
Endogenous microbial contamination during *In vitro* culture of sweet potato [*Ipomoea batatas* (L.) Lam]: identification and prevention

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Endogenous microorganisms mostly bacteria and fungi were observed under microscope. Altogether three bacterial strains and three fungal strains were isolated from the contaminated sweet potato *in vitro* cultures. Among all the bacterial strains one of them was found to be gram positive and the rest were gram negative. *Corynebacterium* was identified as the gram positive rod shaped bacteria and two gram negative strains were identified as *Klebsiella* sp. and *Pseudomonas* sp. All bacterial strains were found to be susceptible to gentamicin. Tetracycline and Ampicillin were found to be satisfactory. Cent Percent contamination free cultures could be obtained by the use of 250 mg/l gentamicin for one hour. Vancomycin was found to be ineffective for eliminating the contaminated microbes. The best medium for shoot development from callus was MS + 1 mg/l IAA + 2 mg/l BAP and average time required was 20-28 days. The good callus was produced on MS supplemented with kinetin at 2 mg/l and also the best for inducing root and shoot formation.

Key words: Sweet potato, *In vitro* culture, *Corynebacterium*, *Klebsiella* sp. and *Pseudomonas* sp.

Introduction

The sweet potato [*Ipomoea batatas* (L.) Lam] is a dicotyledonous plant which belongs to the family convolvulaceae. After Columbus introduced the sweet potato to Spain then it spread to India, Africa, Asia and Oceania. It was introduced in India by Portuguese travelers. It is an important crop and is cultivated in more than 100 countries due to its significant source of calories, proteins and vitamins (Woolfe, 1992). It is the fifth most important food crop on a fresh weight basis after rice, wheat, maize and cassava. Asia is the world's largest sweet potato producing region, with China alone producing 90% of the total world production (CIP, 2008). Nishiyama, (1971) first proposed that the

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sweet potato had an autohexaploid genome derived from the doubling of the genome of a triploid hybrid between *I. littoralis* Blume (4x, or an autotetraploid form of *I. leucantha*) and *I. leucantha* (2x). Approximately among 50 genera and more than 1000 species of this family, only *I. batatas* is a crop plants whose large, starchy, sweet tasting tuberous roots are an important root vegetable. Besides simple starch sweet potatoes are rich in complex carbohydrates, dietary fiber, beta carotene, vitamin C and vitamin B6. In 1992 the center for science in the public interest compared the nutritional value of sweet potato to other vegetables. Considering, fiber content complex carbohydrates, proteins, vitamin A and C, iron and calcium, the sweet potato ranked highest in nutritional value. Sweet potato is used as many eating purposes, mainly leaves and shoots are edible. Sweet potatoes pie is a traditional favorite dish in southern U.S cuisine. It has many industrial uses which include production of starch, alcohol, alcohol fuel etc. and also has many ethomedecal uses like-diabetes, hook worm, hemorrhage, abscesses, asthma etc. India is not far behind in exporting sweet potato as well as many other valuable crops like banana and ornamentals in the international levels.

Sweet potato is propagated either from storage roots or from vine cutting. However, these methods are fruitless for maintaining disease free plants, mainly due to the viral diseases. Conservation of sweet potato germplasm involves the maintenance of field collection which is costly and often result in the loss of desirable genotypes through either the attack by pathogens or other natural disasters or a mixing up to the varieties. In order to prevent the losses of valuable germplasm from the above mentioned causes, it is essential to study and apply *in vitro* culture techniques. The sweet potato is an out-crossing hexaploid and the variation due to somatic mutation and sexual reproduction can be maintained through vegetative propagation. Some progress has been made in *in vitro* culture of sweet potato. On sweet potato *in vitro* culture, both internal and external environmental factors, such as sources of plant part and growth regulators are very important. Plant cell growing *in vitro* are considered to be under some degree of stress and may be predisposed to direct infection, even by bacteria not normally pathogenic to them (Bradbury, 1970). The plant tissue culture medium contain many different bacterial nutrients. Thus pathogens, epiphytes, endophytes and incidental contaminate may occur and may interfere with growth of the plant tissue (Bradbury, 1988). Almost all pathogenic bacteria develop in the soil/partly in plant debris as saprophytes and mostly in plants as parasites.

Bacterial contamination is a great problem for *in vitro* micro propagation of sweet potato. At the base of the explants the microbial contamination was observed within 10 to 15 days after inoculation. In the culture media bacterial

growth was also observed around the explants. Due to endogenous bacteria many explants were destroyed in the culture medium. The present investigation deals with the *in vitro* culture of sweet potato, isolation and characterization of these endogenous bacteria/microbs associated with the stem and callus of sweet potato and measure to control them.

Materials and method

Collection and preparation of explants

Ipomoea batatas (L) Lam. was used as experimental materials. The shoot tips along with a portion of stem were used for *in vitro* cultures. The explants used in the study were collected from the orchard of Orissa University of Agriculture Technology, Bhubaneswar, Orissa, India.

Disinfection procedure

The surface sterilization procedure began with dissection of explants material in to manageable units. Stem section containing axillary buds were treated by initially removing the small leaflets and cleaning away surface detritus under running tap water for 1 to 2 minute. A plastic vessel was used for treatments with sterilant solution. Sterilization was undertaken for 6 minute using 0.1% HgCL₂. Explants were transferred to a separate vessel for the washing phase in three changes of sterile distilled water.

Isolation and identification of endogenous microbial contaminants

Transverse section of stem of sweet potato was observed under the microscope to determine microbial contamination. From the contaminated tissue culture bottles, emerging microbes and fungi were isolated by inoculating them into nutrient agar medium and potato dextrose medium for bacteria and fungal contaminates respectively. All the isolated contaminants were purified by serial dilution techniques (Collins and Lyne, 1984). Pure cultures of bacteria and fungi were observed under microscope after proper staining (Gram, and spore staining) and for fungus (lacto phenol cotton blue staining). Morphological and microscopic characteristics of isolated fungi were demonstrated (Table 2). Morphological, biochemical and physiological test were carried out for identification of isolated bacteria. Essential biochemical tests including oxidase, arginine hydrolase and Voges proskauer test etc. which were carried out as per standard methods (Krieg and Holt, 1984; Sneath *et al.*, 1986). For

identification, characterized bacterial strains were compared with the standard strains of Bergey's Manual of Bacteriology.

Culture and sensitivity (CS) test of contaminated bacteria

Antibiotic sensitivity test of the identified bacteria were performed 3 times for each strain using 6 different antibiotics (Hi Media) by disc diffusion method (Bauer *et al.*, 1966). Muller-Hinton agar and six antibiotic disks viz. vancomycin, ampicillin, cephradine, chloramphenicol, tetracycline and gentamycin were used. The disks containing antibiotics were placed after inoculation of the test organisms. The inoculated plates were incubated at 37°C for 24 hours. The developed inhibition zone around the disks was measured in terms of zone inhibition (mm).

Immersion of the surface sterilized explants in different antibiotics

In this experiment, the surface sterilized explants were immersed in screened antibiotics (Gentamycin and Ampicillin) for different duration of time to ensure contamination free cultures.

Regeneration of shoots

The callus of sweet potato was heated to a temperature which the callus could withstand. The excised explants, callus and the shoot tip of sweet potato after sterilization were inoculated on MS Medium with varying concentration of growth regulators for shoot and root regeneration. For shoot multiplication, the regenerated single shoots decapitated with a view to induce auxiliary budding. The decapitated shoots were inoculated on MS Medium with varying concentration of auxin and cytokinin separately. They were sub cultured after every 30 days in the same fresh medium for multiple shoot production. After rooting the plantlets were transferred to small polythene bags containing loam soil and cow dung (1:1) for hardening for 15 days. Finally after hardening the plantlets were transferred to field.

Result and discussion

In the present investigation the presence of bacteria was detected in the transverse section of the rhizome under the microscope. Three bacterial strains and four fungi were isolated from the contaminated culture. All the three distinguished bacteria were gram positive and rod shaped. The three isolated bacteria were *Corynebacterium* sp., *Klebsiella* sp. and *Pseudomonas* sp.

according to the results of (Table 1). The result of morphological and biochemical tests were presented in Table 1.

Table 1. The Morphological and biochemical test of three bacterial isolates

No of isolates	Vegetative cells	Gram staining	Spore test	Arginine hydrolase test	Methyl red test	Starch hydrolysis	Kovacs oxidase test	Fluorescent Pigment test	Casein hydrolysis	Gelatin hydrolysis	Voges Proskauer test	Name of bacteria
B1	Rod shaped	+ve	Spore not present	+ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	<i>Corenebacterium</i>
B2	Rod shaped	-ve	Spore not present	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	<i>Klebsiella sp.</i>
B3	Rod shaped	-ve	Spore not present	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	<i>Pseudomonas sp.</i>

Some morphological and microscopic characteristics of isolated fungi are revealed in Table 2. Yeast colonies are pink, moist with unbroken, even edges. Microscopic appearance of the cells is oval, colour less and reproduces by budding.

Table 2. Morphological and microscopic characteristics of isolated fungi

Isolate No.	Colonial morphology	Microscopic appearance
F1	White colonies become greenish blue, black or brown as the culture matures	Conidia in chains developing at the end of the sterigma arising from the terminal bulb of the conidiospore. long conidiophore arises from a septate mycelium
F2	Rapidly growing white colored fungus, swarms over entire plate, aerial mycelium cottony and fuzzy	Spores are oval. colourless, non septate mycelium gives rise to straight sporangiophores that terminate with black sporangium containing a columella, root like hypha penetrate the medium
F3	Woolly, white, fuzzy colonies changing colour to pink	Multicelled spores (conidia) are oval and attached to the conidio spores arising from a septate mycelium

Those cells which were completely inhibited, failed to resist the action of corresponding antibiotics against them and so they became sensitive to the antibiotics. Antibiotic sensitivity testing revealed that the antibiotic that were the most effective against the contaminating microbes are gentamycin. The two antibiotics, tetracycline and ampicillin were found to be satisfactory. Of this, vancomycin was found to be ineffective for eliminating the contaminated microbes. The antibiotic sensitivity tests of the three bacterial isolates were presented in Table 3. Different concentrations of antibiotics applied to infected cultures at different time intervals and its result revealed that the cent percent of the contamination free cultures could be obtained by the use of 250 mg/l gentamicin

for one hour and finally when these antibiotic treated explants were cultured in MS medium, they produced healthy shoots. In case of oxacillin, the bacterial growth was inhibited up to some time. In a report by Falkiner (1990) who mentioned that the agents act specifically on bacterial cell walls would be more suitable to control infection in plant tissue cultures. It is well established that gentamicin and ampicillin inhibit bacterial cell wall synthesis. This observation also corroborates with the reported results of Reed *et al.* (1995) and Habiba *et al.* (2002). The explants treated with antibiotic were cultured in MS supplemented with different concentration of IAA+ BAP. The best medium for shoot development from callus was MS + 1mg/l IAA + 2 mg/l BAP and average time required was 20-28 days.

Table 3. Culture and sensitivity test of the selected isolates

Name of the antibiotic	Width of inhibition (cm)				Strength(μ g)
	B1	B2	B3	Mean	
BVVancomycin	0	0	0	0	30
Ampicillin	2.3	2.2	1.2	1.9	10
Cephadrin	0	1.9	1.8	1.23	30
Chloramphenicol	2.9	2.4	1.0	2.1	30
Tetracycline	2.0	1.6	1.6	1.73	30
Gentamycin	1.9	1.8	2.0	1.9	10

The good callus was produced on MS supplemented with kinetin at 2mg/l and the best for inducing root and shoot formation. Kinetin has been used more widely for sweet potato tissue culture *in vitro*. The MS with NAA at 10mg/l was most effective to produce excellent callus after 13 days, however failed to produce roots and shoots. The importance of kinetin for inducing callus from cultured explant of sweet potato were described by Kobayashi and Shikata (1975) and Tsay and Lin (1973) who reported that both 2, 4-D and kinetin were necessary for callus induction. In essence, the present study clearly demonstrated the identification and prevention of bacterial contamination during *in vitro* culture of sweet potato and proves that gentamicin is very effective for elimination of the three bacteria like *Corynebacterium*, *Klebsiella* sp. and *Pseudomonas* sp. This information generated here will be of great use for sustainable of plant tissue culture industry

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