Elimination of Bacterial Contaminants from Sweetpotato in In Vitro Cultures - OP53

TITLE	**Elimination of Bacterial Contaminants from Sweetpotato in In Vitro Cultures - OP53**
OWNER	Head_IVU
APPROVER	Head Genebank
APPROVAL DATE	
ISSUE DATE	Sep 17, 2010 15:58
CONTRIBUTORS	
CITATION	
KEYWORDS	head_ivu , head_genebank , procedure , non-accredited
NEXT REVISION	
DOCUMENT ID	OP53
VERSION NUMBER	22

INTERNATIONAL POTATO CENTER - CIP

Version	Date	Author	Comment
22	Sep 17, 2010 15:58	Luis Rojas	Changed page structure for bilingual format
21	Aug 05, 2010 09:10	Edwin Rojas	Formatted and Internal error fixed
20	Feb 17, 2010 15:06	Luis Rojas	Changes given by Ana Panta
19	Feb 02, 2010 20:51	Alison Light	Scope, Safety, Materials sections missing
18	Jan 29, 2010 16:49	Alison Light	inoculated changed to inserted

INTRODUCTION

Controlling internal or superficial bacterial contamination from sweet potato *in vitro* cultures can be difficult. Contaminants are not easy to detect visually, and most methods reported for bacteria elimination recommend the use of several antibiotics. Intensive use of these chemicals results toxic to the plants and frequently generates resistant strains. At the International Potato Center (CIP), a large sweetpotato *in vitro* collection is held (5,499),and bacterial contaminants have been detected in the collection. Several treatments applying antibiotics alone or in combination with meristem/shoot tip culture have been tested. The majority of these methods were unsuccessful. Currently, about 200 sweetpotato clones per year are cleaned from bacteria using a method based on reducing or eliminating the contaminant by transferring the plants to soil and re-introducing to *in vitro* culture.

SCOPE

This procedure covers the elimination of bacterial contaminants from sweetpotato in vitro cultures.

SAFETY

No specific requirements above the normal laboratory safety procedures.

MATERIALS

Equipment	
Autoclave	Medium dispenser
PHmeter	Analytical balance
Laminar flow chamber	Shakers
Refrigerator	Cultivation growth chamber
Oven	Microwave oven
Other materials	
Glass test tube	Cotton
Petri dishes	Alcohol
Forceps	Burner
Blades	Sterilizer
Saran wrap or Parafilm	

PROCEDURE

Transferring of *in vitro* plantlets to greenhouse
1. Select the healthiest plantlet, transplant to soil (jiffy #7) treated with 0,5 ml/L of Dimanin® (chloride of bencill-dimetil alquil amonium, Bayer).
2. Store for 7 days in the growing chamber under 22-24º C and spray Dimanin 0,5 ml/L weekly.
3. After 30 days, transplant into a 6 inch pot grown under greenhouse conditions (28º - 30º C).
4. After 2 months, when the plant is grown, it should be re-introduced to *in vitro*.
Re-introduction to *in vitro*
5. At the greenhouse, remove the leaves using a sterile blade for each plant.
6. Cut the stem into uninodal segments.
7. Move the samples to the laboratory and place them into a disinfectant solution (1 g/L hexythiazox (Nissorunâ), 1 mL/L azocyclotin (Peropalâ), 1 g/L benomil (Farmatheâ), and 3-4 mL/L of Tween 20) for 10 minutes.
8. Rinse four times with running water. Rinse with alcohol 70% for 30-60 seconds.
9. Under aseptic conditions, place the samples into 2.5 % sodium hypochlorite (NaClO) for 15 minutes.
10. Move the samples from the hypochlorite solution and rinse five times with sterilized water . Leave in sterilized water containing ascorbic acid 100 mg/L in order to prevent explants oxidation.
11. Excise nodal segments with 1-2 buds and place them on MIB culture medium (Table 1) using 13 mm test tubes. Explants should be cultured one per vessel.
In vitro* culture bacteria testing
12. After 30 days of culture, test possible bacterial contamination, culturing plant segments into Nutritive Broth (NB) (Table 2) for detecting bacteria growth.
13. Select one test tube per accession and under aseptic conditions, take the plantlet off and place on a petri dish.
14. Remove the leaves and roots and cut the stem into 2-3 nodal segments.
15. Transfer isolated explants to 18 x 150 mm test tubes containing conservation medium (MCB) (Table 1).
16. Cut residual material (basal part of plantlet) finely to obtain a mince of plantlet leaves, stems and roots.

17. This mince of residual material and a little portion of culture medium is inserted into a 16 x 150 mm test tube containing 3 ml of NB. Include a positive control test tube containing original bacteria-contaminated accession, and a negative control test tube containing only NB.
18. Incubate the cultures at 30 ^{o}C for 72 hours. Evaluate bacteria presence at 24, 48 and 72 hours. Negative cultures follow additional culture at room temperature for a further 3 week period.
19. Cultures showing bacteria growing (cloudiness) in NB indicates the clone is bacteria positive. Bacteria negative clones must be returned to *in vitro* genebank.
20. When tested clone results bacteria positive, testing is repeated with another clone of the same accession.

Table 1. Media composition for sweetpotato re-introduction to *in vitro* culture

	MIB	MCB
MS salts (g/L)	4.3	4.3
Ascorbic acid (g/L)	0.1	0.2
Calcium nitrate (g/L)	0.1	0.1
Calcium panthotenate (mg/L)	2	2
Gibberellic acid (mg/L)	10	-
L-Arginine (g/L)	0.1	0.1
Putrescine-HCl (mg/L)	20	20
Sucrose (g/L)	30	30
Coconut milk (mL/L)	10	
Agar (g/L)	---	
Phytagel (g/L)	2.8	3
pH	5.7	5.7
Conditions		
Temperature º C	23-25	
Light Intensity Lux	3,000	
Photoperiod h/l	16	

Table 2. Nutritive broth composition for detection of bacterial contaminants in sweetpotato *in vitro* culture*

Peptone (g/L)	5
Beef extract (g/L)	1
Yeast extract (g/L)	2
Glucose (g/L)	10
Sodium chloride (g/L)	5
pH	7.0

***Preparation**

Mix the ingredients in 1 liter of distilled water
Stir until completely dissolved.
Measure the pH
Distribute 3 ml in 16x150mm test tubes
Sterilize in autoclave at 121^{o}C for 15 minutes

Store at 4^{o}C

INTRODUCTION

Controlling internal or superficial bacterial contamination from sweet potato *in vitro* cultures can be difficult. Contaminants are not easy to detect visually, and most methods reported for bacteria elimination recommend the use of several antibiotics. Intensive use of these chemicals results toxic to the plants and frequently generates resistant strains. At the International Potato Center (CIP), a large sweetpotato *in vitro* collection is held (5,499),and bacterial contaminants have been detected in the collection. Several treatments applying antibiotics alone or in combination with meristem/shoot tip culture have been tested. The majority of these methods were unsuccessful. Currently, about 200 sweetpotato clones per year are cleaned from bacteria using a method based on reducing or eliminating the contaminant by transferring the plants to soil and re-introducing to *in vitro* culture.

SCOPE

This procedure covers the elimination of bacterial contaminants from sweetpotato in vitro cultures.

SAFETY

No specific requirements above the normal laboratory safety procedures.

MATERIALS

Equipment	
Autoclave	Medium dispenser
PHmeter	Analytical balance
Laminar flow chamber	Shakers
Refrigerator	Cultivation growth chamber
Oven	Microwave oven
Other materials	
Glass test tube	Cotton
Petri dishes	Alcohol
Forceps	Burner
Blades	Sterilizer
Saran wrap or Parafilm	

PROCEDURE

Transferring of *in vitro* plantlets to greenhouse
1. Select the healthiest plantlet, transplant to soil (jiffy #7) treated with 0,5 ml/L of Dimanin® (chloride of bencill-dimetil alquil amonium, Bayer).
2. Store for 7 days in the growing chamber under 22-24º C and spray Dimanin 0,5 ml/L weekly.
3. After 30 days, transplant into a 6 inch pot grown under greenhouse conditions (28º - 30º C).
4. After 2 months, when the plant is grown, it should be re-introduced to *in vitro*.
Re-introduction to *in vitro*
5. At the greenhouse, remove the leaves using a sterile blade for each plant.

6. Cut the stem into uninodal segments.
7. Move the samples to the laboratory and place them into a disinfectant solution (1 g/L hexythiazox (Nissorunâ), 1 mL/L azocyclotin (Peropalâ), 1 g/L benomil (Farmatheâ), and 3-4 mL/L of Tween 20) for 10 minutes.
8. Rinse four times with running water. Rinse with alcohol 70% for 30-60 seconds.
9. Under aseptic conditions, place the samples into 2.5 % sodium hypochlorite (NaClO) for 15 minutes.
10. Move the samples from the hypochlorite solution and rinse five times with sterilized water . Leave in sterilized water containing ascorbic acid 100 mg/L in order to prevent explants oxidation.
11. Excise nodal segments with 1-2 buds and place them on MIB culture medium (Table 1) using 13 mm test tubes. Explants should be cultured one per vessel.
In vitro* culture bacteria testing
12. After 30 days of culture, test possible bacterial contamination, culturing plant segments into Nutritive Broth (NB) (Table 2) for detecting bacteria growth.
13. Select one test tube per accession and under aseptic conditions, take the plantlet off and place on a petri dish.
14. Remove the leaves and roots and cut the stem into 2-3 nodal segments.
15. Transfer isolated explants to 18 x 150 mm test tubes containing conservation medium (MCB) (Table 1).
16. Cut residual material (basal part of plantlet) finely to obtain a mince of plantlet leaves, stems and roots.
17. This mince of residual material and a little portion of culture medium is inserted into a 16 x 150 mm test tube containing 3 ml of NB. Include a positive control test tube containing original bacteria-contaminated accession, and a negative control test tube containing only NB.
18. Incubate the cultures at 30 ^{o}C for 72 hours. Evaluate bacteria presence at 24, 48 and 72 hours. Negative cultures follow additional culture at room temperature for a further 3 week period.
19. Cultures showing bacteria growing (cloudiness) in NB indicates the clone is bacteria positive. Bacteria negative clones must be returned to *in vitro* genebank.
20. When tested clone results bacteria positive, testing is repeated with another clone of the same accession.

Table 1. Media composition for sweetpotato re-introduction to *in vitro* culture

	MIB	MCB
MS salts (g/L)	4.3	4.3
Ascorbic acid (g/L)	0.1	0.2
Calcium nitrate (g/L)	0.1	0.1
Calcium panthotenate (mg/L)	2	2
Gibberellic acid (mg/L)	10	-
L-Arginine (g/L)	0.1	0.1
Putrescine-HCl (mg/L)	20	20
Sucrose (g/L)	30	30
Coconut milk (mL/L)	10	
Agar (g/L)	---	
Phytagel (g/L)	2.8	3
pH	5.7	5.7
Conditions		
Temperature º C	23-25	
Light Intensity Lux	3,000	
Photoperiod h/l	16	

Table 2. Nutritive broth composition for detection of bacterial contaminants in sweetpotato *in vitro* culture*

Peptone (g/L)	5
Beef extract (g/L)	1
Yeast extract (g/L)	2
Glucose (g/L)	10
Sodium chloride (g/L)	5
pH	7.0

***Preparation**

Mix the ingredients in 1 liter of distilled water
Stir until completely dissolved.
Measure the pH
Distribute 3 ml in 16x150mm test tubes
Sterilize in autoclave at 121^{o}C for 15 minutes
Store at 4^{o}C