Malaysian Journal of Microbiology, Vol 13(4) December 2017, pp. 289-297

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Identification and prevention of microbial contaminants of potato culture in temporary immersion bioreactor (TIB) system

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Received 8 March 2017; Received in revised form 16 April 2017; Accepted17 April 2017

ABSTRACT

Aims: Temporary Immersion Bioreactor (TIB) system is an advanced technology for commercial mass production of potato microtubers. Despite of several advantages, this system possess a great risk of culture loss at any stage of micropropagation due to microbial contamination. The aims of this study were to identify microbial contaminants isolated during potato shoot growth in the TIB system, evaluate the efficacy of antimicrobial agents to prevent them, to investigate the effect of those agents *in vitro* on growth and morphology of potato plantlets.
Methodology and results: Six bacteria namely *Pseudomonas*, *Staphylococcus*, *Klebsiella*, *Corynebacterium*, *Proteus*, *Bacillus* and five fungi *Aspergillus*, *Penicillium*, *Mucor*, *Fusarium and Rhizopus* were isolated from the TIB system. We examined the effect of three antibacterial (Gentamycin, Vancomycin and Tetracycline) and four antifungal agents (Mencozeb, Propiconazole, Bavistin and Copper oxychloride) on the contaminants and on potato shoot growth. Results show that Gentamycin (50 mg/L) and Propiconazole (0.15%) were most effective against the isolated bacteria (35 mm inhibition zone) and fungi (100%) respectively, whereas Gentamycin in combination with Bavistin showed better performance on potato shoot and root development.
Conclusion, significance and impact of study: Present study will provide useful guidelines to reduce or eliminate the risk of contamination during micropropagation.

Keywords: Antimicrobial agents, contamination, potato, Temporary Immersion Bioreactor

INTRODUCTION

The application of plant tissue culture technology is contributing a lot towards the *in vitro* mass production, multiplication and maintenance of disease free potato plants for commercial and other purposes. With this technology a large number of quality plantlets can be produced within a short period (Naik and Karihaloo, 2007). For commercial production of potato microtubers through *in vitro* culture, bioreactor system is an important and advanced method (Rahman *et al.*, 2015). By this time different bioreactor systems have been developed such as RITA™ (Recipient with ATM- automatized temporary immersion), BCB- Bubble Column Bioreactor, BTBB- Balloon Type Bubble Bioreactor (Takayama, 1991; Alvard, *et al.*, 1993; Paek, *et al.*, 2005).

Bioreactor system offer many advantages, including better control of the culture conditions; optimal supply of nutrients and growth regulators; renewal of the culture atmosphere; monitoring of nutrient uptake; replacing the medium during culture period according to the developmental stage of cultured plant material and filtration of the medium for exudates without disturbing the whole culture system (Grigoriadou and Leventakis, 2003; Ziv, 2005). Despite these advantages, bioreactor systems possess a huge risk of culture loss due to microbial contamination. Although different sterilization techniques such as chemical and moist-heat or filter sterilization are used at different stages of micropropagation to ensure clean culture, contaminants may be introduced in the culture with explants, during manipulation in the laboratory, by micro arthropod vectors or endophytic microbes (Tanprasert and Reed, 1997; Leifert and Cassells, 2001; Sharaf-eldin and Weathers, 2006). The principal microbial contaminants frequently reported in plant *in vitro* cultures are bacteria and fungi (Cassells, 1990; Reed, *et al.*, 1995; Pereira, *et al.*, 2003). Major bacterial contaminants are *Pseudomonas syringae*, *Bacillus licheniformis*, *B. subtilis*, *Corynebacterium* sp. and *Erwinia* sp., whereas the fungi *Alterneria tenius*, *Aspergillus niger*, *A. fumigatus* and *Fusarium culmorum* are frequently observed in plant tissue culture by Odutayo, *et al.* (2007). Once established in the cultures, the microbes grow fast, depletes nutrient of the medium and

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produce toxic substances within the medium which causes the mortality, tissue necrosis or variable growth of cultured plantlets resulting in partial or total loss of culture (Kane, 2003). However, the choice is either discarding potentially valuable cultures or attempting to eliminate the contamination using antimicrobial agents. Previously, different antimicrobial agents have been extensively tested for their ability to inhibit or prevent the growth of microbes with varying success in banana (Msogoya *et al.*, 2012); *Lilium* (Altan *et al.*, 2009); *Saraca asoca* (Vichitra *et al.*, 2014); sugarcane (Wagih, *et al.*, 2009); *Pelargonium* sp. (Wojtania *et al.*, 2005) and *Ipomoea* sp. (Jena and Samal, 2011) and potato (Venkatasalam *et al.*, 2013).

Pollock, *et al.* (1983) mentioned that the ideal antimicrobial agents for micropropagation should be water soluble and stable because they should not react with medium components and remain unaffected by pH. Such agents should be safe for the plants and stimulates growth. They should also be usable in combination; with minimum scope of resistance and a wide range of activity against contaminants. Prevention of microbial contamination is a very important issue since it is a major challenge in developing bioreactor systems for large scale production through micropropagation. Under these circumstances this work has been undertaken to identify bacterial and fungal contaminants isolated during potato shoot growth in the TIB system, evaluate the efficacy of antibiotics and antifungal agents to prevent these contaminants, to investigate the effect of antimicrobial agents *in vitro* on growth and morphology of potato plantlets. Results of this study will help to prevent microbial contaminations in temporary immersion bioreactor system and improve the quality of cultured potato plantlets.

MATERIALS AND METHODS

Temporary Immersion Bioreactor

This system was of the "twin-flasks" type (Figure 1A), based on the model described by Jiménez *et al.* (1999). Two screw capped glass bottles (Schott Duran, 500 mL) were modified as the TIB system. One of the bottles was used as medium reservoir unit while the other bottle was used as culture unit. The bottles were connected with autoclavable silicone tube (6 mm ID) along with caps that were fitted with a 0.22 µM sterile filter for ventilation. Air is supplied inside the bottles using diaphragm pump at 0.012 Mpa pressure to transfer liquid medium from the bottles. Two (2) solenoid valves (3 ways) connected with a programmable digital timer to control the nutrient immersion frequency and duration in the TIB system. Medium was transferred by air pressure from the reservoir unit to the culture unit or through opposite direction by opening one valve or the other. Each system had 200 mL multiplication medium in the reservoir unit and 30 single nodal segments the culture unit. Then the TIB system was incubated in illuminated conditions (50 µmol/m^2.s, 16-h photoperiod) for shoot growth.

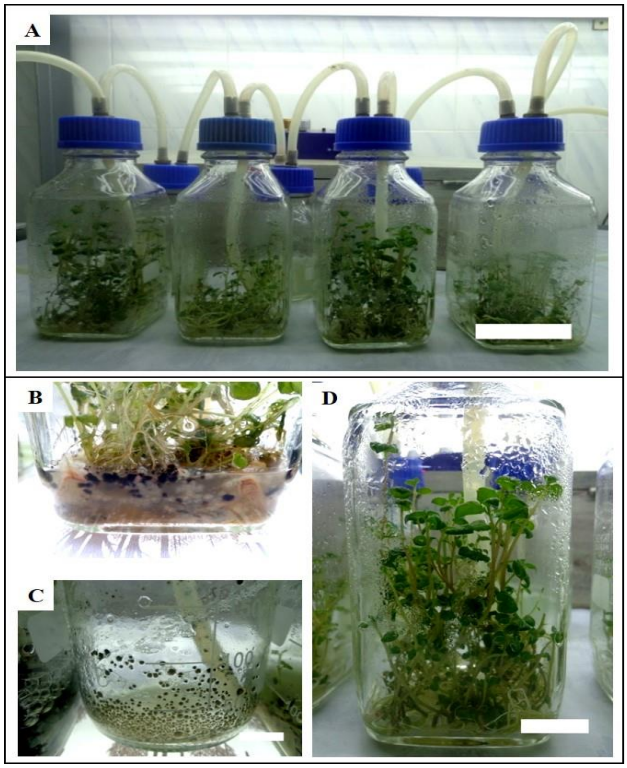

Figure 1: (A) Temporary Immersion Bioreactor (TIB) system, (B) Contaminated culture vessel, (C) Contaminated medium reservoir, (D) Antimicrobial mediated shoots grown in the (TIB) system.

Culture medium and growth conditions

Liquid MS medium was used in the TIB systems. For shoot growth 30 g/L sucrose was added to the medium and the pH was adjusted to 5.8 before autoclaving (121 °C, 15 psi). The culture systems were incubated under 50 µmol/m^2.s light intensity for 3 weeks at 25±1 °C in 16 h day/ 8 h night cycle for shoot growth and multiplication.

Isolation and identification of bacterial and fungal contaminants

The microbial contaminants from the TIB systems were isolated and identified following the method of Msogoya *et al*. (2012). A single colony of contaminants was aseptically picked with a sterile loop and streaked onto nutrient agar medium and incubated for 24 h at 28 °C for growth. These isolates were further purified by serial dilution and repeated sub-culturing and identified them based on their morphological characters (shape of vegetative cells, presence or absence of spores and gram reaction) as well as some biochemical tests e.g. indole production, methyl red, Voges-Proskauer, citrate, urease, catalase and oxidase production (Collins and Lyne 1985; Krieg and Holt, 1984; Sneath *et al.*, 1986). Fungal contaminants were also isolated and purified by the

similar protocol and incubated at 24 °C in PDA medium for 1-2 weeks. These isolates were prepared by lactophenol cotton blue and examined by microscope and morphologically identified based on the appearance of conidiophores and conidia (Barnett and Hunter, 1998).

Culture susceptibility tests of bacterial contaminants

In this experiment, the identified bacteria were tested for susceptibility against three antibiotics *viz*. Gentamicin, Vancomycin and Tetracycline by disc diffusion method (Bauer *et al.*, 1966). Disks of 10, 30 and 50 ppm of each antibiotic were placed onto Mueller-Hinton (1941) agar medium right after inoculation of pure bacterial isolates. After 24 h of incubation at 28 °C, the inhibition zone developed around each disk was measured. Inhibition zone diameter 9-14 mm considered as resistant, 15-19 mm meant intermediate resistant and >20 mm referred to susceptible to the antibiotics (Kneifel and Leonhardt, 1992).

Fungal bioassay

Bioassay of four fungicides *viz*. Mencozeb, Propiconazole, Bavistin and Copper oxychloride with three different concentrations (0.05, 0.10 and 0.15%) were performed against the identified fungi by the method of Vichitra *et al.* (2014). Here, PDA medium supplemented with aforesaid concentration of each fungicide and without any of them, were inoculated with 6 mm plug of pure fungal isolates. After 7 days of incubation period at 25 °C the rate of inhibition for each colony was calculated by the following formula:

$$\% \text{ inhibition} = \frac{T - C}{C} \times 100$$

T= Diameter of fungal colony on fungicide supplemented medium, C= Diameter of fungal colony on control.

Reconstruction experiments

In vitro potato shoot cultures were transferred using four bioreactor systems. Here, thirty explants were placed in each culture vessel. And after one week of incubation period, the centre of each culture vessel was re-inoculated with loop full bacterial and fungal contaminants as mentioned in Tables 1 and 3. These intentionally contaminated cultures were incubated for 1-7 days with different concentrations of antibacterial and antifungal combination as mentioned in Table 4. The frequency of contaminated cultures was determined 3 weeks later.

Effect of different concentration of antimicrobial combinations on shoot and root development

The MS (Murashige and Skoog, 1962) basal medium was supplemented with four different combination of antimicrobials *viz*. Gentamycin + Bavistin, Gentamycin + Propiconazole, Tetracycline + Bavistin and Tetracycline + Propiconazole in three different concentrations (mg/L) *viz*. 50 + 50, 75 +75 and 100 + 100. Gentamycin and Tetracycline were added to the autoclaved medium under aseptic conditions after filter sterilization. The explants (thirty nodal segments per culture vessel) were cultured on above mentioned media composition along with the control (MS medium). Cultured tubes were incubated at 25±1 °C under 16 h photoperiod (50 µmol/m^2.s). After three weeks of culture initiation plant height (cm); number of nodes and roots and its length (cm) were recorded.

RESULTS AND DISCUSSION

Bioreactor is an advanced system for *in vitro* mass production of quality potato seed and plant materials but microbial contamination is a major problem with this system and it is one of the most prevalent causes of culture and economic loss (Rahman *et al.*, 2015). However, the importance of contamination has been reported by Cassels (1990), Guan *et al.* (2005) and Sharaf-eldin and Weathers (2006).

Eleven microbial contaminants were isolated from *in vitro* bioreactor culture of potato. The bacterial contaminants were identified according to the biochemical tests as mentioned in Table 1. Bacterial contaminants were three gram positive, i.e. *Staphylococcus*, *Corynebacterium* and *Bacillus* sp. and three Gram negative i.e. *Pseudomonas, Klebsiella* and *Proteus* sp. It was observed that all of the isolates, except *Bacillus,* formed no spores. The bacterial isolates from this study have been reported as contaminants by several authors in previous studies (Kneifel and Leonhardt 1992; Msogoya *et al.*, 2012). All of the bacterial isolates are exogenously found in soil, water and on plant surfaces except *Klebsiella* sp. which has been reported as endophytic contaminant in internal tissues of banana, maize, wheat and sweet potato (Jena and Samal, 2011; Msogoya *et al.*, 2012). On the other hand, the identified fungi isolates were *Aspergillus* sp., *Penicillum* sp., *Mucor* sp., *Fusarium* sp. and *Rhizopus* sp. All of the fungal contaminants isolated during this study are exogenous. However, *Fusarium* sp. has also been reported as an endophytic fungus in banana and pumpkin plants while *Penicillium* sp. and *Aspergillus* sp. were found in internal tissues of mallow plants (Cassels, 1997; Odutayo *et al.*, 2007). The occurrence of exogenous microbial contaminants in this study was probably due to insufficient asepsis during operation and can be eliminated easily. Endophytic microbes are beneficial to host plants as they enhance plant defense against diseases but becomes problematic in micropropagation where total asepsis is required (Guan *et al.*, 2005). The elimination of endophytic microbes through surface sterilization is usually ineffective except when stronger and systemic sterilants are used (Danso *et al.*, 2011).

Table 2 showed the CS (culture susceptibility) test of the selected bacterial isolates and found that all bacteria were susceptible to Gentamycin.

Table 1: Morphology and biochemical characteristics of the bacterial contaminants isolated during bioreactor culture of potato.

No. of isolates	Vegetative cells	Spores	Gram reaction	Indole production test	Methyl red test	Voges-Proskauer test	Citrate test	Urease test	Catalase test	Oxidase test	Name of isolates
B1	Short rod	NP	-	-	-	-	+	-	+	+	*Pseudomonas* sp.
B2	Cocci	NP	+	-	+	+	-	-	.+	-	*Staphylococcus* sp.
B3	Rod	NP	-	-	-	+	+	+	+	-	*Klebsiella* sp.
B4	Rod	NP	+	-	-	-	-	-	+	-	*Corynebacterium* sp.
B5	Rod	NP	-	+	+	-	-	+	+	-	*Proteus* sp.
B6	Rod	NP	+	-	-	+	-	-	+	-	*Bacillus* sp.

+, positive; -, negative; NP, Not present.

This antibacterial agent exhibited highest zone of inhibition against four isolates at 50 mg/L (24, 29, 35 and 25 mm for *Staphylococcus* sp., *Corynebacterium* sp., *Proteus* sp. and *Bacillus* sp., respectively). Similar dose of Tetracycline showed highest activity against *Pseudomonas* sp. and *Klebsiella* sp. with the inhibition zone of 26 and 27 mm, whereas at 10 mg/L it was not effective against *Bacillus* sp. Vancomycin was found also non-effective against all isolates except *Bacillus* sp. This is in line with several authors who reported the effectiveness of Gentamycin to suppress bacterial contaminants such as *Klebsiella*, *Erwinia*, *Proteus*, *Staphylococcus*, *Pseudomoans*, *Corynebacterium*, *Bacillus* and *Cellulomonas* (Keskitalo *et al.*, 1998; Habiba *et al.*, 2002; Msogoya *et al.*, 2012). According to Falkiner (1990), to be the most effective and suitable agent for plant tissue culture, an antibacterial should act specifically on bacterial cell wall. Gentamycin is a broad spectrum bactericide that suppresses both Gram-positive and Gram-negative bacteria by inhibiting the bacterial cell wall protein synthesis (Pollock *et al.*, 1983; Reed *et al.*, 1995).

Fungal bioassay experiment (Table 3) showed that Propiconazole at 0.15% concentration was 100% effective against all the identified fungal contaminants, namely *Aspergillus* sp., *Penicillium* sp., *Mucor* sp., *Fusarium* sp. and *Rhizopus* sp. However, Copper oxychloride was found to be less effective against all of the fungal isolates (14-39%). The findings of our investigation have positive correlation with those of Vichitra *et al.* (2014) who reported the effectiveness of propiconazole against *Fusarium luteum* and *Colletotrichum gloeosporoides* during *in vitro* propagation of *Saraca asoka*. Propiconazole has been used on many crops. It is a broad spectrum systemic fungicide from trizole group and appears to act by inhibiting ergosterol production, which is an essential sterol in the membranes of most fungi (Rouabhi, 2010).

In order to test the effectiveness of antifungal and antibacterial agents against microbial contamination, *in vitro* bioreactor cultures were intentionally inoculated with the isolates and treated for 1-7 days with different concentrations and combinations of antibiotics (Table 4). The combination of gentamycin (50 mg/L) and propiconazole (0.15%) was found most effective for contamination prevention at 3-5 days treatment but plantlet mortality was observed at longer duration (7 days). Similar combination of Gentamycin and Bavistin also prevented bioreactor contamination when treated for 5-7 days. It was observed that lower concentration of antibiotics combination was less effective whereas higher concentrations led to plantlet mortality. In a previous study, Kritzenger and Vuuren (1998) eliminated fungal and bacterial contaminants of *Zandedeschia aetheopica* rhizomes using fungicide and antibiotic combinations. In accordance with this report, the present investigation also demonstrated the positive effect of Gentamycin and Propiconazole against intentional contamination (Table 4). The combined effect of Gentamycin and Bavistin was found satisfactory too. According to Altan *et al.* (2010), most antimicrobial agents alone have a narrow target spectrum, but in combination with another compatible antimicrobial agent the spectrum widens and thus their effectiveness increase. .

Table 2: Culture Susceptibility (CS) test of the isolated bacterial contaminants as measured in diameter (mm).

Name of the antibiotic	Strength (mg/L)	Width of inhibition (mm)					
		B1	B2	B3	B4	B5	B6
Tetracycline	10	23 (S)	16 (I)	19 (I)	17 (I)	18 (I)	0 (R)
	30	25 (S)	17 (I)	23 (S)	17 (I)	21 (S)	9 (R)
	50	26 (S)	20 (S)	27 (S)	18 (I)	24 (S)	16 (I)
Vancomycin	10	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	13 (R)
	30	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	22 (S)
	50	0 (R)	8 (R)	0 (R)	11 (I)	0 (R)	23 (S)
Gentamycin	10	21 (S)	21 (S)	23 (S)	25 (S)	29 (S)	20 (S)
	30	24 (S)	23 (S)	25 (S)	28 (S)	30 (S)	22 (S)
	50	25 (S)	24 (S)	26(S)	29 (S)	35 (S)	25 (S)

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

Table 3: Bioassay of the isolated fungal contaminants as measured in diameter (mm).

Name of the antifungal	Strength (%)	Percentage of inhibition				
		Species				
		Aspergillus	*Penicillium*	*Mucor*	*Fusarium*	*Rhizopus*
Mencozeb	0.05	75	86	56	67	74
	0.10	78	88	76	69	67
	0.15	79	89	78	75	73
Propiconazole	0.05	81	96	88	92	94
	0.10	91	93	93	95	97
	0.15	100	100	100	100	100
Bavistin	0.05	88	73	89	91	87
	0.10	91	77	91	94	88
	0.15	93	79	93	97	95
Copper oxychloride	0.05	16	19	14	28	32
	0.10	22	23	18	35	34
	0.15	26	31	24	38	39

Table 4: Effect of different combinations and concentrations of antimicrobial agent treatment at different duration of days to ensure contamination free culture.

Name of antimicrobials	Duration of treatments (days)	Concentration of antimicrobials				
		10 mg/L + 0.05%	30 mg/L + 0.10%	50 mg/L + 0.15%	75 mg/L + 0.20%	100 mg/L + 0.25%
		Percentage of contamination				
Gentamycin + Bavistin	1	100^{fb}	100^{f}	100^{fb}	75^{f}	75^{fb}
	3	100^{fb}	25^{fb}	50^{f}	75^{fb}	25^{b}
	5	75^{fb}	50^{f}	0	0	0^{m}
	7	50^{f}	25^{f}	0	0^{m}	0^{m}
Gentamycin + Propiconazole	1	75^{fb}	100^{fb}	25^{f}	75^{f}	50^{f}
	3	50^{f}	75^{fb}	0	25^{f}	75^{fb}
	5	100^{fb}	25^{f}	0	0	25^{f}
	7	75^{fb}	25^{f}	0^{m}	0^{m}	0^{m}
Tetracycline + Propiconazole	1	75^{fb}	100^{fb}	75^{fb}	50^{fb}	75^{b}
	3	75^{fb}	25^{f}	25^{f}	0	0^{m}
	5	50^{f}	25^{f}	0	25^{b}	0^{m}
	7	25^{f}	50^{f}	0^{m}	0^{m}	0^{m}

b, bacterial contamination; f, fungal contamination; m, plantlet mortality.

Table 5: Effect of different concentration of antimicrobial cocktails on shoot and root growth of potato plantlets after 3 weeks culture in bioreactor (Mean ± SE).

Antibacterial agent	Strength	Plantlet height (cm)	Number of nodes	Number of roots	Length of roots (cm)
Control	0	5.2±0.35	4.3±0.20	4.2±0.28	4.4±0.56
Gentamycin + Bavistin	50 mg/L + 0.15%	5.3±0.39	3.9±0.40	4.4±0.42	4.2±0.41
	75 mg/L + 0.20%	6.1±0.35	4.9±0.76	5.3±0.56	4.8±0.28
	100 mg/L + 0.25%	4.7±0.41	4.0±0.65	2.5±0.39	3.4±0.76
Gentamycin + Propiconazole	50 mg/L + 0.15%	5.4±0.65	4.1±0.42	4.3±0.29	4.0±0.29
	75 mg/L + 0.20%	4.9±0.56	3.8±0.41	4.2±0.31	3.8±0.40
	100 mg/L + 0.25%	5.1±0.29	4.3±0.76	4.4±0.29	3.3±0.42
Tetracycline + Bavistin	50 mg/L + 0.15%	5.9±0.28	4.5±0.65	4.2±0.42	3.9±0.76
	75 mg/L + 0.20%	5.5±0.39	4.3±0.35	3.3±0.40	4.1±0.28
	100 mg/L + 0.25%	nil	nil	nil	nil
Tetracycline + Propiconazole	50 mg/L + 0.15%	4.2±0.56	3.2±0.41	4.1±0.35	3.9±0.28
	75 mg/L + 0.20%	nil	nil	nil	nil
	100 mg/L + 0.25%	nil	nil	nil	nil

The effect of combined antimicrobial agents on the growth of *in vitro* cultured potato plants were evaluated and the results showed that with 75 mg/L Gentamycin + 0.20 % Bavistin increased plants height (6.1 cm), number of nodes (4.9), roots (5.3) and length of roots (4.8 cm) (Table 5). On the other hand, combination of Tetracycline and Propiconazole resulted in reduced growth but at higher doses plant mortality was observed. Tiwari *et al.* (2012) reported that the combination of Bavistin and Trimethoprim increased the shoot number and length in *Bacopa monniera*. The growth promoting effect of Bavistin were also reported by Tripathi and Ram (1982) in Carrot, Patnaik and Debata (1996) in *H. indicus*, Sahoo and Chand (1998) in *V. negundo* (L), Panathula *et al.* (2014) in *Centella asiatica*. This effect of the fungicide may be due to molecular structure of bavistin which has some similarity with the molecular structure of kinetin, adenine, and many other adenine based cytokinins as adenine thiosulphate (Panathula *et al*., 2014).

CONCLUSION

Microbial contamination could occur at any stage of *in vitro* micropropagation systems. In the TIB system, it may cause a decrease of the quality and quantity of plantlets or total loss of the culture. It is very important to standardize an optimal method to reduce or eliminate the risk of contamination without minimizing the growth of the cultured plantlets. The present study clearly demonstrated about the prevention method of microbial contamination during culture of potato plantlets in TIB system and proved that Gentamicin in combination with Propiconazole or Bavistin is effective for prevention of contamination. Whereas, the combination of Gentamycin and Bavistin improved shoot and root development of potato.

The results presented in this study will provide useful guidelines to reduce or eliminate the risk of contamination, and will prove useful in the implementation of the TIB and other large scale culture system for commercial micropropagation for potato and other crops.

ACKNOWLEDGEMENT

Authors are gratefully acknowledges to the Institute of Biological Sciences, University of Rajshahi and BCSIR Laboratories Rajshahi for providing fellowships and technical supports for this study.

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