Plant Cell Biotechnology and Molecular Biology 5(1&2): 51-58

EFFECT OF FUNGICIDES AND ANTIBIOTICS TO CONTROL MICROBIAL CONTAMINATION IN *HEVEA* CULTURES

R.G. KALA*, LEDA PAVITHRAN AND A. THULASEEDHARAN

Biotechnology Division, Rubber Research Institute of India, Kottayam - 686 009, India
[*For Correspondence: E-mail:rgkala@rubberboard.org.in)

ABSTRACT

***In vitro* toxicity and microbial contamination control efficiency of three fungicides - benomyl, bavistin and fytolan and three antibiotics - nystatin, amphotericin-B and streptomycin were assessed by incorporating them in the *Hevea* shoot culture medium. Of the different compounds tested no single compound could effectively control contamination of cultures. Among the different combinations tried, nystatin (25 mg/l) along with streptomycin (10 mg/l) was found to be non-toxic to *Hevea* cultures and also controlled microbial growth. In this combination, growth enhancements as well multiple shoot induction of nodal cultures and also shoot elongation with new flushes were also noticed. This study indicates the need of fungicides/bactericides as adjuvant in tissue culture medium for obtaining sterile and viable shoots.**

Key words: Antibiotics, Contamination control, Fungicides, *Hevea brasiliensis*, Phytotoxicity

INTRODUCTION

In the tissue culture of woody plant species, establishment of viable plant regeneration systems are often hampered by various constraints namely recalcitrance of the explants, irreversible juvenile-mature phase shift, stress conditions such as phenol exudation, microbial contamination etc. Contamination of plant tissue cultures by micro-organisms often occur either by their presence in the explant or arise as laboratory contaminants. In *Hevea* tissue culture also, contamination of cultures due to micro-organisms such as fungi and bacteria is a major problem. When explants are taken from nursery or field grown trees, the contamination rate is more and recovery of sterile cultures is less than 10 per cent. Surface sterilization of the explants could prevent initial contamination, however, micro organisms often find their way into the tissues causing delayed emergence of contaminants, sometimes even after subculture. Though, exogenous micro-organisms can be effectively eliminated by surface sterilants, it is very difficult to eliminate endogenous contaminants without tissue damage (Bonga, 1982). Hence, this is a serious problem with explants of mature origin and it often hampers interpretation of experimental results. Similar phenomenon is frequently observed in shoot tip/axillary bud cultures of *Hevea* posing problems in aseptic culture establishment. However, the response of clonal materials derived from mature field grown plants to *in vitro* cultures is very poor and persistent microbial contamination often causes constraints in interpretation of results. Surface sterilization with mercuric chloride could produce sterile cultures but recovery percentage of viable

Corresponding Editor: Dr. P.K. Saha

cultures was very low. This confirmed the need for incorporation of fungicides/antibiotics in the culture medium at concentrations, which are not lethal to the growth of the explants.

Antibiotics are frequently used to remove tissue contamination in animal and plant tissue culture (Pollock et al., 1983; Shields et al., 1984; Wilson and Power, 1989; Carew and Patterson, 1970). Wilson and Power (1989) have screened a few antibiotics in relation to tissue stress and callusing capacity in stem explants for initiating protoplast cultures in *Hevea*. Accordingly, the incorporation of erythromycin, streptomycin, tetracyclin and nystatin at bactericidal levels could prevent contamination of stem sections for protoplast preparation without adversely affecting callusing capacity. Pollock et al. (1983) have examined the toxicity of over twenty antibiotics to plant cells of *Nicotiana*. The anti-fungal agents nystatin and amphotericin B were found to be nontoxic to plant protoplasts at concentrations that controlled fungal growth (Watts and King, 1973; Anonymous, 1961). In shoot tip and nodal cultures of *Hevea*, fungal contamination was more pronounced than bacterial. So an ideal anti-fungal agent that would be fungicidal in plant tissue culture medium and nontoxic to plant cells with support of an ideal antibiotic need to be identified. This paper describes the development of protocols to control contamination in shoot tip/nodal cultures of *Hevea* by incorporating fungicides and antibiotics in the initial culture medium.

MATERIALS AND METHODS

Explants

Nodal segments and shoot tips taken from greenhouse grown seedlings were used as explants for various experiments. Explants were also taken from elite clones of *Hevea*, clone RRII 105 and RRIM 600, raised in poly bags and maintained in greenhouse. The results of seedlings were compared with clonal materials and necessary modifications were made to get optimum results. Explants were collected on sunny days during December to April since recovery percentage of sterile cultures was more during this period.

Surface Sterilization

Both fungal and bacterial contamination occurs during initiation of *in vitro Hevea* cultures, causing tissue damage leading to death of explants. To overcome this, surface sterilization experiments were carried out with different concentrations of ethanol (40-80%), sodium hypochlorite (0.1-0.5%) and mercuric chloride (0.1-0.3%) for different time intervals. Shoot tips of about 2 cm in length, with two newly formed leaves, were taken from greenhouse grown plants. Stem cuttings with single nodes were used for initiating nodal cultures. For shoot tip and nodal culture, ideal sterilant, optimum concentration and sterilizing time, for effective sterilization with minimal phytotoxicity were identified separately (Table 1). This inference was used for further optimization experiments. Surface sterilization was done uniformly to all the explants. Shoot tips and nodal cuttings were washed thoroughly in running tap water, sterilized with different concentrations of $HgCl_2$ containing one drop of tween 20 for 2 and 3 minutes and rinsed five times in sterile distilled water. Sterilized explants were kept for 5-8 min. on sterile filter paper in a laminar flow hood to remove water particles. Explants were then cultured in the initial culture medium after cutting the base and the petiole ends. In control, no antibiotics were added.

Culture medium

WPM (Lloyd and McCown, 1981) containing ascorbic acid 100 mg/l, sucrose 40 g/l and 3% charcoal was used for nodal cultures. Thidiazuron (TDZ - 0.8 mg/l), benzyl adenine (BA -1.5 mg/l), indole-3 acetic acid (IAA -0.2 mg/l and gibberellic acid (GA_3 - 1.0 mg/l) was incorporated as phytohormones. For shoot tip cultures, the medium used was MS (Murashige and Skoog, 1962) with modified major nutrients, ascorbic acid 100 mg/l, sucrose 30 g/l and charcoal 0.3% (Asokan et al.,1988). Culture medium also contained the hormones TDZ (0.2 mg/l), BA (0.5 mg/l), IAA (0.2 mg/l) and GA_3 (0.3 mg/l). In both cases, the PH of medium was adjusted to 5.6 prior to autoclaving at 120° C for 10 min. Phytohormones were filter sterilized, added to the autoclaved medium and vortexed. Culture tubes containing 10-15 ml medium were used for culturing nodal explants and shoot tips. Rate of contamination and growth performance of

axillary buds and shoot tips were evaluated after 9 days and 3-4 weeks.

Contamination control

Different concentrations of three antibiotics-nystatin, amphotericin-B and streptomycin and three fungicides - benomyl, bavistin and fytolan alone and in combinations were used for the study. For both shoot tip and nodal cultures, same concentrations of fungicides and bactericides were used and were added to the autoclaved medium after filter sterilization, as an adjuvant. Tissue response was assessed for 3 weeks at weekly intervals with respect to contamination, phytotoxicity and extent of chlorosis. All cultures were maintained under 25±2°C and 18 hrs. Photoperiod. The experiments were repeated thrice with 10 replications.

RESULTS

Contamination control and phytotoxicity

Among three sterilants tried for surface sterilization of shoot tips, ethyl alcohol was ineffective in controlling contamination of cultures (Table 1). Though higher concentrations (>0.25%) of sodium hypochlorite could give contamination free cultures, the explants showed gradual yellowing followed by tissue death. Lower concentrations of mercuric chloride (<0.25%) could control surface contamination more effectively than other sterilants with minimum phytotoxicity (<50%). Gradual emergence of contaminants occurred at the cut ends of the explants and from dead leaves or petiole ends in about 50% of the sterile and viable cultures obtained. The effect was similar with nodal cultures also.

In experiments with single compounds, none could control growth of all microbial contaminants effectively. Incorporation of fungicides/bactericides in the culture medium reduced contamination and lethality. The results shows that fungicides as well as the fungal antibiotic, nystatin could reduce fungal contaminants and antibiotics such as amphotericin B and streptomycin that of bacterial contaminants. There were no major differences between the growth rate of explants in control and fungicides/bactericide containing media when applied at lower concentrations. Streptomycin and nystatin at con.>50 mg/l caused a retardation of growth, while amphotericin B at concentrations above 25 mg/l was lethal to the cultures. The fungicides benomyl, bavistin and fytolan also retarded growth at concentrations above 25 mg/l, which controlled fungal contamination in the medium. The effect of the individual antibiotics and fungicides in controlling contamination and their phytotoxic effects in seedling explants is given in Table 2. The ideal concentrations identified with respect to contamination control and phytotoxicity of seedling-derived explants were compared with explants of clonal origin. Regarding contamination control, the response of clonal explants was similar to that of seedling derived shoots. The effect of these compounds on the growth rate of different types of explants used i.e. seedlings, clone 105 and RRIM 600 varied markedly. Even the very juvenile seedling could withstand high concentrations of the above compounds exhibiting continued growth. The response of nodal cultures of clones RRIM 600 was in between seedling derived nodal cultures and RRII 105. In the case of shoot tip cultures also the trend was the same. The leaves of RRII 105 shoot tips were very sensitive to sterilization treatments (data not shown).

Since no single compound could effectively control both fungal and bacterial contamination, and combination of compounds was tried. This did not have any negative effect on the growth of cultures but could help more in controlling contamination (Table 3). The compounds nystatin, streptomycin and bavistin, which showed optimum response in terms of both contamination control and phytotoxicity were used in combination for a more effective contamination control. Of the different concentrations of the above combinations tried, nystatin 25 mg/l and streptomycin 10 mg/l could give optimum contamination control of above 80% causing minimum phytotoxicity.

Multiple shoot induction

Multiple shoot induction was observed in shoot tips of seedlings, and clonal materials when cultured in nystatin containing medium (Figs.1 A-D). The medium, which contained nystatin 25

Table 1 Effect of different sterilants in controlling surface contamination seedling derived shoot tips

Sterilant	Con. (%)	% Contamination during diff. time intervals			% phytotoxicity during sterilization for diff. time.		
		2 min	3 min	Row Mean	2 min	3 min	Row Mean
Mercuric chloride	0.10	63.3	46.6	60.0	16.0	18..3	17..6
	0.15	46.6	43.3	45.0	16.6	20.0	18.0
	0.20	40.0	30.0	35.0	20.0	25.0	22.5
	0.25	33.3	26.6	30.0	43.3	46.6	45.0
	0.30	26.6	23.3	25.0	56.6	60.0	53.3
Sodium hypochl.	0.10	76.6	70.0	73.0	30.0	33.3	31.6
	0.20	66.6	66.6	66.0	43.3	53.3	48.3
	0.30	60.0	60.0	60.0	40.0	46.6	43.3
	0.40	40.0	33.3	36.0	63.3	60.0	61.6
	0.50	43.3	30.0	36.0	76.6	73.3	75.0
Ethyl alcohol	40	83.3	83.3	83.0	30.3	33.3	31.6
	50	76.6	76.6	76.0	33.3	40.0	36.6
	60	73.3	73.3	73.0	43.3	46.6	45.0
	70	76.6	76.6	76.0	43.3	43.3	43.3
	80	70.0	66.6	68.0	50.0	50.0	50.0
Column mean		5.84	5.44		4.6	5.13	
CD (0.05)		8.2		5.3	10.7		3.9

Values given are mean of percentages of 10 samples repeated thrice.

Table 2. Effect of different antibiotics and fungicides in combating contamination and phytotoxicity of seedling derived cultures.

Treatment			Shoot tip culture		Nodal culture	
Fungicidal/ Bactericidal agent (mg/l)			Contamination (Mean)	Phytotoxicity (Mean)	Contamination (Mean)	Phytotoxicity (Mean)
Amphotericin	10	T_1	42.99 (46.66)	53.15 (63.33)	57.00 (70.00))	41.15 (43.33)
	25	T_2	33.00 (30.00)	50.85 (60.00)	32.78 (33.33)	41.07 (43.33)
	50	T_3	21.14 (13.23)	62.78 (63.33)	26.92 (33.33)	50.85 (60.0)
	100	T_4	13.93 (6.88)	68.85 (63.00)	35.22 (33.33)	62.58(63.33
Nystatin	10	T_5	28.78 (23.33)	17.22 (16.66)	11.07 1(0.00)	0.00 (0.00)
	25	T_6	12.29 (66.66)	18.78 (13.33)	15.00 (10.00)	0.00 (0.00)
	50	T_7	18.86 (16.66)	32.22(36.66)	12.29 (6.66)	35.00 (33.00)
	100	T_8	0.00 (0.00)	38.85 (43.66)	11.14 (10.33)	39.93 (36.66)
Strep	10	T_9	45.00 (50.0)	28.78 (23.33)	0.00 (0.00)	0.00 (0.00)
	25	T_{10}	37.22 (36.66)	35.01 (33.33)	0.00 (0.00)	00.00 (0.00)
	50	T_{11}	35.22 (33.33)	37.22 (36.66)	15.00 (10.00)	15.00 (10.00)
	100	T_{12}	15.00 (10.00)	8.86 (6.66)	17.71 (13.33)	21.14 (13.33)
Bavistin	10	T_{13}	33.00 (30.00)	41.15 (43.33)	0.00 (0.00)	0.00 (0.00)
	25	T_{14}	31.00 (26.66)	37.22 (36.66)	11.07 (10.00)	19.93 (16.66)
	50	T_{15}	17.71 (13.33)	31.00 (26.66)	33.00 (30.00)	12.29 (6.66)
	100	T_{16}	6.14 (33.33)	28.29 (23.33)	43.08(46.66)	33.00 (30.00)
Benomyl	10	T_{17}	31.00 (26.66)	37.22 (36.66)	0.00 (0.00)	0.00 (0.00)
	25	T_{18}	28.78 (23.33)	39.15 (40.00)	41.15 (43.33)	11.07 (10.00)
	50	T_{19}	31.00 (26.66)	35.22 (33.33)	37.22 (36.66)	35.22 (33.33)
	100	T_{20}	23.86 (16.66)	48.85 (56.66)	41.15 (43.33)	33.00 (30.000)
Fylotan	10	T_{21}	43.08 (46.66)	45.00 (50.00)	8.86 (6.66)	8.86 (6.66)
	25	T_{22}	45.00 (50.00)	33.00 (30.00)	37.14 (36.66)	22.14 (20.00)
	50	T_{23}	37.22 (36.66)	33.00 (30.00)	37.22 (36.66)	35.22 (33.33)
	100	T_{24}	33.00 (30.00)	50.85 (60.00)	33.00 (30.00)	37.22 (36.66)
Control		T_{25}	43.08 (46.66)	23.08 (22.66)	31.00 (26.66)	28.78 (23.33)
CD (0.5)			13.67	14.68	12.57	15.84

Values given are percentage of mean of 10 samples repeated thrice.

Table 3. Effect of combination of compounds in controlling contamination and phytotoxicity

Treatment		Shoot tip culture		Nodal culture	
Fungicide/ Bactericide (mg/l)		% Contamination (Mean)	% Phytotoxicity (Mean)	% Contamination (Mean)	% Phytotoxicity (Mean)
Nyst.10+ Bavistin					
10	T_1	15.2 (23.3)	12.8 (16.6)	16.3 (26.6)	11.4 (13.3)
25	T_2	14.1 (20.0)	34.1 (30.0)	17.3 (20.0)	31.4 (33.3)
50	T_3	16.3 (16.6)	45.2 (43.3)	17.2 (23.0)	33.8 (32.0)
Nyst. 25 +Bavistin					
10	T_4	11.4 (13.3)	11.4 (13.3)	13.8 (20.0)	12.8 (16.6)
25	T_5	12.8 (16.6)	12.8 (16.6)	12.8 (16.6)	16.3 (26.6)
50	T_6	11.4 (13.3)	16.3 (26.6)	12.8 (16.6)	19.1 (36.6)
Nyst.10 + Strep.					
10	T_7	23.8 (26.6)	10.0 (10.0)	18.8 (20.0)	10.0 (10.0)
25	T_8	11.4 (13.3)	20.00 (15.0)	12.4 (13.3)	28.0 (10.0)
50	T_9	10.4 (13.3)	32.8 (36.6)	11.4(10.6)	35.2 (33.3)
Nyst. 25+ Strep					
10	T_{10}	11.4 (13.3)	15.0 (18.0)	13.8 (20.0)	15.0 (18.0)
25	T_{11}	13.8 (12.0)	14.7 (10.60)	11.4 (13.3)	18.5 (16.6)
50	T_{12}	11.4 (13.3)	23.8 (20.0)	15.8 (16.6)	12.8 (26.6)
CD (0.05)		4.0	5.1	5.3	6.6

Values are expressed as the mean of percentages of 10 samples repeated thrice

Table 4. Effect of Nystatin and Thidiazuron on multiple shoot induction, elongation and maturation

Treatment		%culture showing multiple shoot induction	Elongation (%)	Maturation (%)
Nystatin10mg/l +TDZ				
0	T_1	23.30	23.30	23.30
0.2	T_2	26.70	20.00	27.00
0.4	T_3	53.00	53.30	53.30
0.6	T_4	76.70	66.70	60.00
0.8	T_5	43.00	76.70	26.70
1.0	T_6	33.00	33.30	36.70
Nystatin25mg/l +TDZ				
0	T_7	47.00	53.30	43.30
0.2	T_8	56.70	50.00	56.70
0.4	T_9	50.00	56.70	93.30
06	T_{10}	77.00	80.00	63.30
0.8	T_{11}	83.30	76.70	76.70
1.0	T_{12}	78.70	73.30	83.30
Nystatin50mg/l +TDZ				
0	T_{13}	40.00	36.70	36.70
0.2	T_{14}	46.70	50.00	40.00
0.4	T_{15}	53.30	53.30	46.70
0.6	T_{16}	56.70	50.00	50.00
0.8	T_{17}	56.70	53.30	46.70
1.0	T_{18}	46.70	53.30	46.70
CD		15.90	13.40	12.90

Values are the mean of 10 samples repeated thrice.

Figure 1. Shoot tip cultures of *Hevea*. A & B : Multiple shoot induction . C&D : Shoot maturation E & F : Sprouting of axillary bud, G : Shoot maturation, H : Rooted plant.

mg/l along with hormones TDZ and GA3, showed the tendency of multiple shoot induction, elongation and maturation. (Table 4). Presence of TDZ along with nystatin was found to enhance multiple shoot induction. 25 mg/l nystatin along with 0.8 mg/l TDZ and GA_3 (0.5 mg/l) helped in induction of multiple shoots. TDZ at a concentration of 0.6 mg/l was ideal for elongation of shoots along with GA_3 0.5 mg/l. Maturation of shoots needed a concentration of 0 4 mg/l TDZ. The rate of multiple shoot induction was more than 90 per cent in seedling explants while with clonal shoot tips it was less than 20 per cent. The multiple shoots induced in shoot tips elongated and matured in the initial medium or when subcultured into fresh medium. Rate of elongation of shoots induced in clonal shoots was lower than that in seedling shoots. The mature seedling shoots rooted in ½ MS medium containing 1 mg/l IBA and 0.5 mg/l IAA.

Above all, the fungal antibiotic nystatm was found to show a growth promoting effect on *Hevea* cultures. Dormant apical buds of shoot tips and axillary buds sprouted showing continued growth (Figs. 1E-H). Compared to control cultures, explants cultured in nystatin containing medium showed a faster growth rate.

DISCUSSION

The aim of this work was to see whether antibiotics and fungicides could be safely used in *Hevea* shoot culture medium to get rid of microbial contamination. Compared to animal tissue cultures where bacterial contamination is more prominent, plant tissue cultures are often destroyed by fungal contaminants. As a result, a large number of fungicides and fungal antibiotics were developed which are active against fungal pathogens (Katherine et al., 1979; Brown et a.l, 1982; Shields et al., 1984; Muralı et al., 2001). To be effective, an ideal anti-fungal agent should be fungicidal in plant tissue culture medium, nontoxic to plant cells and have a broad spectrum of fungicidal activity when supplemented in the plant tissue culture medium. Many of the fungicides have been tested for their effect in plant tissue cultures (Brown et al., 1982; Shields et al., 1984; Pollock et al., 1983). Wilson and Power (1989) have studied the effect of incorporation of antibiotics to control systemic contamination of rubber stem tissues to produce sterile protoplast cultures of *Hevea*. They have also tested the phytotoxic effect of a number of antibiotics on rubber stem tissues.

Sensitivity of the rubber stem tissue to antibiotics/fungicides was explant specific. The antibiotic amphotericin B retarded growth in all types of explants cultured at concentrations above 25 mg/l and contamination control was ineffective. At concentrations above this irreversible phytotoxicity occurred, thereby making it unsuitable for *Hevea* cultures. Though, higher concentration of nystatin showed occasional phenolic exudation, concentration below 50 mg/l was found to increase the growth rate of cultures by 30% (Frank et al., 1958-59; Anonymous; 1961). Regarding contamination control, this would be achieved up to 80% when used alone up to a concentration of 50 mg/l. The other compounds tried such as bavistin, fytolan and benomyl could also give contamination control up to 50%. Prolonged exposure of cultures to these was causing deleterious effects.

Endogenous contamination in stem cultures of *Hevea* usually occurred after 10 days culture while surface contamination appeared after 3-5 days. No single compound could effectively control both fungal and bacterial contamination in *Hevea* cultures. Endogenous contamination in nodal and shoot cultures could be controlled effectively using a combination of nystatin (25 mg/l) and streptomycin (10 mg/l) as adjuvant in the initial culture medium. Incorporation of these antibiotics in the culture medium could produce sterile cultures without affecting further growth of the explants. This is in accordance with the observation of Wilson and Power (1989) in controlling contamination of rubber stem explants and protoplast preparations. Similar observations have been made by Pollock et al, (1983) and Frank (1958-59). According to them combining non-toxic levels of amphotericin or nystatin with N-substituted imidazoles would provide broad spectrum antifungal activity. Washing the explants in antibiotic solutions prior to culture was also ineffective. Nystatin belongs to the group of polyenes, which are clinically important group of fungicides and have been used in animal and plant cell cultures (Hammond et al., 1974; Watts and king, 1973). These agents appear to function by increasing fungal cell wall permeability.

Several antibiotics have been reported to have stimulatory effect on *in vitro* plant cell growth (Carew and Patterson, 1970; Skene, 1972 and Thomas 1973).

We have also observed a similar effect by the presence of antibiotics in the medium. Both shoot and nodal cultures showed enhanced growth compared to controls and multiple shoot induction also occurred. The growth stimulatory effect of cultures in presence of antibiotics and fungicides have been reported for kanamycin which promote morphogenesis in plant tissues (Owens, 1979) and benomyl exhibiting cytokinin like activity (Williams, 1975; Zutshi, 1975). The growth stimulatory effect seen in *Hevea* cultures may be due to the effect of combination of antibiotics as has been seen in the case of rubber stem tissues (Wilson and Power, 1989)

Though antibiotics are no substitute for good sterile technique, their use may be considered in disinfection of hard-to sterilize tissues. Routine use of antibiotics in initial culture medium of rubber explants would help in the establishment of viable and sterile cultures which would further help in increasing the efficiency of micro propagation systems.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Dr. N.M. Mathew, Director of Research, RRII, for his support and encouragement. The authors also wish to thank Mr. Ramesh B. Nair for the help rendered in statistical analysis.

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