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Effect of Antibiotics and Fungicides on the *in Vitro* Production of *Citrus limonia* Osbeck Nodal Segment and Shoot Tip Explants

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ABSTRACT

Among the several rootstocks, Rangpur lime (*Citrus limonia* Osbeck) is commercially employed in India, particularly in Andhra Pradesh state, for the propagation as a rootstock for sweet orange cultivar 'Sathgudi' on account of its disease tolerance, drought resistance and overall better performance over other cultivars. The *in vitro* propagation of plants is always under threat of microbial and fungal contamination either from the environment or from the explants. To overcome the surface contamination of explants, several experiments were conducted to evaluate the efficacy of different antibiotics and fungicides. The treatments consist of benomyl (0.01%), bavistin (0.1%) and antibiotics viz., ampicillin, streptomycin and tetracycline at 150, 200 and 250 mg/L. The growth medium used was Murashige and Skoog (MS) supplemented with 3% sucrose and 0.5 mg/L benzylaminopurine (BAP). Benomyl (100 mg/L) and streptomycin (250 mg/L), when added to MS medium after surface sterilization, resulted in 93.33 and 100% bacterial and fungal-free explants, respectively.

Keywords: benomyl, clean culture, contamination, phytotoxicity, Rangpur lime, tissue culture

INTRODUCTION

Citrus is one of the most ancient genera of the globe, dating back to 800 B.C and is cultivated in over 100 countries in tropical and subtropical climatic regions of the world. In India, citrus is grown in 7.12.000 ha with a production of 59.96.001 tons (Center for Monitoring, Indian Economy 2007). Among the different cultivated citrus species, Andhra Pradesh occupies number one position in area and production with respect to acid lime and sweet orange in India. It is reported by several groups that the rootstock employed for a particular variety has a profound effect on the longevity, growth, yield and quality of fruits. In Andhra Pradesh, the rootstock Rangpur lime is commercially used for production of sweet orange cv. 'Sathgudi' as its overall performance is reportedly better than other rootstocks (Kusuma Grace *et al*. 2005).

At present the Rangpur lime rootstock is propagated by seed which results in wide variability in the performance of scion varieties on account of sexual and asexual (polyembryonic) seedlings (Batchlor and Webber 1948; Randhawa and Srivastava 1986; Vijayakumari 2001; Davies and Albrigo 2003). This problem can be overcome by propagating Rangpur lime rootstock vegetatively. However, the surface of an explant may carry a wide range of microbial contaminants. To avoid infection, the tissue must be thoroughly sterilized before inoculating it onto nutrient medium. To disinfect plant tissues, various sterilizing agents such as hypochlorite solutions (sodium or calcium), mercuric chloride, silver nitrate, ethanol and antibiotics have been reported to be effective in different plants (Bhojwani and Razdan 2005). It is important to realize that a surface sterilant is also toxic to plant tissue if used at higher concentrations. Therefore, the concentration of the sterilizing agent and duration of treatment should be standardized to minimize tissue death. The inability to control contamination levels is the primary reason for the failure of commercial laboratories. Benomyl has been used in plant tissue culture with little toxicity (Niedz and Bausher 2002). In this context many workers in the tissue culture field reported that collecting explant material from field-grown plants either in citrus or other woody plants is almost always accompanied with a high level of bacterial and fungal contamination which resulted in extreme difficulty in producing clean culture explants (Niedz and Bausher 2002; Santana *et al.* 2003; Chandra *et al*. 2004; Pence 2005; Jholgiker *et al.* 2006; Rastgoo 2008; Papafotiou and Martini 2009). Hence the present study was conducted to evaluate the efficacy of different antibiotics and fungicides to overcome microbial contamination of explants of field-grown Rangpur lime rootstock plants.

MATERIALS AND METHODS

Collection of explants

Four-year-old field Rangpur lime trees (*Citrus limonia*) were used as the source of nodal segments and shoot tip explants for experiments. Forty-days-old new shoots measuring 20-30 cm in length with 5-7 nodes were collected in polythene bags and brought to the laboratory for further treatments.

Sterilization of explants

In all the experiments, the explants were washed under running tap water and dipped in 0.1% Tween 20 (Rolex-Laboratory-Reagent, Mumbai, India). Shoot tip and nodal segment explants measuring 1-1.5 cm in length were excised under laminar air flow (LAF) and inoculated onto culture medium after the explants were treated with surface sterilants and then washed three times with sterile distilled water.

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Preparation of culture medium

The nutrients consisted of Murashige and Skoog (1962) salts and the medium was solidified by 7% agar agar (Qualigens, bacteriological grade, Mumbai, India) supplemented with 3% sucrose and 0.5 mg/L 6-benzylaminopurine (BAP) (Molychem, Mumbai, India). The pH of the medium was adjusted to 5.8 before gelling with agar. The MS medium was then dispensed in 250-ml culture bottles and autoclaved for 20 min at 121°C. All cultures were incubated at 25 ± 2°C under a 16-hr photoperiod under cool white fluorescent light (37.5 μmol m^{-2} s^{-1}).

Statistical analysis

All three experiments were conducted in a factorial completely randomized design (FCRD) with three replications each with 10 explants per replication. Data obtained on various parameters were subjected to statistical analysis accordance to Gomez and Gomez (1983) and Sastry (2007).

Experiment to determine the effect of streptomycin and bavistin on surface sterilization

The experiment consisted of 12 treatment combinations i.e. two explants (nodal segment and shoot tip), two bavistin (BASF, Carbendazime 50% WP, Mumbai, India) concentrations (0 and 0.1%) and three streptomycin sulphate (Himedia, Mumbai, India) concentrations (0.1, 0.5 and 1.0%). The explants were washed under running tap water for 1 hr and dipped in 0.1% Tween 20 for 1 hr. Further, they were washed three times for 2-3 min and then immersed in a 0 or 0.1% solution of bavistin alone or in combination with streptomycin sulphate at 0.1, 0.5 and 1.0% for 15 min. The explants after treatment were again washed three times for 1-2 min with sterile distilled water and inoculated onto MS medium and sealed by Parafilm® (Chicago, IL, USA).

Experiment to determine the effect of dipping explants in antibiotics on surface sterilization

The experiment consisted of 18 treatment combinations i.e. two explants (nodal segments and shoot tips), three antibiotics viz., ampicillin (Amp) (Cipla, Mumbai, India), streptomycin (Strep) and tetracycline (Tet) (Nicholas Piramal India Ltd., Gujrat, India) and three antibiotic concentrations (150, 200 and 250 mg/L). The explants were washed under running tap water for 15 min and dipped in 0.1% Tween 20 for 20 min. The nodal segments and shoot tips were further washed with distilled water three times and then immersed in 70% ethanol (Changshu Yangyuan Chemical, analytical reagent, China) for 60 and 30 sec, respectively and again washed with distilled water three times for 1-2 min. After washing, the explants were dipped in Amp, Strep and Tet solutions at 150, 200 and 250 mg/L for 2 hrs and again washed with distilled water three times for 1-2 min. Under LAF, nodal segments and shoot tips were again submerged into 0.1% $HgCl_2$ (Qualigens) for 10 and 5 min, respectively and then washed with sterile distilled water three times for 1-2 min. Finally the explants were inoculated onto MS medium containing 0.01% benomyl (Coromandel Fertilizers Ltd., 50% W.P, Tamilnadu, India). In this experiment, the Rangpur lime plants grown in the field were sprayed with 1 g/L bavistin mixed with 200 mg/L K-cyclin (Insecticide (India) Ltd., Jammu & Kashmir, India) (an antibiotic to control bacterial contamination) four days before collecting the explants.

Experiment to assess the effect of adding antibiotics to culture medium on surface sterilization

The experiment consisted of 18 treatment combinations i.e. two explants (nodal segment and shoot tip), three antibiotics (Amp, Strep and Tet) at three concentrations (150, 200 and 250 mg/L). The procedure as mentioned in experiment No. 2 was followed. The nodal segment and shoot tip explants were inoculated onto MS medium containing Amp, Strep and Tet mixed with benomyl.

Evaluation of cultures

1. Contamination

The number of explants showing bacterial or fungal contamination was counted and expressed as a percentage for the first experiment whereas in experiments No. 2 and 3, bacterial and fungal contamination was separately determined. The data on contamination were recorded 7-14 days after culturing the explants.

2. Phytotoxicity

The number of explants showing complete yellowing or browning (drying) was counted and expressed as a percentage.

3. Clean culture

The number of explants free from contamination were counted and expressed as a percentage.

RESULTS AND DISCUSSION

The technique and protocols adopted in this study include the use of antibiotics and fungicides for field-grown Rangpur lime plants followed by disinfection of explants with different surface sterilants in the laboratory and also by adding different antibiotics and fungicides to the culture medium. An active and efficient protocol for the establishment of contamination-free explants (nodal segments and shoot tips) was standardized. At first, Rangpur lime plants were sprayed with 1 g/l bavistin mixed with 200 mg/l K-cyclin for 2-3 times four days before collecting the explants. The explants were treated with disinfectant chemicals in the laboratory viz., ethanol and $HgCl_2$ and finally following the addition of antibiotics and fungicides to the culture medium). Our protocol contrasts to the methods reported by earlier studies, which use only either method, but not both. Rastgoo (2008) also used Certimide, $HgCl_2$ and NaOCl separately to overcome contamination in nodal segments of field-grown pummelo (*Citrus grandis* L. Osbeck) explants. Similarly, Symal *et al.* (2007) used ethanol, NaOCl + $HgCl_2$ to disinfect the shoot tip explants of Kagzi lime. Niedz and Bausher (2002) attempted ethanol + H_2SO_4 + $NaHCO_3$ + NaOCl (standard procedure) along with sodium dichloroisocyanurate (NaDCC) and Plant Preservative Mixture (Isothiazolonse) to overcome contamination in field-grown explants of sweet orange (*Citrus sinensis* cv. 'Valencia'). The success rate of eliminating contamination varied from 0 to 58% (Rastgoo 2008), 75% (Symal *et al.* 2007) and 90% (Niedz and Bausher 2002). As contamination was found to be very serious in field-grown explants of Rangpur lime, different methods with different chemicals were attempted in our study to achieve success.

Effect of bavistin and streptomycin sulphate on surface sterilization

No significant differences in contamination were observed between explants, bavistin concentrations and among streptomycin sulphate concentrations except for phytotoxicity between explants and bavistin concentrations (**Table 1**). Higher phytotoxicity was noticed with shoot tip explants (15.8%) than nodal segment explants (0%). Further, bavistin at 0.1% resulted in higher phytotoxicity (10%) than the control (5.8%). Furthermore, contamination was maximum (100%) in all treatments (**Figs. 1A, 1B**). This could be attributed to higher surface contamination of field-grown explants.

Rastgoo (2008) also reported 100% contamination when nodal segment explants of field-grown pummelo were collected and treated with 0.1 to 0.2% $HgCl_2$ for 3-10 min. Even with an increase in concentration of $HgCl_2$ from 0.3 to 0.4% for 3-6 min the contamination percentage was still reported to be 100%. Rastgoo further reported that using NaOCl from 1 to 4% with dipping between 15 and 30 min

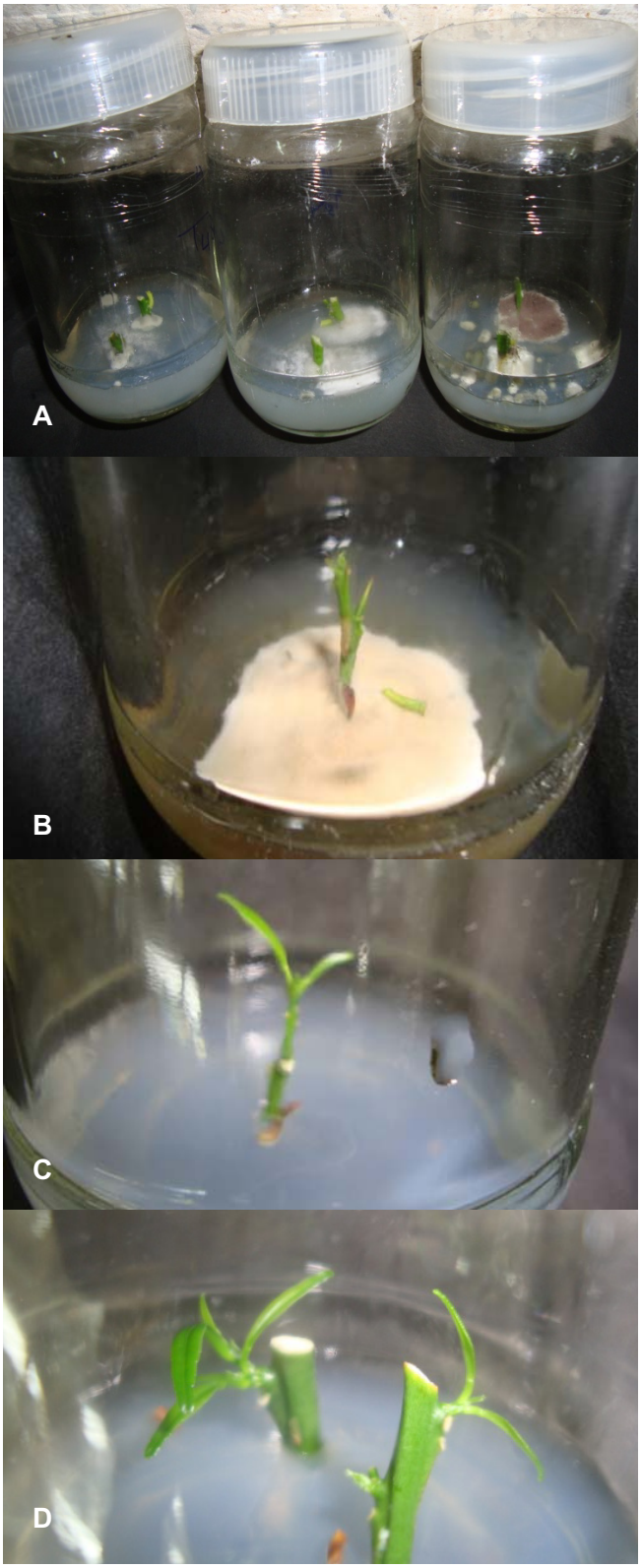

Fig. 1 (**A**) Contaminated nodal segment explants (0.1% bavistin + 0.5% streptomycin); (**B**) contaminated shoot tip explants (0.1% bavistin + 1.0% streptomycin); (**C**) contamination-free culture (shoot tip explant) when 100 mg/L benomyl + 250 mg/L streptomycin were added to culture medium; (**D**) contamination-free culture (nodal segment explants) when 100 mg/L benomyl + 250 mg/L streptomycin were added to culture medium after 14 days of culture.

could also not control explant contamination (100-83.33%).

Chandra *et al.* (2004) also reported high contamination when mango (*Mangifera indica* L.) green bud explants were collected from the field even when treated with 0.05% Amp + 0.01% bavistin for 2 hrs and 0.1% $HgCl_2$ for 4 min. Jholgiker *et al.* (2006) reported 100% fungal contamination with

Table 1 Percentage of contamination, phytotoxicity and clean culture when explants were dipped in bavistin and antibiotics.

Treatment	Contamination[y] (Mean)* (%)	Phytotoxicity[y] (Mean ± SD)* (%)	Clean culture[y] (Mean)* (%)
Explants (E)			
Nodal segment	100.00 a	0.00 ± 0 b	0.00 a
Shoot tip	100.00 a	15.83 ± 0.02 a	0.00 a
Bavistin concentrations (BC) (%)			
0.0	100.00 a	5.80 ± 0.08 b	0.00 a
0.1	100.00 a	10.00 ± 0.81 a	0.00 a
Streptomycin concentrations (SC) (%)			
0.1	100.00 a	3.33 ± 0.01 a	0.00 a
0.5	100.00 a	8.33 ± 0.02 a	0.00 a
1.0	100.00 a	8.33 ± 0.2 a	0.00 a

*Similar letters indicate means which are not significantly different (*LSD, P = 0.05*), comparisons are made in each column within E, BC and SC, values represent mean ± Standard Deviation (SD).
[y]Data were recorded during 7-14 days after inoculating explants.

field-grown mango current season and mature shoots explants in MS medium 10 days after transferring to fresh medium when a conventional procedure of chemical disinfectants was employed, namely, dipping in 0.2% bavistin and again dipping in 0.01% Strep sulphate solution with mechanical stirring for 20 min.

Effect of dipping explants in antibiotics on surface sterilization

Different antibiotics with different concentrations significantly affected bacterial contamination and clean culture (**Table 2**). Higher bacterial contamination was observed in nodal segments (66.7%) than in shoot tips (43%). Higher clean culture was observed with shoot tips (40%) than with nodal segments (23%). Among the antibiotics, no significant difference in contamination was observed between Strep and Tet but both antibiotics showed a higher percentage contamination than Amp. A higher clean culture was observed with Amp (41.1%) than with Sterp (26.7%) or Tet (26.7%).

Symal *et al.* (2007) reported 100% contamination when shoot tip explants of Kagzi lime (*Citrus aurantifolia* Swingle) were immersed in 1% NaOCl for 3-5 min and 61.6% when dipped in a solution containing NaOCl + $HgCl_2$ for 5 min. They also reported 80. 8% aseptic cultures and 32.7% survival of cultures treated with a solution of NaOCl at 1% for 20 min and $HgCl_2$ at 0.1% for 10 min.

Effect of adding antibiotics to culture medium on surface sterilization

Antibiotics at different concentrations significantly affected bacterial contamination and clean culture (**Table 3**). Bacterial contamination decreased significantly with an increase in the concentration of antibiotics from 150 mg/L (27.8%) to 250 mg/L (6.7%). Among explants, shoot tips showed higher clean culture (85.9%) than nodal segments (77%). Strep recorded higher clean culture (86.7%) than Amp (78.8%) and Tet (78.9%). The percentage clean culture increased significantly as the concentration of antibiotics increased from 150 mg/L (70%) to 250 mg/L (91.1%). The interaction between explants, antibiotics and antibiotic concentration revealed that 100% clean culture was possible with shoot tips followed by 93.3% in nodal segment explants treated with streptomycin at 250 mg/L (**Table 4, Figs. 1C, 1D**). The findings reported by several groups on the tissue culture of explants from woody plants revealed that surface sterilization was not sufficient to produce clean culture explants, but when an antibiotic (Strep) and fungicide (benomyl) were added to the culture media best results in controlling bacterial and fungal contamination were possible. Niedz and Bausher (2002) achieved 53% clean culture of sweet orange explants by using a standard procedure.

Table 2 Percentage of bacterial and fungal contamination, phytotoxicity and clean culture when explants were dipped in antibiotics.

Treatment	Contamination (%)[y] (Mean ± SD)*		Phytotoxicity[y] (%) (Mean ± SD)*	Clean culture[y] (%) (Mean ± SD)*
	Bacteria	Fungi		
Explants (E)				
Nodal segment	66.66 ± 2.65 a	10.37 ± 0.02 a	0.00 ± 0 a	22.96 ± 0.06 b
Shoot tip	42.96 ± 0.19 b	17.03 ± 0.03 a	1.48 ± 0.02 a	40.00 ± 2 a
Antibiotics (A) (%)				
Ampicillin	41.11 ± 0.02 b	17.77 ± 0.02 a	1.11 ± 0.02 a	41.11 ± 0.45 a
Streptomycin	62.22 ± 0.02 a	11.11 ± 0.06 a	1.11 ± 0.04 a	26.66 ± 0.04 b
Tetracycline	61.11 ± 0.04 a	12.22 ± 0.02 a	0.00 ± 0 a	26.66 ± 0.01 b
Antibiotic concentrations (AC) (mg/L)				
150	58.88 ± 0.02 a	0.00 ± 0 a	0.00 ± 0 a	26.66 ± 0.03 c
200	56.66 ± 0.03 a	0.00 ± 0 a	0.00 ± 0 a	33.33 ± 0.33 b
250	48.88 ± 0.02 a	16.66 ± 0.03 a	2.22 ± 0.02 a	34.44 ± 0.02 a

*Similar letters indicate means which are not significantly different (*LSD, P = 0.05*), comparisons are made in each column within E, A and AC, values represent mean ± Standard Deviation (SD).
[y]Data were recorded at 14 day after inoculating explants
100 mg/L benomyl was added to MS media

Table 3 Percentage of bacterial and fungal contamination, phytotoxicity and clean culture when explants were inoculated onto MS media containing antibiotics.

Treatment	Contamination (%)[y] (Mean ± SD)*		Phytotoxicity[y] (%) (Mean ± SD)*	Clean culture[y] (%) (Mean ± SD)*
	Bacteria	Fungi		
Explants (E)				
Nodal segment	19.25 ± 0.02 a	3.70 ± 0.2 a	0.00 ± 0 b	77.03 ± 0.03 b
Shoot tip	11.85 ± 0.05 a	2.22 ± 0.02 a	4.44 ± 0.04 a	85.88 ± 0.02 a
Antibiotics (A) (%)				
Ampicillin	15.55 ± 0.05 a	5.55 ± 0.05 a	1.11 ± 0.01 a	78.83 ± 0.03 b
Streptomycin	12.22 ± 1 a	1.11 ± 0.02 a	3.33 ± 0.03 a	86.66 ± 0.03 a
Tetracycline	18.88 ± 2 a	2.22 ± 0.04 a	2.22 ± 0.04 a	78.88 ± 0.022 b
Antibiotic concentrations (AC) (mg/L)				
150	27.77 ± 1 a	2.22 ± 0.02 a	0.00 ± 0 a	70.00 ± 1 c
200	12.22 ± 1 b	4.44 ± 0.04 a	2.22 ± 0.01 a	83.27 ± 0.02 b
250	6.66 ± 0.03 b	2.22 ± 0.07	4.44 ± 0.01 a	91.11 ± 0.01 a

*Similar letters indicate means which are not significantly different (*LSD, P = 0.05*), comparisons are made in each column within E, A and AC, values represent mean ± Standard Deviation (SD).
[y]Data were recorded at 14 days after inoculating explants
100 mg/L benomyl was added to MS media

Table 4[y] Percentage of clean culture when explants were inoculated onto MS media containing antibiotics.

Explants	Explants x antibiotics (Mean ± SD)*			Explants x concentrations		
	Antibiotics			Concentration (mg/L)		
	Ampicillin	Streptomycin	Tetracycline	150	200	250
Nodal segment	75.55 ± 0.04 d	86.66 ± 0.03 b	68.88 ± 1 e	64.44 ± 1 e	80.00 ± 2 c	86.66 ± 3 b
Shoot tip	82.11 ± 1 c	86.66 ± 0.04 b	88.88 ± 0.04 a	75.55 ± 0.05 d	86.55 ± 2 b	95.55 ± 0.05 a

Antibiotics	Antibiotics x concentrations		
	Concentration (mg/L)		
	150	200	250
Ampicillin	66.66 ± 0.02 g	79.83 0.03 e	90.00 ± 1 b
Streptomycin	76.66 ± 0.01 f	86.66 ± 0.03 c	96.66 ± 0.01 a
Tetracycline	66.66 ± 0.01 g	83.33 ± 0.03 d	86.660.04 c

Explants	Antibiotic	Explants x antibiotics x concentrations		
		Concentration (mg/L)		
		150	200	250
Nodal segment	Ampicillin	66.66 ± 002 f	73.33 ± 0.03 e	86.66 ± 0.02 c
	Streptomycin	80.00 ± 2 d	86.66 ± 1 c	93.33 ± 0.03 b
	Tetracycline	46.66 ± 0.04 g	80.00 ± 1 d	80.00 ± 3 d
Shoot tip	Ampicillin	66.66 ± 0.01 f	86.33 ± 0.01 c	93.33 ± 0.02 b
	Streptomycin	73.33 ± 0.03 e	86.66 ± 3 c	100.00 ± 0 a
	Tetracycline	86.66 ± 0.06 c	86.66 ± 0.01 c	93.33 ± 0.04 b

*Similar letters indicate means which are not significantly different (*LSD, P = 0.05*), comparisons are made in each interaction, values represent mean ± Standard Deviation (SD).
[y]Data were recorded at 14 days after inoculating explants.
100 mg/L benomyl was added to MS media.

Rastgoo *et al.* (2009) reported 100% clean culture when explants of pummelo were treated with 0.2% cetrimide solution (a broad-spectrum bactericide) containing 0.1% Tween 20 for 2 hrs, dipped in 95% ethanol for 1 min and then treated with 1% silver nitrate for 10 min.

Haldeman *et al.* (1987) reported that MS medium supplied with 1 g/L benomyl resulted in 100% clean culture of *Camellia sinensis* shoot tip explants after using a chemical disinfectant (70% ethanol + 20% Clorox). Sharma *et al.* (1998) reported 12.3% bacterial contamination in papaya explants in MS media supplemented with 100 mg/L Strep. In the present investigation, antibiotics such as Amp, Strep and Tet at 150, 200 and 250 mg/L, when added to MS medium, reduced bacterial contamination significantly.

Antibiotics, when used with leaf discs of *Lonicera maakii*, effectively reduced bacterial contamination with low percentage of toxicity (Pence 2005). Cefotaxime, a broad spectrum antibiotic has been used to eliminate bacterial

contamination from plant tissues *in vitro* when collected from the field. The fungicide benomyl, which has been widely used in agriculture, effectively lowered the percentage of fungal contamination of leaf cultures. In aqueous solution, benomyl breaks into two compounds, methyle-2-benzimidazolecarbamate and butyl isocyanate, which are effective against fungi (Pence 2005).

The mechanism of action of benomyl is by strongly inhibiting DNA synthesis or its related process of cell division of fungi (Vyas 1993; Pence 2005).

CONCLUDING REMARKS

This study revealed that dipping of explants (nodal segments and shoot tips) in solution of Amp, Strep and Tet at 100, 150, and 250 mg/l for 2 hours ineffectively overcame contamination at a commercial level, i.e. ≤40%. However, adding benomyl (100 mg/L) to culture medium in combination with Strep at 250 mg/L produced 100% contamination-free shoot tips; for nodal segments, the value was 93.3%.

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