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RESEARCH ARTICLE

Investigation, characterization and prevalence assessment of various fungal contaminants in sugarcane *in vitro* cultures

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Abstract

Microbial contamination poses a significant challenge during the tissue culture process. The current investigation focuses on examining fungal contaminants in *in vitro* cultures of two distinct sugarcane varieties, CoN 13073 and CoN 707, aiming at their detection, isolation, and identification. The study employed the fungal tip isolation method to isolate and identify fungal contaminants. Six different fungal species were identified, including *Aspergillus flavus* Link, *Penicillium* sp., *Macrophomina phaseolina* (Tassi) Goid., *Curvularia* sp., *Rhizopus* sp., and an aseptate sterile fungus, based on their cultural and morphological characteristics from contaminated cultures of the two sugarcane varieties. The frequency of fungal contaminants associated with *in vitro* cultures of sugarcane was assessed in both varieties at 15-day intervals from explant inoculation to 60 days. Among the six isolates, *A. flavus* (21.62-33.33%) was the most prevalent across all time intervals in both varieties, followed by *Penicillium* sp. (14.81-31.58%), *Curvularia* sp. (0-26.32%), *M. phaseolina* (0-21.62%), and *Rhizopus* sp. (15-19.05%). The least frequent fungal contaminant observed was the aseptate sterile fungus (4.76-11.76%) at all time intervals in both varieties.

Keywords: Sugarcane; Contamination; Tissue culture; Micropropagation; Fungal frequency

Introduction

Sugarcane (*Saccharum officinarum* L.) is an important cash crop of India. It is traditionally cultivated over centuries to prepare various sweeteners and to some extent for direct consumption. Sugarcane produces a range of food products including sugar, molasses and golden syrup (Viswanathan 2018). Conventional propagation of sugarcane suffered from low propagation rates, expensive labour, time-consuming and potential transmission of pathogens through seed cane from generation to generation, which limits the efficiency of this method. Vegetative propagation methods are associated with a high risk of spreading various parasites and pathogens that reduce the viability and productivity of the crop (Siddiqui et al. 1994). Plant tissue culture (Micro propagation) is a tool for obtaining maximum population and rapid regeneration of disease-free planting material, true-to-type plants and quality planting material of sugarcane. Micro propagated sugarcane planting materials are of high quality and produce phytosanitary planting materials at a faster rate in a short period with good sugar yield (Snyman et al. 2007). Micropropagation using shoot tip or apical meristem culture has been widely used to produce virus-free plants with rejuvenation and mass production of true-to-type and uniform planting materials from old diseased sugarcane plants (Tolera et al. 2014). Microbial contamination is one of the major challenges especially faced by commercial and large-scale tissue culture facilities. Microbial contamination in micropropagation affect tissue culture media and explant during different stage of culture processes such as culture initiation and sub culturing. The

principal cause of microbial contamination in tissue culture and micropropagation facilities are poor aseptic techniques, continuous changing of tissue culture media and conditions favorable for microbial growth (Nalavade et al. 2017). The present investigation aimed at detection, isolation and identification of different fungal contamination and their frequency of contamination in two different varieties (CoN 13073 and CoN 707) of *in vitro* cultures of sugarcane.

Materials and Methods

In vitro culturing of sugarcane

Healthy shoots of 3 to 5 months old spindles (1.5cm diameter and 5-8cm long) of sugarcane cultivar procured from Main Sugarcane Research Station, NAU, Navsari. The collected shoots were pretreated in a mixture solution of 0.5 per cent ascorbic acid, 0.5 per cent citric acid, 0.1 per cent streptomycin and 0.1% Carbendazim for 15 to 20 min in a beaker. The pretreated explants were surface sterilized with 0.2 per cent mercuric chloride solution for 3 to 4 minutes and washed with sterile distilled water for 3 to 4 times inside the laminar flow. Nodal portion of spindle was discarded and shoot tip portion was separated and the outer two or three whorls of leaves were removed using a sharp sterile scalpel blade. Nearly 1 to 1.5cm shoot tip portions were prepared. Thereafter, the explants were inoculated with the help of forceps onto the medium over the flame of a spirit lamp in the sterile culture bottles having 20ml of sterile MS medium (Murashige and Skoog, 1962). The inoculated culture bottles were transferred to the tissue culture chamber having controlled environment conditions such as temperature 25± 2°C and relative humidity (RH) 50 per cent to 80 per cent. The continuous light of about 2 kilo lux was maintained through tube lights. The *in vitro* developed shoots were sub-cultured on the new media at 15-day intervals up to the regeneration stage (up to 100 DAI).

Detection and collection of contaminated cultures

From the above-mentioned procedure of initiation of *in vitro* cultures, contaminated culture bottles were selected. Samples were collected based on visual fungal colonies present on growth medium. Sampling was done at every 15-day intervals during the growth stage period. Selected samples were brought to the laboratory of the Department of Plant Pathology for the isolation and identification of fungi. The frequencies of occurrence of the different types of fungi associated with sugarcane *in vitro* cultures were determined. The percentage of fungal frequency was calculated with the following formula (Ebele, 2011).

$$\text{Fungal frequency} = \frac{\text{Number of times a fungal sp. encountered}}{\text{Total no. of fungal sp. isolated}} \times 100$$

Isolation and identification of fungal contaminants

The fungal contaminants present in the culture bottles were isolated using aseptic techniques within a laminar airflow chamber, employing the fungal tip isolation method. A small portion of each fungal mycelium was carefully extracted from the culture bottle using sterile forceps and transferred onto separate Petri dishes containing PDA (Potato Dextrose Agar) medium. These plates were then incubated at a temperature of 27 ± 1°C for seven days. The isolated fungi were subsequently cultured on PDA slants for further examination. Preliminary identification of the pure fungal cultures was conducted based on their cultural and morphological characteristics, which were assessed through both visual inspection and microscopic examination. Cultural traits, such as colony appearance and sporulation patterns (including asexual or sexual spores or fruiting structures), were observed visually. A detailed morphological analysis of the fungal isolates was

carried out using a compound microscope, and their identification was confirmed by comparing them with relevant literature (Barnett and Hunter 1972).

Results and discussion

A total of six fungal species were isolated from *in vitro* cultures of sugarcane variety CoN 13073 and CoN 707 *viz., Aspergillus flavus*, *Penicillium* sp., *M. phaseolina*, *Curvularia* sp., *Rhizopus* sp. and aseptate sterile fungus by fungal tip isolation method. After purification, each of the isolates was identified based on their cultural and morphological characteristics. The cultural and morphological characteristics of the isolated fungi are described here as under.

Aspergillus flavus

In *in vitro* culture of sugarcane, the fungus appears dark green in colour. The fungal growth was observed as contaminant and attached to the explant *i.e.,* at shoot tip of sugarcane. Colony on PDA was brownish green spreading with creamy white border. Surface of colonies were velvety to woolly in texture with a floccose center. Conidiophores were terminated in a vesicle with conidia bearing cells in long chains. There were one or two layers of conidia-bearing cells. Conidiophores were less than 1.5 mm length and 10 -22 µm diameter. Conidia were one-celled, rough or smooth walled, hyaline or pigmented, typically spherical to sub-spherical shaped and measured about 3.0- 3.6 µm.

***Penicillium* sp.**

In *in vitro* culture of sugarcane, dark green to brown colour patchy fungal growth was observed. The fungal growth was not attached with the explant**.** The fungus growth on PDA media was velvety blue green in colour with white to creamy white border. On reverse side appeared pale to yellowish brown. Single or branched conidiophores were brush like and cluster of spores that were terminated by clusters of flasks shaped phialides. Conidia (2.5 to 5.0 µm in diameter) were round to ovoid, pale green, unicellular and visualized as unbranched chains at the tip portion of phialides.

***Curvularia* sp.**

In *in vitro* culture of sugarcane, initially pinkish fungal growth was observed. The whole media and explant were fully covered with the fungal growth. Fungus produced rapidly growing and woolly colonies on potato dextrose agar. Initially, the colour of the colony was white to pinkish gray initially and turned to olive brown as the colony matures. From the reverse, it was dark brown to black. It produced septate brown hyphae and simple or branched conidiophores. The conidia (8-14 x 21-35 µm) were pyriform, brown and multi septate. The septa were transverse and the central cell was typically darker and enlarged compared to the end cells in the conidium. The swelling of the central cell usually gave the conidium a slightly curved appearance.

M. phaseolina

In *in vitro* culture of sugarcane, the greyish white mycelial growth was observed and had covered the whole explant. On PDA, initially the fungus produced dirty white mycelial growth which later changed to brownish black in centre due to the formation of numerous small sclerotia, which were black in colour. The mycelium was hyaline to brown, branched somewhat at right angles, septate and 1.63 to 6.62 µm in width. The sclerotia formed in culture were black, hard and measured around 62.60 to 117.22 µm in diameter.

***Rhizopus* sp.**

The entire *in vitro* culture/explant was covered with greyish fungal growth. Growth rate and sporulation was faster on PDA. Mycelium was

formed on stolons, rhizoids and unbranched sporangiospores. Sporangiospores were mainly globose, ellipsoidal and angular shaped. The sporangiospores were 2-2.5 mm in length and 20-22 µm in diameter. The spores were ovate, polygonal or angular shaped.

Aseptate sterile fungus

In *in vitro* culture of sugarcane, whitish to brown fungal growth was observed and the fungal growth was attached at the base of the explant. The isolated fungus on PDA produced fast growing white colour colonies. The mycelium was aseptate, coenocytic and there was no production of conidia or spores.

Nalavade et al. (2017) isolated and identified *Aspergillus* spp. and *Rhizopus* spp. from *in vitro* cultures of sugarcane based on cultural and morphological characteristics. Msogaya et al. (2012) isolated and identified *Aspergillus* sp. and *Penicillium* sp. from *in vitro* cultures of banana based on cultural and morphological characteristics. Thus, the present results are more or less similar in confirmation with earlier results.

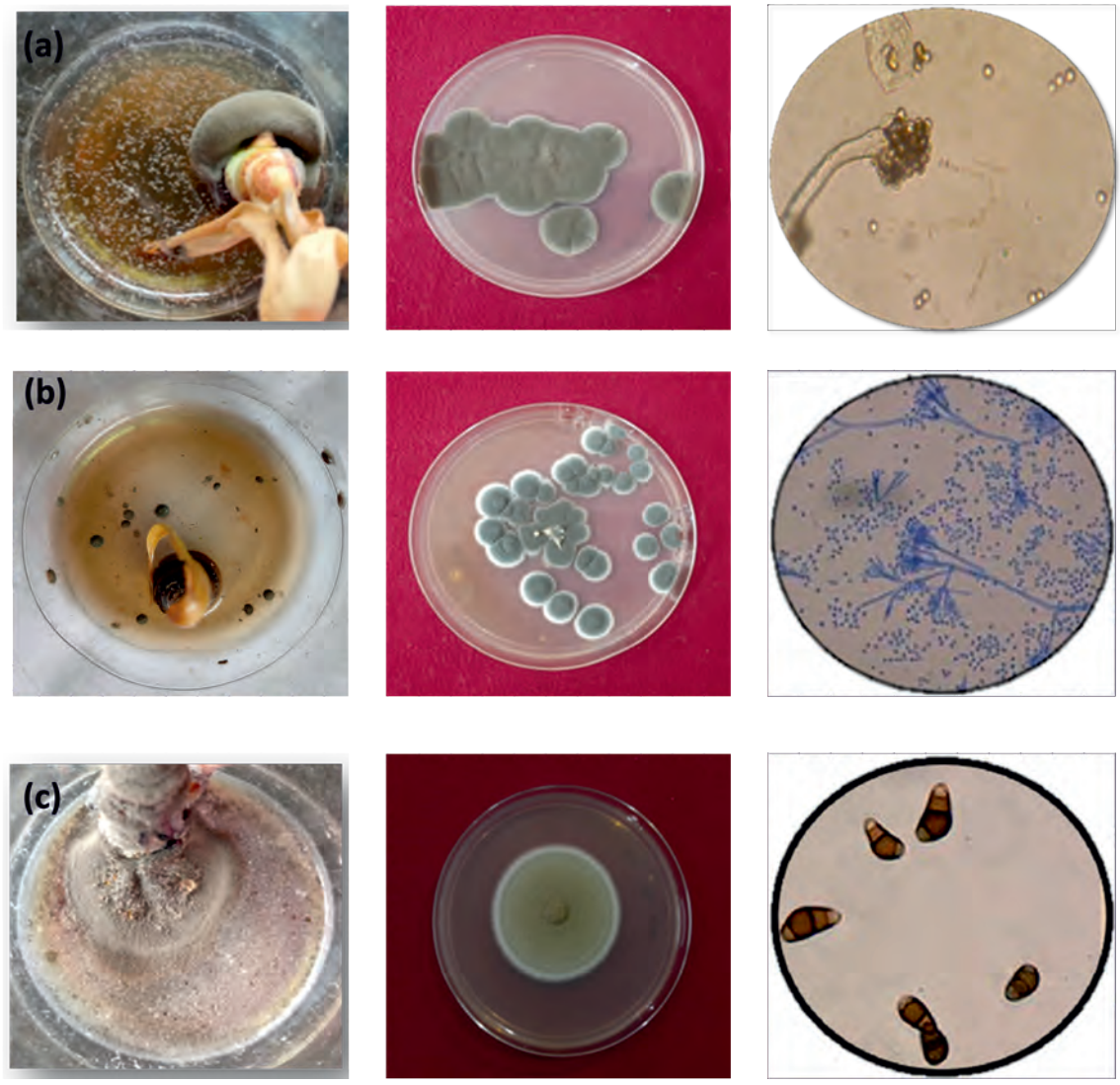

Figure 1A. Culture bottle, pure culture and microscopic view of different fungal contaminants of sugarcane *in vitro* cultures. a) *Aspergillus favus*, b) *Penicillium* sp., c) *Curvularia* sp.

Figure 1B. Culture bottle, pure culture and microscopic view of different fungal contaminants of sugarcane *in vitro* cultures. a) *Macrophomina phaseolina*, b) *Rhizopus* sp., c) Aseptate sterile fungus

Frequency of fungal contaminants of *in vitro* cultures of sugarcane

The frequency of fungal contaminants associated with *in vitro* cultures of sugarcane was recorded in two varieties *i.e.*, CoN 13073 and CoN 707 as these varieties are highly susceptible to fungal contamination.

CoN 13073

Among the fungal contaminants isolated at 15 days after inoculation (DAI), the most frequent fungal contaminant was *A. flavus* (22.22%) followed by *M. phaseolina* (20.37%), *Rhizopus* sp. (16.67 %), *Curvularia* sp. (14.81%) and *Penicillium* sp. (14.81%) while the least frequent fungal contaminant was aseptate sterile fungus (11.11%). At 30 DAI, *A. flavus* (21.74%) was found as more frequent fungal contaminant followed by *Penicillium* sp. (19.57%) and *M. phaseolina* (17.39%) as well as *Rhizopus* sp. (17.39%), while *Curvularia* sp. (13.04%) and aseptate sterile fungus (10.87%) were found less frequent. At 45 DAI, the most dominant fungal contaminants were *A. flavus* (21.62%), *M. phaseolina* (21.62%), *Penicillium* sp. (16.22%), *Curvularia* sp. (16.22%) and *Rhizopus* sp. (16.22%). While, aseptate sterile

fungus was observed with the lowest frequency (8.11%). At 60 DAI, the most frequent fungal contaminants were *Penicillium* sp. (31.58%) followed by *A. flavus* (26.32%), *Curvularia* sp. (26.32%) and *Rhizopus* sp. (15.79%). While, *M. phaseolina* and aseptate sterile fungus were not recorded at all at 60 DAI in this variety.

CoN 707

Among the isolated fungal contaminants at 15 DAI, the most frequent contaminations were *A. flavus* (22.06%) followed by *Penicillium* sp. (17.65%), *M. phaseolina* (17.65%), *Rhizopus* sp. (16.18%) and *Curvularia* sp. (14.71%), while the less frequent contaminant was aseptate sterile fungus (11.76%). At 30 DAI, the most frequent contaminant was *A. flavus* (22.22%) followed by *Penicillium* sp. (20.37%), *Rhizopus* sp. (18.52%), *M. phaseolina* (16.67%) and *Curvularia* sp. (12.96%) while, less frequent contaminant was aseptate sterile fungus (9.26%). At 45 DAI, the most frequent contaminant were *A. flavus* (27.50%) followed by *Penicillium* sp. (20.00%), *M. phaseolina* (17.50%), *Rhizopus* sp. (15.00%) and *Curvularia* sp. *A. flavus* (33.33%), *Penicillium* sp. (28.57%) and *Rhizopus* sp. (19.05%) were found more frequent. While, *M. phaseolina* (14.29%) and aseptate sterile fungus (4.76%) were found less frequent. *Curvularia* sp. was not recorded at all in this variety at 60 DAI. The results on frequency of fungal contaminants associated with *in vitro* cultures of sugarcane revealed that the most frequently detected fungal contaminants were *A. flavus* (21.62-33.33%), *Penicillium* sp. (14.81-31.58%), *M. phaseolina* (0.00-21.62%) and *Curvularia* sp. (0.00-26.32%). Whereas less frequently detected fungi was *Rhizopus* sp. (15.00-19.05%) and aseptate sterile fungus (0.00-11.76%).

Devi and Srinivasan (2006) found that *Aspergillus* sp. and *Penicillium* sp. were the most frequent fungal contaminants of *in vitro* cultures of sugarcane. *Aspergillus* sp. and *Rhizopus* sp. were the most dominant fungal contaminants in sugarcane *in vitro* cultures (Nalavade et al. 2017). Thus, the results of present findings are more or less in agreement with results of above workers.

Table 1. Percentage frequency of fungal contaminants associated with *in vitro* cultures of sugarcane

Sr. No.	Varieties	Percentage frequency (%)							
		CoN 13073				CoN 707			
	Fungi	15 DAI*	30 DAI	45 DAI	60 DAI	15 DAI	30 DAI	45 DAI	60 DAI
1.	*Aspergillus flavus*	22.22	21.74	21.62	26.32	22.06	22.22	27.50	33.33
2.	*Penicillium* sp.	14.81	19.57	16.22	31.58	17.65	20.37	20.00	28.57
3.	*Macrophomina phaseolina*	20.37	17.39	21.62	0.00	17.65	16.67	17.50	14.29
4.	*Curvularia* sp.	14.81	13.04	16.22	26.32	14.71	12.96	12.50	0.00
5.	*Rhizopus* sp.	16.67	17.39	16.22	15.79	16.18	18.52	15.00	19.05
6.	Aseptate sterile fungus	11.11	10.87	8.11	0.00	11.76	9.26	7.50	4.76

* DAI - Days after inoculation of explant

Conclusion

The present study involved isolating fungi associated with contaminated *in vitro* cultures of two sugarcane varieties using the fungal tip isolation method. The results identified six different fungal species: *Aspergillus flavus*, *Penicillium* sp., *Macrophomina phaseolina*, *Curvularia* sp., *Rhizopus* sp., and an aseptate sterile fungus. These fungi were identified based on their cultural and morphological characteristics. The evaluation of the frequency of fungal contaminants associated with *in vitro* cultures of sugarcane was carried out at 15-day intervals up to 60 days. Among the six fungi isolated, *A. flavus* was the most frequently encountered contaminant, with a frequency ranging from 21.62% to 33.33% across all time intervals and in both varieties of sugarcane. *Penicillium* sp. was the next most common contaminant, with a frequency ranging from 14.81% to 31.58%. The least frequent fungal contaminant associated with *in vitro* sugarcane cultures was the aseptate sterile fungus, with a frequency ranging from 0.00% to 11.76% throughout the entire 60-day period in both sugarcane varieties. Additionally, it was noted that the sugarcane variety CoN 707 was more susceptible to fungal contamination compared to CoN 13073, as it encountered a higher number of fungal contaminants. Implementing strict and meticulous precautions is crucial to eliminating contamination in tissue culture, thereby ensuring higher productivity of sugarcane *in vitro* cultures.

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