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Prevalence and Management of Laboratory Microbial Contaminants in Plant Tissue culture:

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ABSTRACT

Microbial contamination is one of the major challenges militating the application of in vitro micro-propagation technique for mass production of disease-free planting malaria at plant tissue culture laboratory of University of Maiduguri out of the 211 invitro culture plantlets examined 118(55.9%) were infected with both bacterial and fungal with 47(22.3%) and 71 (33.6%) respectively. Purified bacterial isolates identified based on negative cell shape, gram reaction, phorescent pigment and biochemical tests indicated that; Bacillis licheniformis as major bacterial contaminants with 17(14.4%), Bacillus subdtilis 10(8.5%) and Erwinia spp 8(6.8%) respectively. Where as fungal contaminants indicated that Aspergillus Niger with infected of 22(30.9%), Penicillium spp 18(25.4%), Aspergillus flavus 16(22.5%) and Candida spp 15(21.1%) respectively. Culture susceptibility test revealed that Gentamycin and Ampicillin at 10µg are highly susceptible and effectively suppressed the growth of all the identified bacterial while Cephradin, Tetracycline, Deoxycycline, Chlorophenicol at 30µ particularly suppressed the grow all identified bacterial. While ketoconazole and Fluconazole at 200mg/litre inhibited the growth of all the identified fungal contaminants. However, partially inhibition were examined in Nystatin 150mg/l and Thiabendazole 100mg/l respectively. The prevalence of bacterial and fungal contaminants can effectively be eliminated by incorporation in the growth media of Gentamycin and Ampicillin at 10 µg and ketoconaazole and Fluconazole at 200mg/litre respectively. Further studies are required to investigate the negative side-effects of these antibiotics and antifungal agents on the growth and genetic stability.

Key words- prevalence; antibiotic treatment; management microbial contaminants; plant tissue culture

INTRODUCTION

Microbial populations dominate the biosphere in terms of metabolic impact and numbers contamination with micro organisms is considered to be the single most important reason for losses during in vitro culture of plants. Such micro organisms include viruses, bacterial, yeast, fungi etc.

(Omamor and Eziashi,2007). Microbial contamination of plant tissue cultures is due to the high nutrient availability in the almost universally used murashige and skoog (1962). These microbes complete adversely with plant tissue cultures for nutrients, resulting increased culture mortality, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane,2003).

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Aseptic conditions are usually implied but many plant cultures do not stay aseptic in vitro and contamination by micro-organisms, especially bacterial, is a continuing problem for commercial and research plant micropropagators. (Cassells,2000 and Debergh1999). Bacterial contamination is a major threat in plant tissue culture, plant tissues cultures could harbour bacterial in a totally unsuspecting manner, either externally in the medium or endophytically. (Pious, 2004). Other major cause of microbial contamination is insufficient sterilization of explants, growth media, working tools and personnal's hands. The principal microbial contaminations frequently reported in plant in vitro cultures are bacterial and fungi. (Omamor and Eziashi,2007). Thus pathogens, edophytes, epiphytes and incidental contaminants may all occur and may interfere with growth of the plant tissue. (Cassells, 1996). The main fungal contaminants in plant tissue cultures are Alterneria tenius, Aspergillus niger, Aspergillus fumigatus and Fusarium culmorum. (Odutayo and Amusa,2004). While major bacterial contaminant include Pseudomonas syringae, Bacillus licheniformis, Penicillium spp, Bacillus licheniformis, Bacillus subtillis, Cornebacterium sp and Erwiria spp.

MATERIALS AND METHODS

Identification and characterization of bacterial and fungal contaminants: Microbial contaminants were isolated from 211 invitro culture plant-lets at plant tissue culture laboratory, University of Maiduguri. Bacterial isolates were aseptically streaked onto sterile nutrient agar (NA) medium and the culture were incubated at 37^{o}c for 24 hours. All the isolated contaminants were purified by serial dilution technique (Collins and Lyne 1984). Whereas, fungal isolates were aseptically transferred onto Petri dishes containing potato dextrose agar (PDA) growth medium and the cultures were incubated at 24^{o}c for 7 days. The purified isolates were stained for morphological characterization based on vegetative cell shape, gram reaction and presence of spores. Below are pictures of the two fungi isolated potatoes dextrose agar. Fig.1 and Fig.2.

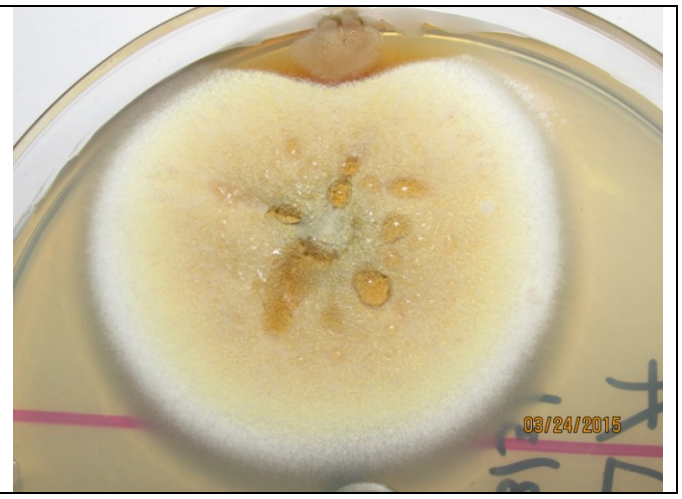

Fig. 1. *Aspergillus spp* **Fig. 2.** *Penicillium spp*

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).

However, standard biochemical tests were carried for bacterial isolates (Collins and Lyne

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1984, Krieg and Holt 1984, Sneath *et al* 1986). The examined results were compared with the standard strains of Bergey's manual (Krieg and Holt 1984), Sneath et al (1986).

Culture and sensitivity test of identified bacterial and fungal contaminants: The susceptibility of bacterial cultures to antibiotics was tested using Kirby-Bauver method (Claus, 1995) Mueller-Hinton agar solidified with the bacterial growth medium was inoculated with the bacterial isolates. Disks singly impregnated with Ampicillin (10 µg), Cephradin (30 µg), Gentamycin (10 µg), Vancomycin (30 µg),Tetracycline (30 µg), Dexycline (30 µg), Chlorophenicol (30 µg) were placed onto the growth medium in 10cm diameter plate after the bacterial inoculation.

Disk containing antibiotics were placed after inoculation of the test organisms. The inoculated plates were incubated inhibition zone around the disks were 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. Likewise, three anti-fungal viz ketoconazole, nystatin and fluconazole were tested for antifungal activities using agar well diffusion method (Trease and Evans, 1983, Ajaiyeoba et al, 1996). The selected 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. The three antifungal with varying concentrations of 100, 150, and 200mg/l for ketoconazole, nystatin and fluconazole and sterile water as a negative control were single filled into the wells with the aid of a pipette. The plates were incubated at 25^{o}c for 7 days and the susceptibility results for each isolates were determined 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. Below is a culture susceptibility test disk containing antibiotics.

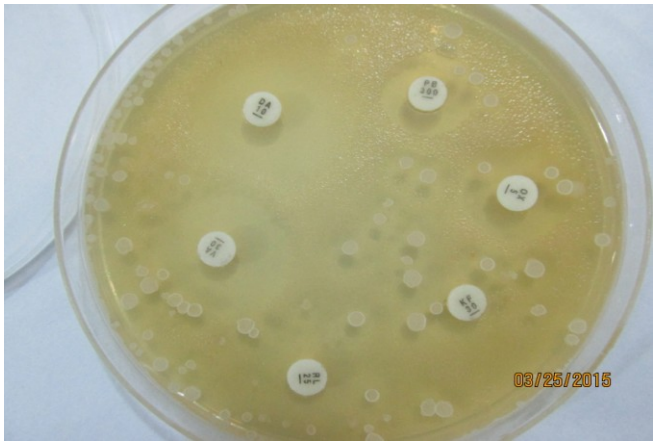

Fig. 3. Antibiotic sensivity test

Results and Discussion

Identification of microbial contaminant in plant tissue culture laboratory: The bacterial contaminants identified in invitro cultured plantlets at plant tissue culture biotechnology center were bacillus subtilis, bacillus licheniformis, erwinia spp and pseudomonas syringae (Table 2). The isolated bacterial contaminants in this study have earlier been reported in plant tissue cultures (Odutayo et al., 2004) while fungal contaminants were aspergillus niger, penicillium spp, aspergilus flavus and candida spp as earlier been reported by (Odutayo et al. 2007).

Prevalence rate of fungal and bacterial contaminants out of the 211 invitro cultured plantlets examined, the result indicated that 118 (55.9%) were infected with both bacterial and fungal contaminants with infected number of 47 (22.3%) bacterial and 71(33.6%) fungi respectively. (Table1). Culture susceptibility test of isolated microbial contaminants: The culture susceptibility test revealed that gentamycin and ampicillin at 10µg are highly susceptible and effectively suppressed the growth of all the identified bacterial. It has been reported by (Reed et al 1995, Habiba et al 2007) gentamicin is a

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broad-spectrum anti-bactericidal agent of gram positive and gram-negative bacterial that suppresses bacterial growth by inhibiting cell protein synthesis. On the other hand, culture susceptibility test revealed that Chlorophenical, Cephradin, Tetracycline, Doxycycline at 30 µg partially suppressed the growth of all identified bacterial whereas, Ketoconazole and Fluconale at 200mg/l inhibited the growth of all the identified fungal contaminants. 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). This study revealed that, Fluconazole at 200mg/L effectively suppressed all fungal contaminants identified. Fluconazole belongs to the azole class of antifungal drugs and is generally considered to be a systematic fungi static rather than fungicidal in standard invitro susceptibility tests (Sheehan et al.,1993). While Nystatin (150mg/L) and Thiabendazole (100mg/L) are susceptible only to Candida spp and Aspergillus flavus respectively.

Recommendations

Further studies are required on the antibiotic and antifungal doses to their effectiveness in eliminating bacterial and fungal contaminants from invitro plantlets. Also, phytotoxicity studies should be conducted to determine the effect of the antibiotic and antifungal agents on the invitro plantlets growth.

Table 1: Prevalence of fungal and bacterial contaminants on Explant identified.

parameters	Microbial contaminants					
Names of microbial contaminants	INFECTED		NOT INFECTED		TOTAL	
	No	%	No	%	No	%
Bacterial	47	22.3	42	19.7	89	42.2
Fungi	71	33.6	51	24.2	122	57.8
Total	118	55.9	93	44.1	211	100.0

Table 2: Distribution of microbial contaminants base of species of organisms

Prevalence of fungal and bacterial contaminants			
Bacterial	No. infected	Fungi	No. infected
(i) Bacillus subtilis	10	Penicillium spp	18
(ii)Bacillus licheniformis	17	Aspergillus niger	22
(iii) Erwinia spp.	8	Aspergillus flavus	16
(iv)Pseudomonas syringae	12	Candida spp	15
Total	47		71

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

Bacterial Genus	Ampicillin (10µg)	Cephradin (30 µg)	Gentamicin (10 µg)	Vancomicin (30 µg)	Tetracycline (30 µg)	Doxycycline (30 µg)	Chlorophenicol (30 µg)
B. subtilis	S	S	S	S	S	S	S
B. licheniformis	S	S	S	S	S	S	S
Erwinia spp.	S	I	S	R	R	R	R
P. syringae	S	I	S	R	I	R	R

R= Resistant, S= susceptible test of the identified fungal contaminants to different antifungal agents.

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

Fungal Genus	Fluconazole Mg/L (200)	Nystatin mg/L (150)	Ketoconazole Mg/L (200)	Thiabendazole (100)
Candida spp	S	S	S	S
Penicillium spp	S	I	S	I
Aspergillus niger	S	I	S	R
Aspergillus flavus	S	S	S	S

R = Resistant, S= susceptible and I = Intermediate Resistant

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