans as a harmless commensal or endosymbiont (Hecror and Domer, 1983), and its incidence in banana *in vitro* cultures in

this study was possibly due to an insufficient asepsis among workers during tissue culture operations.

**Culture susceptibility test of isolated microbial contaminants:** Chloramphenicol, rifampicin and gentamicin each at a concentration of 150mg/litre were effective in the suppression of *Klebsiella* spp., *Proteus* spp., *Erwinia* spp. and *Staphylococcus* spp. (Table 3).

**Table 3:** Culture susceptibility test of the identified bacterial contaminants to different antibiotics

| Bacteria genus | Chloramphenicol (mg/L) | | | Rifampicin (mg/L) | | | Gentamycin (mg/L) | | | Vancomycin (mg/L) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 200 | 150 | 100 | 200 | 150 | 100 | 200 | 150 | 100 | 200 | 150 | 100 |
| *Klebsiella* | S | S | I | S | S | I | S | S | I | I | R | R |
| *Erwinia* | S | S | I | S | S | I | S | S | I | S | I | R |
| *Proteus* | S | S | I | S | S | I | S | S | I | S | R | R |
| *Staphylococcus* | S | S | I | S | S | I | S | S | I | S | S | I |

R = Resistant, S = Susceptible and I = intermediate resistant

**Table 4:** Culture susceptibility test of the identified fungal contaminants to different antifungal agents

| Fungal genus | Ketoconazole (mg/L) | | | Fluconazole (mg/L) | | | Nystatin (mg/L) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 200 | 150 | 100 | 200 | 150 | 100 | 200 | 150 | 100 |
| *Penicillium* spp. | S | S | I | S | I | R | I | I | R |
| *Aspergillus* spp. | S | S | I | S | I | R | I | I | R |
| *Fusarium* spp. | S | I | I | I | R | R | R | R | R |
| *Candida* spp. | S | S | I | S | I | R | I | I | R |

R = Resistant, S = Susceptible and I = intermediate resistant

The effectiveness of gentamicin and rifampicin to suppress both endophytic and epiphytic bacterial contaminants has earlier been reported in dessert banana *in vitro* cultures in which gentamicin and rifampicin suppressed *Klebsiella, Erwinia, Pseudomonas*, *Corynebacterium*, *Bacillus* and *Cellulomonas* (Keskitalo *et al*., 1998; Habiba *et al*., 2002). Gentamicin is a broad-spectrum anti-bactericidal agent of gram positive and gram-negative bacteria that suppresses bacterial growth by inhibiting cell protein synthesis (Falkiner, 1990; Reed *et al*., 1995; Habiba *et al*., 2007). Unfortunately, gentamicin has been reported to have toxic effects to plant cultures for at a dose of 100mg/litre it inhibited shoot initiation from tobacco callus and reduced *in vitro* shoot growth of tansy *(Tanacetum vulgare*) plants (Eichholtz *et al*., 1982; Keskitalo *et al*., 1998; Thomas, 2004). Rifampicin is a bactericidal agent that inhibits nucleic acid synthesis and effectively suppressed bacterial contaminants at 50 mg/litre in artichoke explant cultures without having any adverse effects on plant cell division, differentiation and DNA synthesis (Phillips *et al*., 1981). The effectiveness of chloramphenicol against the identified bacteria in this study is comparable to previous reports (P'eaud-Lenoël and de Gournay-Margerie, 1962; Gholamreza *et al*., 2008). Chloramphenicol is a broad-spectrum bacteriostatic agent that inhibits protein synthesis and is usually effective against a wide range of gram-negative and gram positive bacteria (Gholamreza *et al*., 2008). However, chloramphenicol has been reported to inhibit the uptake of solutes in isolated wheat plant roots (P'eaud-Lenoël and de Gournay-Margerie, 1962).

On the other hand, culture susceptibility test revealed that vancomycin at 200mg/litre was only effective against *Erwinia*, *Proteus* and *Staphylococcus*. Vancomycin at higher concentration of 250mg/litre in combination with cefotaxime at 250 mg/litre effectively eliminated *Erwinia*, *Proteus, Staphylococcus*as and *Agrobacterium tumefaciens* in soybean embryogenic tissues without any significant toxic effects to plant cells (Wiebke *et al*., 2006). However, results on the effectiveness of vancomycin against a wide variety of gram-positive pathogens are still contradictory for vancomycin-resistant enterococci, streptococci and staphylococci strains have continued to evolve (Jones, 2006).

Ketoconazole at a concentration of 200mg/litre was the most effective against all the identified fungal contaminants, namely *Aspergillus* spp*., Fusarium* spp*., Penicillium* spp*.* and *Candida* spp. (Table 4). The effectiveness of ketoconazole in this study supports earlier reports in which it suppressed fungal contaminants in animal cell cultures, especially *Aspergillus fumigatus, Candida albicans* and *Penicillium* spp. (Wyler *et al*., 1979). Ketoconazole is a systemic antifungal agent that interferes with the synthesis of fungal cell membranes as well as certain enzymes' activities (Shepp *et al*., 1985). Although reports on phytotoxic effects of ketoconazole are scanty, the antifungal agent has been reported to suppress larval development in mussel *in vitro* culture (Owen *et al*., 2010). In this study, fluconazole at 200mg/litre effectively suppressed *Penicillium*, *Aspergillus* and *Candida* except *Fusarium* spp. Fluconazole belongs to the azole class of antifungal drugs and is generally considered to be a systemic fungistatic rather than fungicidal in standard *in vitro* susceptibility tests (Sheehan, 1993). The side-effects of fluconazole are hardly known for it has not yet been used to suppress fungal contaminants in plant tissue culture.

## CONCLUSION

*Proteus*, *Erwinia*, *Klebsiella* and *Staphylococcus* are the major bacterial contaminants while *Aspergillus, Fusarium, Penicillium* and *Candida* are the main fungal contaminants of banana *in vitro* cultures at SUA. *Klebsiella*, *Aspergillus, Fusarium* and *Penicillium* occur as both endophytic and epiphytic contaminants while *Proteus*, *Erwinia and Staphylococcus* exist as ephytic contaminants only. Based on culture susceptibility tests, gentamicin, rifampicin and chloramphenicol each at 150mg/litre can effectively suppress all the identified bacterial contaminants while only ketoconazole at 200mg/litre is able to suppress all the isolated fungal contaminants. These findings suggest that the identified microbial contaminants of banana *in vitro* culture can effectively be suppressed by a combination of strategies, including incorporation in banana culture growth media of gentamicin, rifampicin and chloramphenicol and ketoconazole as well as improving surface sterilization and training laboratory operators on general aseptic procedures. Further studies are required to investigate the adverse side-effects of these antibiotics and antifungal agents on the growth and genetic stability of banana *in vitro* cultures.

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