BACTERIAL CONTAMINATION IN *MUSA* SHOOT TIP CULTURES

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Abstract

Banana (*Musa* spp.) shoot tip cultures from the INIBAP (International Network for the Improvement of Banana and Plantain) Transit Centre gene bank are routinely screened for bacterial contamination during initiation and subculturing. The bacteriological test involves streaking of the explant base onto nutrient agar, enriched with 1% glucose and 0.5% yeast extract. Bacteria have been recovered from 4.5% of the 500 tested accessions. Over 60% of the contaminants are identified as *Bacillus* spp.

Culture of 1mm sized meristem tips, isolated from contaminated *in vitro* cultures, proves to be effective for the elimination of bacteria but risks losing explants (33%) due to establishment failure. However, up to 100% of the growing meristem tips tested negative for bacteria.

In another experiment a range of antibacterial agents i.e. rifampicin, tetracyclin, streptomycin, trimethoprim, penicillin and bacitracin were tested for their ability to control *Bacillus pumilis* in banana shoot tips. Their phytotoxicity was also examined. Treatment of shoot tips with rifampicin at 100mg.l^{-1} during 1 month resulted in 100% bacteria-free explants without any phytotoxicity.

1. Introduction

Bacterial contamination is a considerable source of concern in all aspects of plant cell, tissue and organ culture. Tissue culture contaminating bacteria may be natural endophytes or rhizosphere microorganisms colonizing plants in their natural *habitat*. These bacteria are carried over into culture as they usually reside in internal (intra- or intercellular) plant parts and escape the effect of surface desinfectants. Although not necessarily pathogenic or harmful to plants in the field, these bacteria may impose serious problems during *in vitro* culturing. Alternatively, bacterial contaminants can be introduced accidentally during tissue culture manipulations, with unsterile laboratory equipment, culture vessels or ineffective sterilized water. Some of these bacteria e.g. *Bacillus* are particularly persistent and spread easily in cultures as they form heat resistant spores (Trick and Lingens, 1985).

A wide range of gram-positive and gram-negative bacteria including *Bacillus, Corynebacterium, Micrococcus, Pseudomonas, Staphylococcus, Xanthomonas*, have been reported in tissue cultures of various plant species e.g. in *Drosera, Nephrolepis, Spatiphyllum, Syngonium* (Kneifel *et al.*, 1992), *Mentha* (Reed *et al.*, 1995), *Fragaria, Malus* and *Triticum* (Trick and Lingens, 1985). Many of these 'internal' contaminants decreased multiplication rates or killed plant tissue cultures.

The INIBAP *Musa* Germplasm Transit Centre holds under the auspices of FAO, the global *in vitro Musa* collection. Presently, this collection comprises approximately 1,100 accessions, kept *in vitro* for 1 to 20 years. The germplasm is stored under medium term

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storage conditions at low temperature (16°C) and under continuous low illumination ($25\mu E.m^{-2}.s^{-1}$), requiring an annual subculture (Van den houwe *et al.*, 1995). Tissue cultures in storage are monthly inspected for contamination but the presence of latent bacteria is nearly impossible to assess, as there are mostly no signs on the medium or plant tissue. Occasionally, bacteria become apparent, after repeated subculturing when sufficient high levels of contamination are reached or immediately after annual subculture when nutrient and pH conditions are changed and temporarily 'haloes' of bacterial growth are formed around the shoot tip base.

We experienced that internal contaminants usually do not present a serious obstacle to the storage of proliferating shoot cultures nor to the multiplication or regeneration of banana shoots. However, their persistence in tissue cultures hampered the initiation of embryogenic cell suspensions and the cryopreservation of meristematic tissues.

The prevention and control of contamination in the collection consists of the use of clean source plants unlikely to harbor microorganisms, the regular screening of explants for asepsis and the elimination of contaminated cultures.

In 1996, the systematic indexing of the entire collection for the presence of cultivable bacteria was initiated. The detection method used is simple, cheap and non-destructive, and involves streaking of explant base onto a bacteriological medium, prior to culturing. Accessions are screened at tissue culture initiation and at annual subculture. Contaminated explants are systematically rejected, unless this would result in the entire elimination of an accession. Then the entire set of contaminated cultures are subjected to a therapeutic treatment.

In this paper we study the explant size to eliminate bacterial contaminants from banana shoot tip cultures and investigate the ability of a range of antibacterial agents to control the frequently occurring gram- positive *Bacillus* spp. in banana shoot tips.

2. Materials and methods

2.1. Media

Each accession in the collection is represented by 20 proliferating cultures, initially derived from one single shoot tip. Cultures are maintained on Murashige and Skoog (MS) (Murashige and Skoog, 1962), semi-solid medium supplemented with $10mg.l^{-1}$ ascorbic acid, $30g.l^{-1}$ sucrose, $2.25mg.l^{-1}$ 6-benzyladenine (BA), $0.175mg.l^{-1}$ indole-3-acetic acid (IAA) and $2g.l^{-1}$ Gelrite.

In the experiments performed, banana explants were cultured on the same medium but with $0.225mg.l^{-1}$ BA instead. Medium is solidified with $2g.l^{-1}$ Gelrite, unless otherwise mentioned. The pH of the medium is adjusted to 6.2 prior to autoclaving (5.8 after autoclaving).

Musa explants were screened for 'internal' contamination on the bacteriological medium Difco Bacto nutrient agar (NA) enriched with 1% glucose and 0.5% yeast extract, at pH 7.

2.2. Screening for bacterial contaminants

Before culturing, the base of each shoot tip, was streaked three times on plated bacteriological medium. Streaked plates were wrapped with parafilm and incubated in a culture room at 28°C. Streaks were checked after 1 week for fast growing bacteria and again after 4 and 6 weeks for slowly growing contaminants.

Explants of 1mm size were found to be too fragile for streaking onto bacteriological medium and were therefore tested for bacterial contamination after 4 weeks of *in vitro* growth.

2.3. Identification of bacterial isolates

Twenty three bacterial isolates were purified through repeated subculturing of individual colonies on bacteriological medium. Individual colonies were characterized morphologically (size, shape and color). Standard bacteriological tests (Gram stain, heat test (15 min at 80°C) to confirm the presence of spores, oxidase and katalase tests) were performed. API 50 CHB test kits (API Analytical Products, Plainview, NY) and the BIOLOG System (Hayward, CA) were used to identify bacterial isolates.

2.4. Localization of bacteria in the banana plant

Bacillus circulans contaminated *in vitro* plantlets of banana cultivars La (AAA) and Pisang Palembang (AAB) were transversely sliced into 1mm thin slices. Instruments (forceps and scalpel) were flamed thoroughly after each manipulation. Tissue slices were cultured for endophytes on bacteriological medium and inspected after 3 and 7 days.

Tissue pieces were isolated from different plant parts of 6 months old greenhouse plants of the cultivar NBB11 (AA), nursed from *B. circulans* contaminated *in vitro* cultures. Cubes of 2-3 cm^3 were excised from the roots, cortex and central cylinder of the corm, the area of the apical meristem and pseudostem. They were immersed for 30 sec in ethanol (70%) and subsequently surface sterilized for 20 min in a 2% sodium hypochlorite solution with a small amount of wetting agent (Tween 20). Then, the tissue cubes were rinsed 3 times for 10 min in sterile distilled water. They were re-cut aseptically. From the leaves, pieces of $2cm^2$ were excised, washed in ethanol (70%) for 5 min, and rinsed three times for 10 min in sterile distilled water. Desinfected tissues of each sampled plant part were crushed and plated onto enriched NA. Plates were observed 3 and 7 days after incubation. There were at least 15 replicates for each plant part.

2.5. Elimination of bacteria from plants

2.5.1. Explant size

Apical tips of 10, 5 and 1mm size were isolated from *Bacillus* contaminated cultures of four varieties grown for one month on proliferation medium: Banksii (M. *acuminata*), La (AAA), Tani (M. *balbisiana*) and Pisang Palembang (AAB). Subsequently, 10 and 5mm sized tips were tested on the bacteriological medium as described above, prior to culturing. The meristem tips of 1mm were placed directly onto plant growth medium. They were incubated at 28°C and in order to minimize blackening of the tissue they were placed in darkness for 1 week. Subsequently they were grown for 3 weeks in light with an intensity of 64 $\mu E.m^{-2}.s^{-1}$. After one month, the survival rate was determined and the cultures were screened for bacterial contamination. Bacteria freed explants were monthly re-indexed at subculturing for a period of 8 months.

2.5.2. Screening of antibacterial agents for bactericidal activity

Bacillus pumilis was tested for its sensitivity to six antibiotics, known to be active against gram-positive bacteria: rifampicin, tetracyclin, streptomycin, trimethoprim, penicillin and bacitracin. Stock solutions of antibiotics were prepared with desionized water and filter-sterilized using 0.22µM membrane filters. Liquid plant growth medium (9ml) was supplemented with 0, 25, 50, 100 and 200 $mg.l^{-1}$ of each antibiotic. 1ml of a diluted *B. pumilis* suspension ($cfu=4.1x10^3.ml^{-1}$) was mixed with the medium and incubated for 24 hr at 28°C. Subsequently, the bacterial number was determined for each concentration according to the pour-plate method and minimal bactericidal concentrations (MBC) estimated.

2.5.3. Treatment of shoot tips with antibacterial agents and screening for phytotoxicity

Shoot tips were isolated from *Bacillus pumilis* contaminated banana *in vitro* shoots of cultivar 'Grande Naine' (AAA) and submerged in liquid plant growth medium in the presence of tetracyclin (100mg.l^{-1}), rifamicine (100mg.l^{-1}), streptomycin (100mg.l^{-1}) or a mixture of these three antibiotics in a concentration of 33.3mg.l^{-1} each. They were incubated on a rotary shaker at 28°C and 63μE.m^{-2}.s^{-1} light intensity.

The efficacy of the antibiotic treatments to eliminate *Bacillus* was determined after 4 weeks. Shoot tips were excised from the treated shoots and tested on bacteriological medium. Subsequently the shoot tips were cultured on fresh antibiotic-free semi-solid plant growth medium.

Phytotoxic effects were measured after one month of treatment. The cultures were scored according to the following five tissue culture performance classes: (0) normal (vigorous shoots with normal roots), (1) slow growth (reduced outgrowth of leaves and roots), (2) poor and slow growth (developing leaves, no roots, healthy meristem), (3) very poor growth (no shoot outgrowth but healthy meristem), (4) no growth and chlorosis (intact meristem), (5) dead.

3. Results

3.1. Isolation and identification of bacteria

Bacteria were recovered from 23 out of 500 accessions tested. Isolates were purified and colonies were characterized morphologically. Then they were separated into three groups based on their growth characteristics and diagnostic test results.

Members of the first group, representing 60% of all isolates, form fast growing, large, creamy colonies on enriched nutrient agar. They were characterized as gram-positive, motile rods and formed heat resistant endospores. With the API 50 tests, bacteria in this group were identified to species level as *Bacillus circulans*, *B. pumilis*, *B. sphaericus* and *B. stratothermophilus*.

The second group comprises 7 isolates, characterized by a very slow growth. They form punctiform, raised, creamy, yellow to orange colored colonies, 4-6 weeks after inoculation on bacteriological medium. Other common media failed to increase the growth rate of these bacteria (data not shown). The cells are very small and rod shaped, and stained gram-negative. Since growth of the bacteria could not be achieved under the standardized conditions for BIOLOG, API or fatty acid analysis, they remain unidentified.

The third group counts 2 isolates which produce fast growing, large creamy colored, flat colonies with entire edges. The bacteria cells stained gram-negative. One isolate was identified by BIOLOG as *Acinetobacter* at 88%.

3.2. Localization

Bacillus was recovered from root and corm samples isolated from contaminated in vitro plants. The isolation frequency was 100% and 84% respectively. The apex, stem and leaves of in vitro plantlets appeared to be bacteria-free. Similar results were obtained with 6 months old greenhouse plants, nursed from Bacillus contaminated in vitro cultures. Bacteria colonies overgrew root and cortex tissue, and tissue from the central cylinder within 3 days after plating on bacteriological medium, whereas no contaminants were recovered from the apex, leaf tissue and tissue from the pseudostem, after 7 days of incubation.

3.3. Elimination of bacteria from in vitro cultures

3.3.1. Explant size

The reduction of the explant to a size of 1mm proved to be effective to eliminate bacteria from contaminated tissue cultures. 1mm meristem tips isolated from shoots of banana clones La and Banksii, gave 94 to 100% bacteria-free plants. All larger explants of 5 and 10mm remained contaminated (Figure 1). In the case of 1mm meristems isolated from proliferating bud clusters of P. Palembang and Tani, bacteria- free plants were recovered at a rate of 73% and 50%, respectively. However, because of the small size of the meristem tip, 20 to 33% failed to establish. All bacteria-free explants remained clean over a period of 8 months.

3.3.2. Effect of antibiotics on bacteria

Penicillin and bacitracin which both interfere with the bacterial cell wall synthesis have almost no effect on *B. pumilis* (Figure 2), even at the highest concentration tested.

The protein synthesis inhibitors, trimethoprim, streptomycin, tetracyclin and rifampicin, suppress growth of *B. pumilis* to different extents: trimethoprim at 200mg.l^{-1} reduces growth of *B. pumilis* with 50%, tetracyclin and streptomycin are bactericidal at 100mg.l^{-1} and rifampicin kills *B. pumilis* at 50mg.l^{-1}.

3.3.3. Treatment of contaminated shoot tip cultures with antibiotics.

In banana shoot tips of 10mm rifampicin (100mg.l^{-1}) effectively eliminates *B. pumilis* without plant tissue damage (Figure 3). All shoot tips were found bacteria free after one month of treatment and remained clean for the testing period of 8 months. Both, tetracyclin and streptomycin, incorporated into the plant culture medium at the MBC (100mg.l^{-1}) killed bacteria in respectively 100% and 70% of the explants. However 'Grande Naine' shoot tips died during the treatment. A mixture of rifampicin, streptomycin and tetracyclin at 33.3 mg.l^{-1} each, was not effective to control *B. pumilis* as all treated shoot tips remained contaminated. Moreover, this mixture of antibiotics induced severe toxic symptoms in the banana tissue (Figure 3) which only disappeared after a transfer to an antibiotic-free medium.

4. Discussion

Bacterial contaminants of *in vitro* cultures of banana which appeared to be internal in origin can be detected and isolated by streaking the base of shoot tip explants onto nutrient agar enriched with 1% glucose and 0.5% yeast extract. Twenty three isolates were recovered from accessions maintained for various periods of time (2-12 years) *in vitro*. Endospore-forming *Bacillus* species were found the most frequently occuring contaminants. Leifert *et al.* (1989) found *B. circulans* only in plant materials which had been cultured in glass rather than in irradiated plastic culture vessels and reported that heat resistant endospores of *B. pumilis* can be isolated from standard sterilized (121°C for 20 min) plant growth medium. Their findings indicate that spore forming *Bacillus* species are easily spread in the laboratory due to inefficient sterilization and that these bacteria are likely enriched in tissue cultures over time. This might explain the relatively high isolation frequency of *Bacillus* spp. from banana accessions in the collection.

The distribution of contaminants in various parts of *in vitro* and greenhouse plants was studied and our results strongly suggest that contamination remains localized to roots and corm tissue only. The shoot apical zone was found bacteria-free, indicating that culture of small meristems could eliminate bacterial contaminants from banana cultures.

One millimeter meristem tips, isolated from contaminated cultures, gave 50-100% bacteria-free cultures. Best results were obtained with meristem tips isolated from shoots

whereas their isolation from clusters of naked meristems resulted in 50-73% bacteria-free cultures.

The main advantage of the presented technique is its easiness for application, whatever the bacterial contaminant, although, a relatively high mortality rate of the explants (33%) needs to be considered.

Alternatively, our experiments showed the feasibility of using antibiotics to eradicate identified bacteria from contaminated banana shoot tip cultures. Six products with activity against gram-positive bacteria were evaluated. Among the antibiotics examined, rifampicin, tetracyclin and streptomycin kill *Bacillus pumilis* in shoot tips, but rifampicin ($100 mg.l^{-1}$) is the only agent that successfully eliminates *B. pumilis* without affecting the normal growth of the shoot tips.

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Attachments

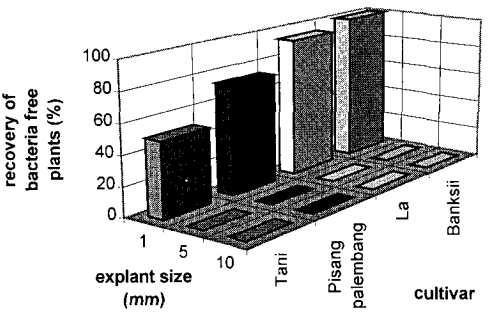

Figure 1 - Percentage of bacteria-free plants as a function of the explant size and cultivar.

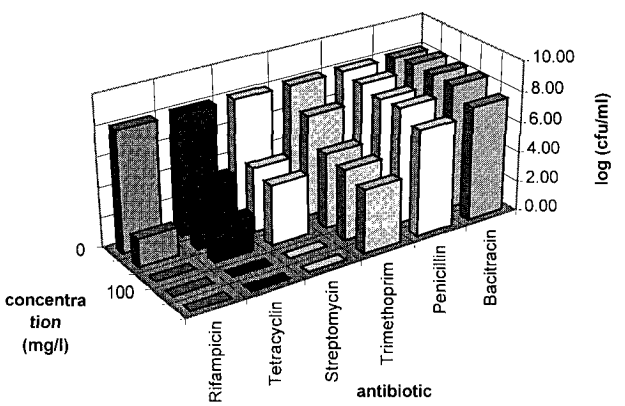

Figure 2 - Effect of different antibiotics on the growth of *B. pumilis*, isolated from contaminated *Musa in vitro* plants. Cfu.ml^{-1} was determined after 24h of incubation at 28°C.

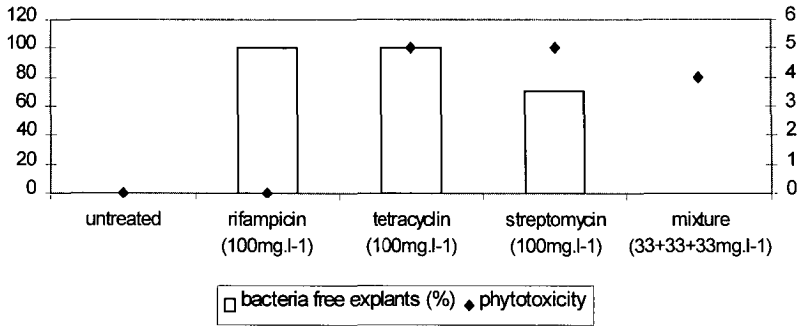

Figure 3 - Effect of different antibiotics, alone or mixed, on *B. pumilis* contaminated shoot tips of cultivar 'Grande Naine' (AAA) after one month treatment in liquid culture. Phytotoxicity rating: (0) normal (vigorous shoots with normal roots), (1) slow growth (reduced outgrowth of leaves and roots), (2) poor and slow growth (developing leaves, no roots, healthy meristem), (3) very poor growth (no shoot outgrowth but healthy meristem), (4) no growth and chlorosis (intact meristem), (5) dead.