

Endogenous Bacterial Contamination of Plant Tissue Culture Materials: Identification and Control Strategy

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Abstract

The endogenous bacterial contamination of plant tissue culture materials and their possible control was studied. Nine bacterial isolates were isolated from the contaminated tissue culture materials *viz.* potato and tea. On the basis of morphology and biochemical characters of nine isolates, seven were identified as Gram positive belonging to *Bacillus alcalophilus*, *B. circulans*, *B. infantis*, *B. lentus*, *B. schlegelii*, *B. pumilus* and *B. subtilis*. Remaining two were Gram negative and identified as *Enterobacter cloacae* sub. sp. *dissolvens* and *Pantoea agglomerans*. Molecular analysis was conducted on the basis of 16S rDNA sequence to confirm three isolates. Culture and sensitivity test was carried out to screen out the antibiotic sensitivity where streptomycin (S-10), polymyxin (PB-300) and gentamicin (CN-120) antibiotics were found to be effective against all bacterial isolates. The culture and sensitivity test reflected the feasibility to control or eliminate the contaminant bacteria during *in vitro* culture of plant which is very much required in the commercial tissue culture production.

Introduction

Micropropagation is a tool which rapidly multiplies the genetically identical plants by using modern techniques and relatively small space, supplies and time. A small piece of explants such as a meristem, stem tip, node etc. is placed in a sterile nutrient medium (usually gel-based) where the inoculated plant parts are multiplied (Daniel 1998). Presently, the micropropagation is practiced comprehensively for the development of plants with pest or stress resistance and pathogen free plants production (Kolomiets et al. 2014, Sharma and Agrawal 2012). Although, the plant materials used in tissue culture are disinfected using

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surfactants to remove microbes and all vessels and media were sterilized properly (George 1993), there are some reports in which microbial contamination in tissue culture is said to restrict the improvement of all *in vitro* techniques (Leifert and Cassells 2001).

Potato (*Solanum tuberosum* L.) has been considering as an important food crop throughout the world. Now, potatoes are cultivated in almost every part of the world, with annual global production exceeding 300 million tons (Camire et al. 2009) and thus it is considered the fourth largest food crop in the world. It is relatively sensitive to environmental stresses (i.e. salinity, drought etc.) where tuber initiation, tuber growth stages of potato had shown susceptibility to drought stress (Evers et al. 2010). The environmental stresses on potato crops were possible to overcome using plant tissue culture. However, some microbial contaminations are noticed in tissue culture of potato plant which need to be avoided for higher yield of potato.

Tea (*Camellia sinensis* (L.) Kuntze) is another popular plant and a major cash crop in most of the developing countries including Bangladesh. Bangladesh cater 3 per cent of global tea production, which plays an important role in building the national economy. Next to water, tea is the most consumed beverage and synthesis a lot of medicinally important secondary metabolites i.e. vitamins (C, E and K), caffeine, polysaccharides, lutein and theanine which are needed for health (Mondal et al. 2004, Lee et al. 2014). The high genetical heterozygosity of existing *Camellia* varieties are hampering in steady production of metabolites which could be overcome by using *in vitro* culture. Secretion of phenolic exudants from the explants attracts microbes and shows susceptibility to pathogens which cause major damage in sapling of tea leading to drastic losses of cultures at each stage of micropropagation which eventually triggering economical losses every year (Mondal et al. 2004). Thus, special attention on microbial control should be given to increase the productivity of *in vitro* culture regardless of microbial contamination.

Nutrient medium is used for *in vitro* culture of plant which is also a good source for microbial growth. The microbes grown in tissue culture media may compete adversely with plant tissue culture for nutrient (Odutayo et al. 2007). As a result, endogenous bacterial contamination is said to pose a great problem for *in vitro* micropropagation of plants including potato and tea species instigating latent infection (tissue necrosis, diminished shoot proliferation etc.) in plant tissue culture (Kane 2003). Eventually, loss of a huge amount of money, man hours and effort spent in culture development and experiment set up are commonly occurring (Jang-Jih et al. 2000). Considering the said situation, the aim of the present study was the isolation, characterization of endogenous bacteria

responsible for contamination of potato and tea shoots as well as to find out the suitable antibiotics that would be most effective for contamination free of tissue culture products.

Materials and Methods

In vitro grown explants, shoots/plantlets of potato and tea and their contaminated culture flasks were used as materials for this study. The contaminated materials were collected from the Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka. The explants were cultured on MS basal medium supplemented with various growth hormones at pH 5.8.



Fig. 1. Endogenous bacterial growth at the base of explants during *in vitro* potato culture.

Isolation and identification of the endogenously contaminant bacteria: The endogenous bacterial contaminants were found in the form of colonies at the base of *in vitro* culture of potato and tea (Fig. 1). The colonies were transferred to nutrient agar medium. The isolated contaminants were purified by serial dilution and repeated streaking technique (Collins and Lyne 1984). The purified bacterial isolates were preserved in a refrigerator at 4°C for further study.

Isolated bacteria were observed under microscope after simple, Gram and spore staining. Major biochemical tests including catalase production, oxidase test, indole production, citrate utilization, urease activity, hydrogen sulphide production, casein hydrolysis, starch hydrolysis and carbohydrate utilization were carried out for provisional identification of the selected isolates (Collins and Lyne 1984, Krieg and Holt 1984, Sneath et al. 1986). Bergey's manual of systematic bacteriology was followed for the identification of aerobic Gram positive bacteria (Sneath et al. 1986) and Gram negative bacteria (Krieg and Holt 1984, WHO 1987).

Molecular analysis: Among nine isolates, three were selected for the sequencing of 16S rDNA to identify specific bacterium. The molecular analyses of the selected isolates were conducted following the standard procedures as described by Marchesi et al. (1998). Bacterial cells were heat lysed in boiling water bath for 5 min and centrifuged for 1 min at 13,000 rpm. The supernatant was used as a source of DNA in the PCR. 16S rDNA gene was amplified using universal primers. The PCR was done with an initial denaturation at 95°C for 5 min. The denaturation at 94°C for 1 min, annealing at 60°C for 30 sec and extension at 72°C for 30 sec were followed by 30 cycles of amplification reactions. The final extension was at 72°C for 5 min. When the PCR was completed, the size of the DNA band was checked by agarose gel (1% agar in 0.5 X TBE solutions) electrophoresis.

DNA amplified by PCR was gel purified using Qiagen kit. The sequences generated by the automated sequencing of PCR amplified DNA was analyzed through BLAST program (<http://blast.ncbi.nlm.nih.gov/>) to find out the correct match of the bacterial isolates. Sequence alignment and phylogeny reconstruction were performed on MEGA 6 using CLUSTAL W and maximum likelihood packages, respectively. The consensus tree generated was tested by bootstrapping (1000 times).

Culture and sensitivity (C/S) test of the selected bacterial isolates: The Kirby-Bauer method was followed for the C/S test (Claus 1995). This test was carried out by inoculating selected isolates in Mueller-Hinton agar medium. The C/S test was performed on test bacterial isolates using seven different antibiotic discs, viz. neomycin (N-30), streptomycin (S-10), gentamicin (CN-120), vancomycin (VA-30), penicillin (P-10), rifampicin (RD-5) and polymyxin (PB-300). The discs containing antibiotics were then aseptically placed on inoculated contaminant bacterial plates. The inoculated plates were incubated at 37°C for 24 hrs. The diameters of the developed inhibition zones around the discs were measured and recorded.

Results and Discussion

Nine bacterial strains were isolated from the contaminated tissue culture flask of potato and tea plants. Microscopic observation revealed that all of the bacterial isolates were rod and short rod shaped (Table 1). Moreover, vegetative cells, spores, sporangia and Gram reaction were critically examined. Gram staining exhibited seven isolates as Gram positive while remaining two were identified as Gram negative. Among seven Gram positive bacteria five were spore former and the remaining two were non-spore former. All of the bacterial isolates showed motile in motility test.

Provisional identification of contaminant bacteria: On the basis of major biochemical tests provisional identification of the selected isolates was presented in Table 2. All the isolates were positive for catalase and L-arabinose test while propionate was found to be negative. Among nine isolates, six were obligate aerobic and the rest three were facultative anaerobic. All the Gram positive bacteria belonged to the genus *Bacillus* which included seven different species viz. *B. alcalophilus*, *B. circulans*, *B. infantis*, *B. lentus*, *B. schlegelii*, *B. pumilus* and

Table 1. Microscopic observation of the endogenous bacterial isolates.

Isolates	Microscopic observation					
	Vegetative cells	Gram reaction	Motility	Spores	Spore type	Sporangium
Pot-1	Rod	+Ve	Motile	Spore former	Ellipsoidal	Swollen
Pot-2	Rod	"	"	"	Oval	"
Pot-3	Short rod	-Ve	"	Non-spore former	-	-
T-1	Rod	"	"	"	-	-
T-2	Short rod	+Ve	"	Spore former	Oval	Swollen
T-3	Short rod	"	"	"	Spherical	"
T-4	Short rod	"	"	"	Oval	"
T-5	Rod	"	"	Non-spore former	-	-
T-6	Short rod	"	"	"	-	-

Pot = Bacteria isolated from potato culture and T = Bacteria isolated from tea culture.

Table 2. Biochemical characteristics of the provisionally identified bacteria.

Iso-lates	Biochemical characteristics										Provisional identification
	VP	DGA	Glu-cose	D-xylose	Casein	Starch	Citrate	Indole	NO ₃ reduction	Tyrosine degradation	
Pot-1	-	OA	-	-	+	+	+	+	+	-	<i>Bacillus infantis</i>
Pot-2	-	OA	+	+	+	+	+	-	-	-	<i>B. alcalophilus</i>
Pot-3	-	FA	+	+	-	+	+	-	-	-	<i>Enterobacter cloacae</i> sub. sp. <i>dissolvens</i>
T-1	+	OA	-	+	-	-	-	-	+	-	<i>Pantoea agglomerans</i>
T-2	+	OA	-	+	-	-	-	+	+	-	<i>Bacillus lentus</i>
T-3	+	FA	-	+	-	-	-	-	+	-	<i>B. schlegelii</i>
T-4	-	FA	-	+	-	-	-	-	+	-	<i>B. circulans</i>
T-5	+	OA	Alk	+	-	-	-	-	-	+	<i>B. pumilus</i>
T-6	+	OA	Alk	+	-	-	-	+	+	+	<i>B. subtilis</i>

DGA = Deep glucose agar, OA = Obligate aerobic, FA = Facultative anaerobic.

B. subtilis. The Gram negative isolates were identified as *Enterobacter cloacae* sub. sp. *dissolvens* and *Pantoea agglomerans*. The identification results had shown that

most of the contaminant bacteria belonged to *Bacillus*. Similar observations were made by Habiba et al. (2002).

Molecular identification: Molecular analysis was carried out to confirm the identification of few selected bacteria found as tissue culture contaminant. Among nine isolates, three were confirmed by the results of 16S rDNA sequences analysis (Table 3). BLAST results of the rDNA sequences of the bacteria and their basic bioinformatic parameters are shown in Table 3. Based on BLAST results, one isolate was identified as the genus *Bacillus infantis* having 98% similarity while other two were detected as *Pantoea agglomerans* and *Enterobacter cloacae* sub. sp. *dissolvens*, respectively.

Table 3. Basic bioinformatic parameters of the bacteria isolated from the tissue culture contaminants.

Isolates	Name and accession number of the bacterial strain having highest similarity		Basis of the bioinformatics parameters				
	Accession number	Strain name	Score	Bits	Expect value	Identities (%)	Gaps (%)
Pot-1	HQ234278.1	<i>Bacillus infantis</i>	989	535	0.0	98	2
Pot-3	HQ434623.1	<i>Enterobacter cloacae</i> sub. sp. <i>dissolvens</i>	926	501	0.0	96	1
T-1	JN036646.1	<i>Pantoea agglomerans</i>	981	531	0.0	98	2

Phylogenetic tree constructed based on the DNA sequences of the isolates are shown in Fig. 2. The phylogenetic tree was generated using MEGA 6.0 (Tamura et al. 2013) software with the Neighbor Joining (NJ) algorithm based on 16S rDNA sequence. Bootstrap values for 1000 replications are indicated.

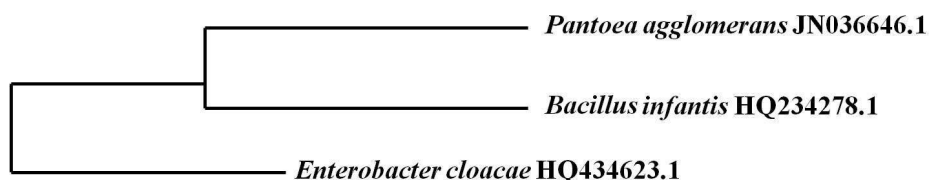


Fig. 2. The phylogenetic tree of the isolated contaminant bacteria found during *in vitro* culture of potato and tea.

Antibiotic sensitivity of the contaminant bacteria: In previous reports the antibiotic was used to control bacterial contaminant of plant tissue culture (Benjama and Charkaoui 1997, Habiba et al. 2002). Another study demonstrated that the addition of antibiotics e.g. streptomycin, penicillin G, tetracycline at 10

$\mu\text{g/ml}$ in the micropropagation media was found to be effective in controlling the growth of the bacterial contaminants in tissue culture (Hennerty et al. 1998). Addition of antibiotic dosages in tissue culture may be beneficial where synergistic action occurs. Therefore, culture and sensitivity test was carried out in the present study (Table 4 and Fig. 3).

Table 4. Culture and sensitivity test of the selected bacterial isolates.

Isolates	Antibiotics and inhibition zone diameter (mm)						
	VA-30	RD-5	N-30	S-10	CN-120	PB-300	P-10
<i>Bacillus infantis</i> (Pot-1)	8.5	11.5	8.0	8.0	11.0	5.0	17.0
<i>B. alcalophilus</i> (Pot-2)	7.0	5.0	6.0	5.0	13.0	3.5	19.0
<i>Enterobacter cloacae</i> sub. sp. <i>dissolvens</i> (Pot-3)	R	2.0	5.0	8.5	11.0	4.0	R
<i>Pantoea agglomerans</i> (T-1)	R	2.5	5.0	3.0	7.0	4.0	R
<i>Bacillus lentus</i> (T-2)	R	2.0	4.0	3.5	6.0	4.0	R
<i>B. schlegelii</i> (T-3)	R	2.5	5.0	3.5	7.5	5.0	R
<i>B. circulans</i> (T-4)	R	3.0	5.0	3.5	7.5	4.5	R
<i>B. pumilus</i> (T-5)	R	2.5	4.0	2.5	6.5	4.0	R
<i>B. subtilis</i> (T-6)	R	R	R	4.0	7.0	4.0	2.0

Disc size = 6 mm, R = Resistance.

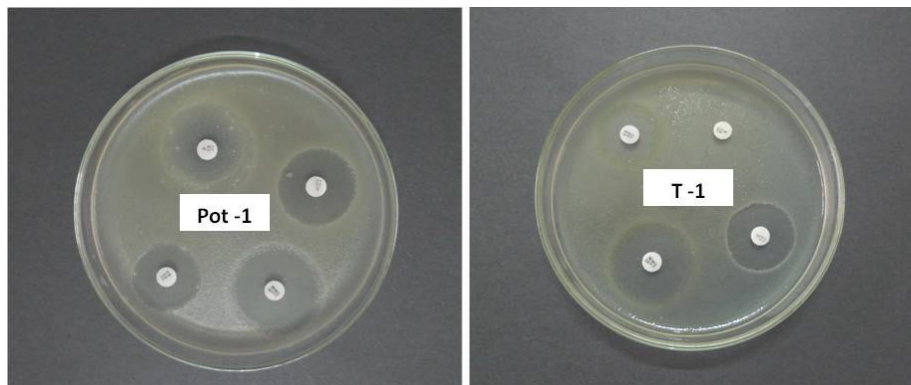


Fig. 3. Culture and sensitivity test using antibiotic discs of the selected isolates.

In another study it was reported that the growth and control of Gram negative bacteria were found to be difficult or unsuccessful by using antibiotics (De et al. 2013). In the present study, the Gram negative bacteria were sensitive to different antibiotics which are good indication to control the contaminants of Gram negative bacteria during *in vitro* culture of plant. Thus, the culture and sensitivity test might help to find out the possible way to control these organisms in tissue culture.

All of the bacterial isolates were found to be susceptible to streptomycin (S-10), polymyxin (PB-300) and gentamicin (CN-120), respectively (Table 4 and Fig. 3), which was in agreement to the previous report (Reed et al. 1995). Neomycin (N-30) and rifampicin (RD-5) had shown antibacterial activities against all bacteria except *B. subtilis*. Among seven antibiotics tested penicillin (P-10) and vancomycin (VA-30) were found to be less effective against the plant tissue culture contaminants. On the other hand, *B. subtilis* showed resistance against three antibiotics, namely vancomycin (VA-30), rifampicin (RD-5) and neomycin (N-30). Among nine bacteria, *Bacillus infantis* and *B. alcalophilus* were found to be susceptible to all of the antibiotics tested in this study indicated that these two bacterial contaminants may be controlled easily. The result clearly suggested that streptomycin, polymyxin and gentamicin could be used to avoid bacterial contamination during *in vitro* culture of plants. Falkiner (1990) mentioned that ampicillin and gentamicin inhibit bacterial growth through resisting the cell wall synthesis of bacteria. Hence, it can be assumed that the antibiotic discs used in this study might have controlled the growth of bacteria through preventing their cell wall development. Uses of specific drugs could play an important role in controlling contaminant bacteria in tissue culture which eventually could lead to increase the culture quality and instigate the growth of tissue culture. Through applying antibiotics, the explants mortality rate also could be reduced in tissue culture which could save money, time and reduce hardship of plant biologists.

Both Gram positive and Gram negative bacteria were found to be associated with the tissue culture contaminants. All of the contaminants were found to be sensitive against streptomycin, polymyxin and gentamicin and they were suggested as effective antibiotics to control contaminant bacteria during *in vitro* culture of potato and tea plants. Moreover, further study could help to find out the specific rate of antibiotic dose for complete suppression of the contaminant bacteria from *in vitro* culture of experimented plants.

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