Plant Tissue Cult. **12**(2) : 117-124, 2002 (December)

PTC

Endogenous Bacterial Contamination During *In vitro* Culture of Table Banana : Identification and Prevention

Ummey Habiba, Sharmin Reza, Mihir Lal Saha, M. R. Khan and Syed Hadiuzzaman

Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

Key words : Endogenous bacteria, *In vitro* culture, Table banana, Prevention

Abstract

The best medium for single shoot development to obtain contamination free culture of the table bananas *Musa sapientum* cv. chini champa and sagar was MS + 4.0 mg/l BAP + 1.0 mg/l Kn. Average time required for shoot development was 15 - 21 days. The best medium for shoot multiplication was MS + 4.0 mg/l BAP + 2.0 mg/l IAA + 13% CW and average time required for production of multiple shoots from single shoot was 40 - 45 days. Half strength MS + 2.0 mg/l IBA was best for root induction in the regenerated shoots. Although initially surface sterilization was successful, microbial contamination at the base of the explant was observed within 7 - 15 days after inoculation which eventually killed the explants. Endogenous microorganisms, mostly bacteria, were observed under microscope. Altogether seven bacterial strains were isolated from the contaminated culture. Four of them were found to be gram positive and the rest were gram negative. The gram positive strains were *Cellulomonas uda, C. flavigena, Corynebacterium paurometabolum* and *Bacillus megaterium*. The gram negative strains were identified as *Klebsiella* sp., *Erwinia cypripedii* and *Pseudomonas* sp. All the strains were found to be susceptible to gentamicin. Cent per cent contamination free cultures were obtained by soaking the explants in 160 mg/l gentamicin for one hr and 40 min.

Introduction

Production of banana plants (*Musa sapientum* L.) through *in vitro* micropropagation has become routine work in many countries. The efficiency of tissue culture came to light when Hwang et al. (1984) reported the production of a total of one million pathogen free plantlets of banana for commercial planting

in Taiwan through meristem culture. Now Singapore is responsible for export of tissue culture derived banana plants in many countries of the world. India, is not far behind in exporting banana as well as many other valuable crops and ornamentals in the international markets. Some private sectors in Bangladesh are now producing banana saplings through tissue culture. But most of these works were hampered by microbial contamination under *in vitro* condition.

Plant cells 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 medium may contain many different bacterial nutrients, both original constituents of the medium and exudates from the plant cells. Thus pathogens, endophytes, epiphytes and incidental contaminants may all occur and may interfere with growth of the plant tissue (Bradbury 1988). Almost all plant pathogenic bacteria develop mostly in plants as parasites and partly in plant debris or in the soil as saprophytes. There are a great differences among bacterial species, in the degree of their development in one or other environment. These bacteria enter plants through wounds made in roots and overwinter in diseased plants or debris, vegetative propagative organs such as potato tubers and banana rhizomes (Agrios 1988).

For *in vitro* micropropagation of banana, bacterial contamination is a great problem. Although initially surface sterilization works, latter on microbial contamination at the base of the explant was observed within 7 to 15 days after inoculation. Bacterial growth was also observed around the explants in the culture media. Huge number of explants were destroyed in the culture due to endogenous bacteria. The present study deals with the isolation and characterization of these endogenous bacteria associated with the rhizome of banana and measure to control them.

Materials and Methods

Collection and preparation of explants : Musa sapientum L. cv. chini champa and sagar were used as experimental materials. The shoot tips along with a portion of rhizomatous tissue were used. The plants were collected from Vawal Modhupur, Trishal, Mymensingh and from the botanical garden of Curzon Hall.

The explants were prepared by removing the outer layer of tissues from suckers with a clean knife. The pale white tissue blocks containing the shoot tip and rhizomatous bases were surface sterilized with 0.1% $HgCl_2$ for 15 min.

Isolation and identification of the selected bacterial isolates: To deter-mine the presence of endogenous bacteria transverse section of rhizome was observed under the microscope. In order to isolate the endogenous bacteria, surface sterilized explants were inoculated in MS medium. The developed bacterial contaminants were transferred to nutrient agar (NA) medium. All the isolated contaminants were purified by serial dilution technique (Collins and Lyne 1984).

Purified bacteria were observed under microscope after proper staining (Simple, Gram and Spore staining). Essential biochemical tests were carried out as per standard methods (Collins and Lyne 1984, Krieg and Holt 1984, Sneath et al. 1986). For identification, characterized bacterial strains were compared with the standard strains of Bergey's Manual (Krieg and Holt 1984), Sneath et al. 1986). The key proposed by Bradbury (1988) was also followed.

Culture and sensitivity (CS) test of the selected bacterial isolates : To test the CS the Kirby-Bauer method was followed (Claus 1995). Mueller-Hinton agar and seven antibiotic disks viz. ampicillin, cephradin, chloramphenicol, gentamicin, vancomycine, tetracycline, doxycycline were used. Disks containing antibiotics were placed after inoculation of the test organisms. The inoculated plates were incubated at 37_C for 24 hrs. The developed inhibition zone around the disks were measured.

Immersion of the surface sterilized explants in different antibiotics: In this test, the surface sterilized explants were immersed in screened antibiotics (ampicillin, gentamicin and tetracycline) for different durations of time to ensure contamination free cultures.

Direct regeneration of shoots : After the treatment with antibiotics the shoot tips of banana were inoculated in MS medium with varying concentrations of hormonal supplements for single shoot regeneration. For the multiplication, the regenerated single shoots were decapitated with a view to inducing axillary budding. These decapitated shoots were incised vertically and placed in MS medium with different concentrations of auxin and cytokinin. The materials were subcultured at 30 days interval in the same fresh medium for the production of multiple shoots.

Induction of roots in the regenerated shoots and their establishment : The regenerated shoots were cultured in rooting media containing half strength MS

supplemented with different concentrations of IBA (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/l). After rooting the plantlets were transferred to small polythene bags containing loamy soil and cowdung (1 : 1) for hardening under cover. Finally the plantlets were transferred to the field.

Results and Discussion

Presence of bacteria was noticed in the transverse section of the rhizome under the microscope. Seven bacterial strains were isolated from the contaminated culture. Four of them were gram positive and the rest were gram negative. The gram positive isolates were *Cellulomonas uda, C. flavigena, Corynebacterium paurometabolum* and *Bacillus megaterium.* The gram negative isolates were *Klebsiella* sp., *Erwinia cypripedii* and *Pseudomonas* sp. All of them were non spore former except *Bacillus megaterium*. Results of the essential biochemical tests are shown in Table 1.

Table 2 shows the CS test of the selected isolates. All the isolated bacteria were found to be susceptible to gentamicin. On the other hand vancomycine was found to be ineffective. Ampicillin and tetracycline were found to be satisfactory. On the basis of this result, ampicillin, gentamicin and tetracycline were used for *in vitro* test of the culture plants. Table 3 shows the effect of different concentrations of antibiotics applied at different length of time. The results show that cent per cent contamination free cultures could be obtained by soaking the explants in 160 mg/l gentamicin for one h and 40 min. When these treated explants were cultured in MS, they produced healthy shoots. In case of tetracycline, the explants did not produce healthy shoots; the plantlets became yellow. In one report Falkiner (1990) mentioned that the agents which act specifically on bacterial cell walls would be more suitable to control infection in plant tissue cultures. It is well established that ampicillin and gentamicin inhibit bacterial cell wall synthesis. Reed et al. (1995) also reported similar results.

The explants treated with antibiotic were cultured in MS supplemented with different concentrations of BAP and Kn. The best medium for single shoot development was MS + 4.0 mg/l BAP + 1.0 mg/l Kn and average time required was 15 - 21 days (Table 4). For the production of multiple shoots regenerated single shoots were cultured in MS with different concentrations of auxin and

Table 1. Morphological and biochemical characteristics of the selected isolates.

No. of isolates	Vegetative cells	Gram reaction	Spores	Methyle red test	Arginine hydro-lase test	Kovacs oxidase test	Starch hydro-lysis	Gelatin hydro-lysis	Casein hydro lysis	Fluorescent pigment test	Voges Proskauer test	Name of isolates
B_1	Short rod	+	Non spore former	–	+	–	–	–	–	–	–	*Cellulomonas uda*
B_2	Short rod	+	Non spore former	–	+	–	+	–	–	–	–	*Cellulomonas flavigena*
B_3	Long rod	+	Non spore former	–	+	+	–	–	–	–	+	*Corynebacterium pauronetabolum*
B_4	Very long rod	+	Spore former	–	–	–	+	+	+	–	–	*Bacillus megaterium*
B_5	Rod	–	Non spore former	–	–	–	–	–	+	–	+	*Klebsiella* sp.
B_6	Long rod	–	Non spore former	+	+	–	–	–	–	–	+	*Erwinia cypripedii*
B_7	Short rod	–	Non spore former	–	–	–	+	–	+	+	+	*Pseudomonas* sp.

+ = positive results, – = negative results.

cytokinin. The highest number of shoots were produced on MS supplemented with 4.0 mg/l BAP + 2.0 mg/l IAA + 13 % CW and 5.0 mg/l BAP + 2.0 mg/l IAA + 2.0 mg/l IBA (Table 5). When MS medium with 5.0 mg/l BAP + 2.0 mg/l IAA + 2.0 mg/l IBA was used, a higher intensity of rooting was observed. As a result multiplication rate was decreased after subculture. Therefore, MS +

Table 2. Culture and sensitivity test of the selected isolates as measured in diameter (mm).

No. of isolates	Name of antibiotics						
	Ampicillin (10 µg)	Cephradin (30 µg)	Chloram-phenicol (30 µg)	Genta-micin (10 µg)	Vancomicin (30 µg)	Tetracycline (30 µg)	Doxycycline (30 µg)
B_1	13 (I)	0 (R)	0 (R)	17 (S)	0 (R)	20 (S)	21 (S)
B_2	9 (I)	0 (R)	0 (R)	21 (S)	0 (R)	22 (S)	20 (S)
B_3	22 (S)	0 (R)	30 (S)	18 (S)	0 (R)	19 (S)	15 (I)
B_4	31 (S)	40 (S)	25 (S)	30 (S)	22 (S)	19 (S)	28 (S)
B_5	22 (S)	21 (S)	23 (S)	18 (S)	0 (R)	15 (I)	15 (I)
B_6	18 (S)	30 (S)	8 (R)	19 (S)	0 (R)	13 (R)	16 (S)
B_7	13 (I)	18 (I)	9 (R)	21 (S)	0 (R)	17 (I)	12.5 (R)

R = resistant, S = susceptible, I = intermediate.

Table 3. Effects of different concentrations of antibiotics immersion at different duration of time to ensure contamination free cultures.

Antibiotics	No. of explants treated	Duration of treatment (mins)	Concentration of antibiotics (mg/l)									
			100		130		160		200		250	
			Contamination free explants regenerated									
			No.	%	No.	%	No.	%	No.	%	No.	%
	15	30	6	40	6	40	6	40	6	40	6	40
Ampicillin	15	60	6	40	6	40	6	40	6	40	9	60
	15	100	6	40	6	40	7	46.66	9	60	12	80
	15	120	6	40	7	46.66	7	46.66	10	66.66	15	100
	15	30	6	40	6	40	7	46.66	8	53.20	9	60
Gentamicin	15	60	6	40	8	53.20	10	66.66	10	66.66	10	66.66
	15	100	7	46.66	10	66.66	15	100	12	80	-	-
	15	120	6	40	10	66.66	15	100	-	-	-	-
	15	30	6	40	6	40	7	46.66	8	53.20	-	-
Tetracycline	15	60	6	40	6	40	8	53.20	10	66.66	-	-
	15	100	6	40	7	46.66	8	53.20	12	80	-	-
	15	120	6	40	7	46.66	9	60	12	80	-	-

4.0 mg/l BAP + 2.0 mg/l IAA + 13 % CW was selected for the production of multiple shoots. 100 % healthy roots were induced from half strength MS with 2.0 mg/l IBA. The regenerated plantlets were successfully established in the soil.

Table 4. Effects of different concentrations of BAP and Kn in MS medium on single shoot development.

Hormonal supplements (mg/l)	No. of explants inoculated	No. of single shoots regenerated	% of explants responded	Average time required (Days)	Average length of shoots (cm)
2.0 BAP + 1.0 Kn	10	2	20	20 - 25	2.0
2.5 BAP + 1.0 Kn	10	3	30	20 - 25	2.5
3.0 BAP + 1.0 Kn	10	5	50	2.0 - 25	2.5
3.5 BAP + 1.0 Kn	10	8	80	15 - 21	2.7
4.0 BAP + 1.0 Kn	10	10	100	15 - 21	3.0
5.0 BAP + 1.0 Kn	10	9	90	15 - 21	3.0
2.5 BAP + 2.0 Kn	10	3	30	20 -21	2.0
3.0 BAP + 2.0 Kn	10	6	60	20 - 25	2.5
3.5 BAP + 2.0 Kn	10	7	70	20 - 25	2.7
4.0 BAP + 2.0 Kn	10	9	90	15 - 21	2.5
4.5 BAP + 2.0 Kn	10	8	80	15 - 21	2.0
5.0 BAP + 2.0 Kn	10	8	80	15 - 21	2.0

Table 5. Effects of different combinations of auxins, cytokinines and coconut water on the production of multiple shoots in MS medium.

Hormonal supplements (mg/l)	No. of single shoots inoculated	No. of single shoot showed multiplication	% of response	Average no. of multiple shoots/explant	Average time required (days)	Intensity of rooting
3.0 BAP + 1.0 Kn + 1.0 IAA	15	2	13	2	45 - 50	-
4.0 BAP + 1.0 Kn + 1.0 IAA	15	5	33	4	45 - 50	-
4.0 BAP + 2.0 Kn + 1.0 IAA	15	7	47	4	45 - 50	-
4.0 BAP + 2.0 Kn + 2.0 IAA	15	11	73	5	40 - 45	-
5.0 BAP + 2.0 Kn + 2.0 IAA	15	12	80	8	40 - 45	-
4.0 BAP + 2.0 IAA + 2.0 IBA	15	14	93	13	40 - 45	+
5.0 BAP + 2.0 IAA + 2.0 IBA	15	14	93	9	40 - 45	+
3.0 BAP + 2.0 IAA + 13% CW	15	12	80	6	45 - 50	-
4.0 BAP + 2.0 IAA + 13% CW	15	14	93	13	40 - 45	-
5.0 BAP + 2.0 IAA + 13% CW	15	14	93	9	40 - 45	-

+ = roots; - = without root.

References

Agrios GN (1988) Plant pathology (3rd ed.), Academic Press Inc. California, U.S.A. pp. 510 - 550.

Bradbury JF (1970) Isolation and preliminary study of bacteria from plants. Rev. Plant Pathol. **49** : 213 - 218.

Bradbury JF (1988) Identification of cultivable bacteria from plants and plant tissue cultures by use of simple classical methods. Acta Hort. **225** : 27 - 37.

Claus GW (1995) Understanding Microbes, (4th ed.),W.H. Freeman and Company, New York. pp. 547.

Collins CH and **Lyne PM** (1984) Microbiological Methods (5th ed.) Butterworths Co. (Publishers). Ltd., London. pp. 56 - 113.

Falkiner FR (1990) The criteria for choosing an antibiotic for control of bacteria in plant tissue culture. IAPTC Newslett, **60** : 13 - 22.

Hwang SC, Chen LC, Lin JC and **Lin HL** (1984) Cultivation of banana, using plantlets from meristem culture.Hort. Science. **19** : 231 - 233.

Krieg RN and **Holt JG** (1984) Bergey's manual of systematic Bacteriology volume 1. Williams and Wilkins Company, Baltimore, U.S.A. pp. 308 - 429.

Reed BM, Bucklay PM and **Dewilde TN** (1995) Detection and eradication of endophytic bacteria from micropropagated mint plants. *In vitro* cell. Dev. Biol. **31** : 53 - 57.

Sneath PHA, Mair NS, Sharpe ME and **Holt JG** (1986) Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins Company, Baltimore, USA. pp. 1120 - 1329.