American Journal of Plant Physiology

ISSN 1557-4539

Academic Journals Inc.

www.academicjournals.com

American Journal of Plant Physiology 1 (2): 205-209, 2006
ISSN 1557-4539

Studies on Various Atmospheric Microorganisms Affecting the Plant Tissue Culture Explants

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Abstract: Bacterial and fungal contaminants create a major problem in plant tissue culture laboratory. Once the explants have been established *in vitro*, it is essential that the culture to be indexed for the presence of microbial contaminants before being multiplied. Besides increased morality, the presence of latent infection can result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting. The percentage occurrence of contaminants in initial and establishment stage of sugarcane tissue culture process was studied. Fungal and bacterial contaminants were isolated from different varieties sugarcane explants. Contaminants were more in the initial stage when compared to the establishment stage. The most predominantly occurring bacteria were *Bacillus* sp. *Serratia* sp. *Pseudomonas* sp. and *Staphylococcus* sp. and the most dominating fungal contaminants were *Aspergillus*, *Candida*, *Cladosporium*, *Fusarium*, *Microsporum* and *Pencillum*. Highest percentage occurrence was recorded for bacterial contaminants.

Key words: Plant tissue culture, contaminants, indexing

Introduction

Plant tissue culture is the technique of growing plant cells, tissues and organs in an artificially prepared nutrient medium, static or liquid under aseptic conditions. Better control of environmental conditions like light, temperature, gas mixtures and nutrients can be achieved for plant tissues growing *in vitro*. This technique has become extremely significant in agriculture. Plants growing *in vivo* may become systematically infected with fungal, bacterial and virus diseases and attacked by insect pests and nematodes. They also invariably carry the superficial contaminants. To survive and grow properly, *in vitro* plant cultures need to be largely free of pests, fungi and most bacterial infections. Contaminants can cause large losses during micro propagation and their control is usually the most frequently encountered problem by micro propagation laboratories. If contamination is to be avoided, it is important to detect and eliminate contaminating organisms before they are transferred to many culture vessels during routine subcultures. Simple surface sterilization does not always remove contamination or infection by bacteria, yeast, virus, viroids, mycoplasmas and rickettsias. Recently Cooper *et al*. (2006) has reported the fungal contaminant *Pythium violae* in *Dacus carota* tissue culture. Dudgale *et al*. (2000) also demonstrated the *Alternaria dauci* contamination in carrot tissue culture. Plant material can be cultured and multiplied without the presence of a microorganism being recognized. The infection is then said to be latent or hidden. It may become troublesome later in the life of the culture or may be transferred to the plantlets, which are produced. The present investigation reports the fungal and bacterial contaminants isolated from sugarcane explants.

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The objectives of the study were to

- Index sugarcane explants
- Isolate contaminants from the initial and establishment stages of tissue culture process.
- Compare the contaminants ratio in different varieties of sugarcane explants and
- Isolate and identify predominantly occurring bacterial and fungal genera.

Materials and Methods

Indexing of Sugarcane Explants for Surface Borne Micro Flora
Initiation

- The first step in obtaining clean culture was to ensure that plants, from which the explants were taken, originated from a healthy batch and did not show obvious disease symptoms.
- Surface sterilization of explants was carried out using sodium hypo chloride solution with a concentration ratio of 1:3 (250 mL NaOCl + 750 mL water) The explants were soaked in the NaOCl solution for 30 min.
- The explants were washed and distilled water for 3 times.

Indexing at Initiation Stage

Explants were placed in tissue culture medium (MS Media) at a pH of 6.2. The explant-inoculated media was incubated at 27-28°C for 7 days. During this period the explants were checked for contaminations.

Contamination appeared as cloudy or flocculent growth in the medium. Standard growing conditions were used in these steps.

Media Preparation and Indexing at Establishment Stage

If no contamination appeared after one week, the plants were taken for further multiplication, the contaminated bottles were removed. The bacterial and fungal contaminants present on the explants were subculture into the sterile media for further studies.

Fungal and Bacterial Indexing Media

SDA medium was used for the culture and growth of fungi including yeast, mould and aciduric microorganisms. Yeast extract dextrose agar medium was used to stimulate bacterial growth. The media were prepared without pH adjustment and dispensed as 60 mL volume into glass bottles covered with autoclavable screw caps. After autoclaving, agar supplemented medium was cooled and solidified at an angle of 45°.

Identification of Bacterial and Fungal Contaminants

Bacteria were recognized by characteristic ooze. The ooze can be in many colours including white, cream, pink and yellow.

Contaminants were purified using standard bacteriological methods and characterized by biochemical tests and staining techniques such as Gram stain, motility, gelatinase, oxidase and O/F (oxidation/Fermentation) (Buckley *et al*., 1995; Klement *et al*., 1990).

Results and Discussion

Percentage Occurrence of Bacterial and Fungal Contaminants During Initiation and Establishment of Explants

The percentage occurrence of contaminants in the initial and establishment stages of the sugarcane tissue culture process was studied from the month of April to August 2002. The fungal and bacterial contaminants were isolated and identified.

The percentage distribution of contaminants is shown in Table 1. It is evident from Table 1 that bacterial contamination was more in the initial as well as in the establishment stage than where else. Total incidence of bacteria in initial stage was 29.78% and in establishment stage it was recorded as 24.08%.

Fungal contamination was less when comparing to bacterial contamination. Percentage contribution of fungi in the initial stage was 1.35% not much and it was recorded as 1.30% in the establishment stage. Contaminants were more in the initial stage difference.

Percentage Distribution of Contaminants in Different Varieties of Sugarcane Explants

Percentages of fungal and bacterial contaminants were recorded for different varieties of sugarcane explants. The data is represented in the Table 1.

Contamination in the different varieties PI982122, CO86032, PI96G4888, COC671, 83R23, CO88025, COV94101 was compared. It is clear from the Table 1 that in all the varieties bacterial contamination was more when compared to fungal contaminants. High percentage occurrence was recorded for PI982122 (40.18%) and CO86032 (36.18%) variety in the initial stage. But in the case of establishment it was different. In CO86032 variety the percentage occurrence was high like that of the initial stage. It was recorded as 25.2%. But the contaminant level was very low in PI982122 variety (20.18%). No contamination was observed in the 83R23 and COV94101 varieties.

Next to CO86032 variety, CO88025 variety was reported. The bacterial percentage was recorded as 29.77% in the initial stage followed by COC671 variety (24.45%). But in the case of establishment, the percentage contribution of contaminants was different. Bacterial contamination was more in COC671 variety (28.29%) and in CO88025 variety (15.07%).

Total percentage of fungi in initial and establishment stage was interpreted. Percentage of fungi was more in the initial stage (1.35%) when compared to establishment stage (1.30%). Fungal incidence was more in the initial stage of PI982122 variety (10.68%) while low percentage occurrence was

Table 1: Percentage distribution of contaminants in different varieties of sugarcane explants

	Average percentage of contaminants											
	April				May				June			
	Initiation		Establishment		Initiation		Establishment		Initiation		Establishment	
Variety	F%	B%	F%	B%	F%	B%	F%	B%	F%	B%	F%	B%
PI982122												
CO86032	0.41	27.77	0.32	22.62	0.35	22.37	1.26	27.83	0.80	39.50	1.84	28.83
PI96G4888												
COC671	0.62	27.77	0.00	5.66	0.00	7.73	1.72	19.46	0.33	25.19	1.78	36.89
83R23												
CO88025									0.00	12.90	0.76	11.45
COV94101												
Total	0.44	27.77	0.31	22.26	0.34	21.79	1.29	27.29	0.73	37.18	1.80	29.05

	July				August				Total			
	Initiation		Establishment		Initiation		Establishment		Initiation		Establishment	
Variety	F%	B%	F%	B%	F%	B%	F%	B%	F%	B%	F%	B%
PI982122					10.68	40.18	0.00	20.80	10.68	40.18	-	20.80
CO86032	2.60	49.33	0.53	36.02	4.59	61.98	2.03	13.97	1.28	36.18	1.10	25.20
PI96G4888					3.66	17.15	0.89	9.11	3.66	17.15	0.89	9.11
COC671			0.52	35.52					0.50	24.45	1.23	28.29
83R23					0.00	0.00			-	-		
CO88025	2.42	3314	4.42	21.93			5.40	12.95	2.02	29.77	4.77	15.07
COV94101					0.00	0.00			-	-		
Total	2.56	46.09	0.91	34.63	2.62	22.63	2.17	14.28	1.35	29.78	1.30	24.08

recorded in the COC671 variety (0.5%). In the establishment stage, the contamination was more in the CO88025 variety (4.77%) and low in PI96G4888 (0.89%). No fungal contaminants were observed in the establishment stage of the PI982122 variety.

Isolation and Identification of Bacteria and Fungi

A total of 9 bacterial and 10 fungal genera were identified. Contaminants are shown in Table 2. The most predominantly occurring bacteria were *Bacillus* sp. *Micrococcus Serratia* sp. *Pseudomonas* sp. and *Staphylococcus* sp. Actinomycetes was also reported. The most dominating fungal contaminants were *Aspergillus*, *Candida*, *Cladosporium*, *Fusarium*, *Microsporum* and *Penticillum*, *Saccharomyces* sp. was recorded. Bacterial contamination was high as compared to fungal contamination in all the varieties. Percentage distribution of contaminants in different varieties was studied.

This was probably due to endophytic bacteria. There are two types, epiphytic bacteria and endophytic bacteria lodge in plant structures and endophytic bacteria localized within the plants at cell junctions and the intercellular spaces of the cortical parenchyma. Disinfections cannot reach this area (Cassells *et al*., 1991; Debergh and Vander Schaeghe, 1988; Leifert *et al*., 1991).

So it would develop on the medium later. It has become increasingly evident that plants can harbour some kinds of bacterial contaminants interstitially between cells or within the vascular system (Litz and Conover, 1981)

Another possible source could have been from the indoor environment at the time of inoculating the media. Contaminants could have gained entry into the medium since most of the bacteria and fungi identified are also known to occur in the indoor environment.

The contaminants were more in the initial stage when compared to establishment because most of the bacteria and fungi could grow within a week. So the contaminant containing bottles were removed. Only the latent infections may occur in the establishment stage. Some bacteria and fungi resistant to disinfection may also grow in the initial stage (Reed *et al*., 1995).

In PI982122 and CO860032 varieties, the contaminants may be more, because of epiphytic and entophytic microbes. These bacteria were difficult to disinfect (Leifert *et al*., 1994).

Predominant Bacteria, Fungi and Yeast

A total of bacteria genera were identified. *Bacillus* sp. was found to be the most dominating bacteria in explants. Pierik (1988) instanced *B. licheniformis* or *B. subtilis* as frequent causes of hidden infections. The second dominating position was occupied by *Staphylococcus* sp. followed by *Psedomonas*, *Serratia*, *Micrococci* and *Actinomycetes*.

A total of 10 fungal genera were identified with the most predominating fungi being *Aspergillus*, *Candida*, *Cladosporium*, *Fusarium*, *Microsporium* and *Penicillium*. The dominating yeast was *Saccharomyces*.

Table 2: Fungal and bacterial contaminants observed in initial and establishment stage of sugar cane tissue culture

Fungi	Yeast	Bacteria
Aspergillus	*Saccharomyces* sp.	*Bacillus* sp.
Alternaria		*Staphylococcus*
Fusarium		*Pseudomonas*
Penicillium		*Micrococcus*
Cladosporium		*Serratia*
Tricothecium		*Klebsiella*
Microsporum		*Agrobacterium*
Acremonium		*Enterobacter*
Helminthosporium		*Lactobacillus*
Candida		*Actinomycetes*

Taking care of the following stages or steps may minimise contamination of tissue cultured material.

- Explants should be cut from the mother plant and brought to laboratory, within the shortest possible time.
- Selection of healthy explants from the clonal nursery.
- Proper care during the preparation of media.
- Strict follow up of Standard operating procedure for culture handling, media bottle handling, storage in growth rooms and further.

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