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Determination of the Sources of Microbial Contaminants of Cultured Plant Tissues

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Abstract: Microbial contamination is a constant problem, which often compromise development of all *in vitro* techniques. This study aimed at investigating the sources of microbial contamination in tissue culture laboratories in southwestern Nigeria. Nineteen microbial contaminants (consisting of eleven bacteria and eight fungi) were found associated with the tissue culture plants and the laboratory environments. The bacterial contaminants includes, *Pseudomonas flourescens*, *Escherichia coli, Proteus* sp., *Micrococcus* sp., *Streptococcus pneumoniae, Staphylococcus aureus, Bacillus cereus*, *Bacillus subtilis*, *Corynebacterium* sp. and *Erwinia* sp., While Fungi isolates were *Alterneria tenius, Aspergillus niger, Aspergillus fumigatus, Cladosporium* sp., *Saccharomyces* sp., *Fusarium oxysporum, Rizopus nigricans* and *Fusarium culmorum.* The rate of occurrence of *Staphylococcus aureus, Bacillus cereus* and *Escherichia coli* were found to be higher (ranging from 36-46%) in human skin than in all other sampled materials. The laboratory walls and tables also harbored most of the contaminating microbes. The laboratory indoor air was found associated with some of the contaminating microbes.

Key words: Micro-propagation, plant tissue culture, bacterial and fungal isolates, contamination

INTRODUCTION

The practice of plant tissue culture has contributed towards the propagation of large number of plant from small pieces of stock plant in relatively short period of time (Daniel, 1998). Basically the technique consists of taking a piece of a plant (such as a stem tip, node, meristem, embryo, or even a seed) and placing it in a sterile, (usually gel-based) nutrient medium where it multiplies. In most of the cases the original plant is not destroyed in the process, a factor of considerable importance to the owner of a rare or unusual plants. The micro propagation has also been used extensively in the improvement of selections of plant with enhanced stress or pest resistance, production of pathogen free plants and somatic hybridizations (Daniel, 1998). 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 embryos for artificial seed.

The nutrient media in which the plant tissue is cultivated is a good source of nutrient for microbial growth. These microbes compete adversely with plant tissue culture for nutrient. The presence of these microbes in these plant cultures usually results in increased culture mortality, the presence of latent infections can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane, 2003).

Although, the tissue culture techniques usually involves growing stock plants in ways that will minimize infection, treating the plant material with disinfecting chemicals to kill superficial microbes and the sterilizing the tool used for dissection, the vessels and media in which cultures are grown (Kane, 2003). However, contamination has been reported as constant problem, which can compromise development of all *in vitro* techniques (George, 1993).

About thirty-one micro-organisms from ten different plant cultivars growing in micro-propagation have been isolated identified and characterized, with Yeasts, *Corynebacterium* sp. and *Pseudomonas* sp. being predominant (Leggatt *et al*., 1994). *Bacillus* sp., *Corynebacterium* sp. and an Actinomycete have also been found contaminating the vitro culture of apple rootstocks (Hennerty *et al*., 1994). Odutayo *et al.* (2004) had also reportedly associated the following bacteria *Pseudomonas syringae* pv *phaseolicoli*, *Bacillus licheniformis*, *Bacillus subtilis*, *Corynebacterium* sp. and *Erwinia* sp. with the contamination of *Hibiscus cannabinus* and *Telfaria occidentalis* in Nigeria.

Therefore since rapid production of pathogen-eradicated plants is a fundamental goal of the micro-

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propagation process, the aim of this study was to investigate and identify sources of microbial contamination of plant tissue cultures in tissue culture laboratories in Nigeria.

MATERIALS AND METHODS

The plant explants used are cassava (*Manihot esculenta*), Kenaf, *(Hibiscus cannabinus)* cowpea (*Vigna unguiclata*) and Banana (*Musa paradisiaca*), tissue cultured vessels, the wall and the air in the tissue culture rooms and transfer rooms and the skin swab of the laboratory staff. The laboratory used includes the tissue culture laboratory at the International Institute of Tropical Agriculture, that of the Cocoa Research Institute of Nigeria, Institute of Agricultural Research and Training and the Plant Quarantine Services Headquarter located in Ibadan Nigeria. The plant tissue culture medium used was Murashige and Skoog (1962) medium and sterilized by autoclaving at 121°C for 15 min.

Acidified Potato Dextrose Agar (APDA) and Nutrient Agar (NA) were exposed to air in the tissue culture laboratories for a period of 30 and 60 sec, respectively in each of the laboratories after which the plates were immediately covered and sealed with cellophane.

Sterile cotton buds were used to swab 3 cm^2 on tissue culture laboratory walls, tables and body skin of the laboratory staff, respectively and kept in sterile bottle.

Sterilization and incubation of plant cultures: The explants were excised and surfaced sterilized by immersion into a 0.75% NaOCl solution for 20 min after rinsing with 70% ethanol for 15 sec. The explants were rinsed in 4 successive changes of sterile distilled water. The excised explants were then aseptically transferred to the culture medium, labeled and incubated at 23±1°C during the day and 19±1°C at night for 3 weeks.

Isolation of microbial contaminants: From the contaminated plant tissue culture tubes, emerging microbes were isolated by inoculating them on Acidified Potato Dextrose Agar (APDA) and incubated for 6 days at 26°C under 12 h photoperiod in the case of fungi and on Nutrient Agar incubated for 3 days at 30°C under 12 h photo-period. Pure isolates obtained from repeated sub-culturing of the isolates were placed in an agar slant in MacCarthney bottles and stored at 4°C in a refrigerator.

Characterization and identification of isolates: The fungal isolates were identified using cultural characters and morphology and by comparison with standards (Barnett and Hunter, 1972). In case of bacteria, beside the morphological characteristics, a number of biochemical and physiological tests were carried out on the isolates. The biochemical tests includes gram staining, spore staining, motility test, catalase production, oxidase test, indole production, citrate utilization, urease activity, hydrogen sulphide production, gelatin hydrolysis, starch hydrolysis and carbohydrate utilization.

RESULTS AND DISCUSSION

Eighteen microbial contaminants (consisting of eleven bacteria and eight fungi) were found associated with the tissue culture plants and the laboratory environments (Table 1) The bacterial contaminants includes, *Pseudomonas flourescens*, *Escherichia coli, Proteus* sp. *Micrococcus* sp. *Streptococcus pneumoniae, Staphylococcus aureus, Bacillus cereus*, *Bacillus subtilis*, *Corynebacterium* sp. and *Erwinia* sp. While Fungi isolates were *Alterneria tenius, Aspergillus niger, Aspergillus fumigatus, Cladosporium* sp. *Saccharomyces* sp. *Fusarium oxysporum, Rizopus nigricans* and *Fusarium culmorum.* The rate of occurrence of bacteria isolates was higher than that of fungal isolates in the plant tissue cultures (Fig. 1). *Pseudomonas flourescens*, *Staphylococcus aureus, Bacillus cereus*, *Bacillus subtilis*, *Corynebacterium* sp. and *Erwinia* sp. were found to be the most prevalent on all the sampled plant tissue material (Fig. 2 and 3). However, the rate of occurrence of *S. aureus* in the plant tissue materials was less than 10% (Table 2).

The rate of occurrence of *Staphylococcus aureus, Bacillus cereus* and *Escherichia coli* were found to be higher (ranging from 36-46%) in human skin than in all other sampled materials (Table 2). The laboratory walls and tables also harbored most of the contaminating microbes (Table 2). The laboratory indoor air was found associated with all the contaminating microbes with the exception of *Erwinia* sp. Microbes are living, biological contaminants that can be transmitted by infected people, animals and indoor air, they can also travel through the air and get inside homes and buildings. Bacteria species like *Staphylococcus* and *Micrococcus*, are found on human skin scales (Trudeau and Fernández-Caldas, 1994). *Pseudomonas*, *Flavobacterium* and *Blastobacter* have been reportedly associated with wet surfaces of air-conditioning systems, cooling coils, drain pans and sump pumps (Trudeau and Fernández-Caldas, 1994). *Staphylococcus aureus* are emitted from the nasopharynx of normally healthy individuals when the person talks and are commonly found in air, water, the skin (Trudeau and Fernández-Caldas, 1994).

Table 1: Frequency of occurrence of microbial contaminants in tissue culture laboratories in southwestern Nigeria

	IAR and T/OAU		IITA		CRIN		PQS	
	A	B	A	B	A	B	A	B
Microbial contaminants	Incidence of occurrence (%)							
Pseudomonas flourescens	15.67ef	9.33ef	9.00de	5.00ef	13.00de	9.00ef	16.67fg	8.00f-h
Corynebacterium sp.	18.33fg	9.00e	10.00e	4.00de	18.00g	8.00d-f	16.00e-g	10.00h
Bacillus subtilis	18.00f	12.00hi	11.00	7.00g	17.00g	8.67ef	13.00d-f	6.00d-f
Bacillus cereus	23.67gh	14.00hi	10.67e	6.00fg	14.00ef	9.00ef	14.67e-g	9.33gh
Erwinia sp.	17.67f	10.00e-h	10.33e	5.67e-g	16.00fg	10.00f	17.00g	10.00h
Streptococcus pneumonia	21.33g	10.00e-h	9.33de	4.00de	18.00g	9.00ef	16.00fg	9.00gh
Streptococcus faecalis	18.33fg	11.00h	10.00e	6.00fg	17.00g	7.33c-e	12.00c-e	5.33c-e
Escherichia coli	18.67fg	12.00hi	10.67e	6.00fg	11.00cd	9.00ef	15.67e-g	9.00h
Proteus vulgaris	11.00cd	5.00bc	9.00de	5.00ef	10.00bc	8.00d-f	9.00bc	4.33b-d
Micrococcus sp.	13.00de	6.67a-d	9.33de	3.00cd	10.00bc	8.67ef	10.00b-d	3.00bc
Staphylococcus aureus	10.00b-d	6.00b-d	6.67cd	3.00cd	10.00bc	6.00a-d	12.00de	6.33d-f
Klebsiella aerogenes	6.00a	5.00a	5.00bc	2.00bc	6.30a	5.00a-d	10.67b-d	5.00c-e
Alternaria tenius	7.67a	4.00a	2.00a	0.00a	9.33a-c	7.00b-d	10.00b-d	8.00f-h
Aspergillus niger	8.00ab	4.00a	5.00bc	2.00bc	7.67ab	4.67ab	7.67b	4.67b-d
Aspergillus fumigatus	10.33b-d	7.00cd	2.00a	0.00a	7.00ab	3.67a	8.00b	5.33c-e
Cladosporium sp.	6.00a	6.00a-d	3.33c	4.00de	8.00bc	8.00d-f	8.00b	5.33c-e
Fusarium oxysporium	11.00cd	7.00cd	4.67bc	0.67ab	10.00bc	6.00a-d	7.00b	2.67b
Rhizopus nigricans	7.33ab	4.00a	5.33bc	2.67cd	8.00a-c	5.67a-d	2.00a	0.33a
Fusarium culmorum	9.67bc	6.00a-d	5.33bc	2.67cd	6.67a	4.67ab	7.00b	5.33c-e
Saccharomyces sp.	14.33e	10.00e-g	9.33	5.00ef	14.00ef	6.67b-e	13.67d-g	8.00f-h

Figures followed by same alphabet along the columns are not significantly different at p=0.05 Using Duncan's Multiple Range test. A= preparatory room, B= incubating room

Table 2: The incidence of occurrence of microbial contaminants in tissue culture laboratory

	Incidence of occurrence (%)				
Microbial contaminants	Plant tissue culture	Human skin	Laborat-ory wall /table	Laborat-ory ind-oor air	Hand gloves
Pseudomonas flourescens	35.67f	18.00c	1.00a	0.00a	6.00b
Corynebacterium sp.	28.33e	0.00a	4.00ab	3.00a	9.00bc
Bacillus subtilis	25.00d	9.00b	4.00ab	0.00a	6.00b
Bacillus cereus	9.67c	36.00d	9.67b	6.00b	8.00b
Erwinia sp.	17.67f	4.00ab	8.33b	5.67ab	6.00b
Streptococcus pneumonia	0.00a	31.00d	6.33b	9.00a	4.00a
Streptococcus faecalis	0.00a	21.00c	7.00b	0.00a	7.00b
Escherichia coli	10.67c	38.00de	12.67bc	0.00a	10.00b
Proteus vulgaris	11.00c	8.00b	6.00b	0.00a	6.00b
Micrococcus sp.	4.00a	18.67c	2.33a	3.00a	2.00a
Staphylococcus aureus	0.00a	46.00f	16.67cd	12.00bc	8.00b
Klebsiella aerogenes	6.00b	0.00a	1.00a	0.00a	3.30a
Alternaria tenius	12.67c	0.00a	2.00a	0.00a	2.33a
Aspergillus niger	28.00e	8.00b	5.00ab	11.00bc	2.67a
Aspergillus fumigatus	21.33d	1.00a	0.00a	8.00b	3.00a
Cladosporium sp.	16.00d	0.00a	3.33a	8.00b	3.00a
Fusarium oxysporium	21.00d	0.00a	6.67b	12.67bc	8.00b
Rhizopus nigricans	27.33e	4.00ab	5.33ab	21.67d	7.00b
Fusarium culmorum	39.67f	1.00a	6.33b	12.67bc	5.67ab
Saccharomyces sp.	24.33e	6.00b	0.33a	0.00a	0.00a

Figures followed by same alphabet along the columns are not significantly different at p=0.05 Using Duncan's Multiple Range test

It was discovered that the microbial population is higher in the preparatory room than the incubating rooms. This might be unconnected with the fact that more people frequent the preparatory room. Flannigan and Morey (1996), reported that presence of bacteria in a room indicate the presence of people and their levels may get high when the building is heavily populated.

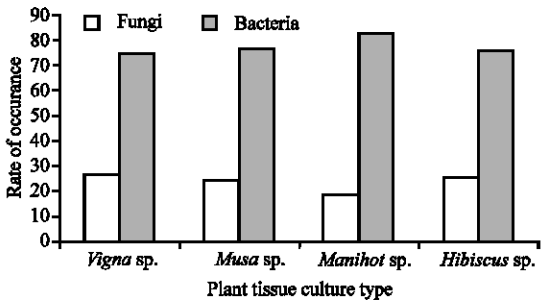

Fig. 1: Rate of occupance of microbial contaminant in plant tissue culture

Fungal contaminants were also found associated with the indoor air, tables/walls and human skin. Typically, fungi make up two-thirds of all of airborne, living organisms. Miller *et al.* (1988) had earlier reported *Cladosporium*, *Penicillium*, *Aspergillus* and Alternaria as the most common indoor fungi. Regularly used furniture has been reported as a major source of fungal spores (Miller *et al.*, 1988). Marked shade around the house has also been reported to increase indoor fungi counts fivefold (Seltzer, 1995). Fungi grow anywhere indoor, where there is moisture and a food source. Many building materials consist of cellulose materials that are particularly suitable for fungi growth when they are wet. Other materials that also support fungi growth include dust, paints, wallpaper, insulation materials, drywall, grease, soap scum, carpet (especially those backed with jute which is a plant fiber), carpet pads, draperies, fabric

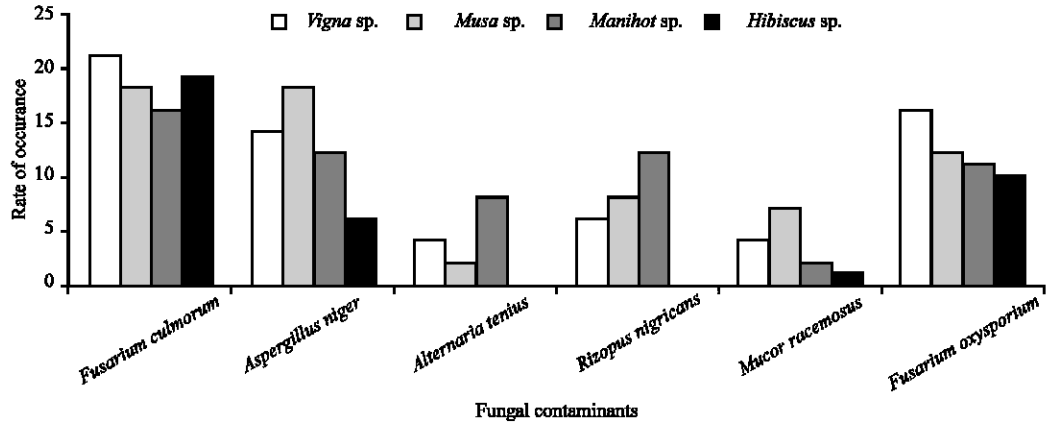

Fig. 2: The rate of occupance of fungal contaminants in plant tissue culture

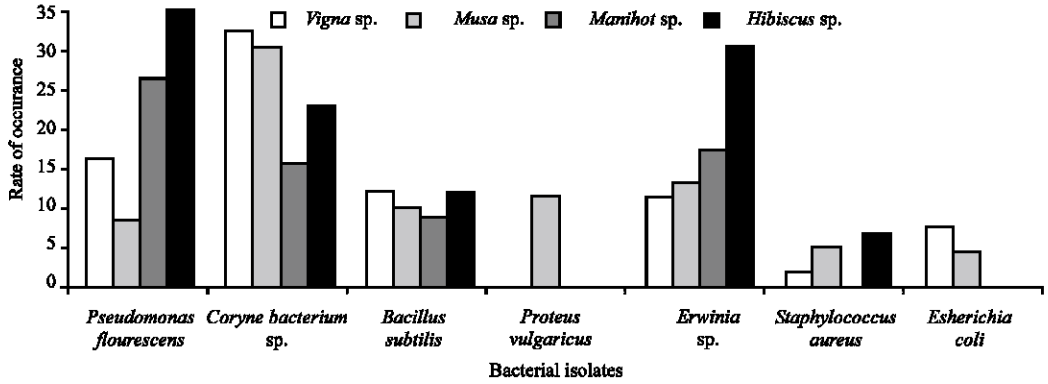

Fig. 3: The occupance of bacteria conterminats in plant tissue cultures

and upholstery (Flannigan and Morey, 1996). Fungi generally need a relative humidity of at least 60% to give them enough moisture to survive or significant moisture intrusion, regardless of humidity.

Sources of indoor moisture that often support fungal growth includes leaky roofs, damp basement or crawl spaces, house plants watering can generate large amounts of moisture, constant plumbing leaks, carpet directly on cement floors, air-conditioners, drain pans/drip pans under cooling coils (as in refrigerators) and steam from cooking (Flannigan and Morey, 1996).

The microbial contaminant found associated with tissue culture plants includes *Pseudomonas flourescens, Corynebacterium* sp., *Bacillus subtilis, Proteus vulgaricus, Erwinia* sp., *Staphylococcus aureus, Escherichia coli Fusarium culmorum, Aspergillus niger, Alternaria tenius*, *Rizopus nigricans* and *Mucor racemosus.* Odutayo *et al.* (2004) had earlier reportedly isolated the following contaminants from plant tissue cultures in Nigeria *Pseudomonas syringae pv. phaseolicoli*, *Bacillus licheniformis*, *Bacillus subtilis*, *Corynebacterium* sp. and *Erwinia* sp., While fungal contaminants includes *Alterneria tenius, Aspergillus niger, Aspergillus fumigatus* and *Fusarium culmorum.* Leggatt *et al.* (1994) reported the isolation and characterization of thirty-one microorganisms from ten different plant cultivars growing in micro-propagation, with yeasts, *Corynebacterium* sp. and *Pseudomonas* sp., being predominant. Hennerty (1994) reportedly identified *Bacillus* sp., a *Corynebacterium* sp. and an Actinomycete as contaminants in the M29 rootstocks. Fungal contaminants of plant tissue cultures have also been reported (Kane, 2003).

Most of these bacteria contaminants have been reported to increase culture mortality; the presence of latent infections can result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane, 2003).

Although some of these contaminants might be endogenously embedded in the plant tissues (Pierik, 1988) , some might also have emanated from contaminated tools, which were not investigated. Boxus and Terzi (1987)

reported that the spread of bacterial contamination was caused by insufficient flaming of contaminated tools and by survival of bacteria in 96% ethanol for a few hours. While flaming for 5 sec or more (till the inoculating tools become red hot) did eliminate the spread of bacterial contamination at transfers (Boxus and Terzi, 1988). The use of bacti-cinerator during 12 sec by inserting inoculating tools in the middle of the heating element, not on the edges has also proved effective (Singha *et al*., 1987).

Tissue culture vessels are always closed with loose-fitting caps in order to allow gaseous exchange with the external environment. However, mites and thrips carrying fungal spores and bacteria in and on their bodies, often gain entry through this loose fittings and travel from one vessel to another thereby contaminating the cultures. Blake (1994) had earlier reported that fungal contamination of cultures is usually the first sign of a mite or thrip infestation. Hence proper sanitation and effective use of appropriate pesticides to control mites and thrips in tissue culture laboratories will be desirable.

Blake (1994) has reported that thorough disinfections and strict hygiene in the laboratory have achieved effective control of microbial contaminants. Movement of people within the preparatory and incubating rooms in tissue culture laboratory should be reduced significantly to avoid the spread of contaminants. Since bacterial concentrations may be high at both low and high levels of relative humidity; therefore, it is advisable to maintain indoor humidity levels between 40 and 60% (Flannigan and Morey, 1996). Leaked pipes and roofs should be repaired within 24 h of detection; the basement floor should be drained, cleansed and disinfected regularly.

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