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Identification of Best Surface Sterilization Treatment and Control of Endophytic Bacterial Contamination in *Annona squamosa* L.

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Authors' contributions

This work was carried out in collaboration among all authors. Author DM carried out research work and drafted manuscript. Authors HD and GP provided guidance during research work. Author SK contributed in manuscript drafting. All authors read and approved the final manuscript.

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ABSTRACT

Surface sterilization is most important step in plant tissue culture protocol. In the present investigation, an attempt was made to eliminate microbial and fungal contaminants from the surface and interior of plant material, thus obtaining axenic culture with highest survival rate. Sequential surface sterilizations of hypocotyl, leaf, shoot tip and mature node were carried out to investigate its effectiveness in controlling surface contamination with satisfactory survival of explants. Combination of different surfactant were used for surface sterilization treatments. The least contamination was obtained when hypocotyl explants were treated with 200 ppm cefotaxime and 500 ppm carbendazim along with 0.1% $HgCl_2$ with best survival percentage. Treatments consisting of alcohol treatment, carbendazim (2000 ppm) followed by 1000 ppm cefotaxime, 500 ppm kanamycin, 2% sodium hypochloride and 0.1% $HgCl_2$ sequentially resulted in complete elimination of surface contaminants from shoot tip, soft node and hard node obtained from field grown mature

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tree. Optimal elimination of bioburden from young leaf (77.38%) were obtained using 1000 ppm carbendazim, 500 ppm cefotaxime, 500 ppm kanamycin and 0.1% $HgCl_2$. Gentamicin used in the medium was able to control the endophytic bacterial bioburden completely in the first cycle of 15 days itself at higher concentration of 96 mol/l to remove endophytic bacterial contamination with out effecting plant growth.

Keywords: Annona squamosa; antibiotic assay; auxiliary nodes; endophytic bacteria; hypocotyls; surface sterilization.

1. INTRODUCTION

Annona squamosa L. is known as sugar apple and also popularly known as Sitaphal. It's diploid chromosome number is 2n=2x=14. It is a favourite table fruit of common man in the Indian subcontinent. It belongs to the family *Annonaceae*. Sugar apple is cultivated throughout the tropical and subtropical regions of the world for its delicious and nutritive fruits. High level of segregation and genetic recombination ratio along with difficulty in propagation by seed and lack of improved varieties have made its commercial cultivation limited [1]. Propagation of sugar apple through grafting and budding have some limitations *viz*., differences in growth rates of rootstocks, susceptibility to water stress including water logging condition and root rot infection [2]. While reduced rooting capacity of *Annona squamosa* makes conventional vegetative propagation difficult [3]. *In vitro* clonal propagation through tissue culture is referred to as micropropagation. Micropropagation on commercial base of improved genotypes with high yielding and free of bacterial and viral diseases is needed to be carried out for the production of uniform planting materials with high yielding. Micropropagation has been used in other species of *Annona viz*. *Annona Cherimola* [4,5] and *Annona muricuta* [6]. Surface sterilization is the first most important step in micropropagation. Mercuric chloride ($HgCl_2$) and its soluble salts are efficient sterilants used in surface sterilization. Nodal portion of *Annona muricata* were treated with fungicide bavistin (0.5-1.0% w/v) and antibiotic streptomycin (0.5-0.1% w/v) followed by $HgCl_2$ (0.1% w/v) [7]. 70% ethanol and 1% sodium hypochloride were used to remove surface contamination of nodal stem segments of two years-old juvenile plants of *Annona glabra* [8]. The nodal explants of *Annona emarginata* were immersed in 70% ethyl alcohol (v/v) for 30 seconds, followed by sodium hypochlorite treatment (1%) for 15 minutes [9].

2. MATERIALS AND METHODS

This research work was done in Center for Advanced Research in Biotechnology, Anand Agricultural University, Anand (India) during the month of May in summer season.

2.1 Mother Plant Selection

The five year old trees of sugar apple genotype (Anand selection-1) was selected on the basis of high reproductive vigour (yield), regular bearing, round to heart shape fruits which was procured from the Horticulture farm, Anand Agricultural University, Anand, Gujarat, India. Fresh seeds of sugar apple (Anand selection-1) were taken from ripe fruits and grown in green house for hypocotyl explants.

2.2 Explant Isolation

Three to four cm long shoot tip (one, two or multiple nodes) and nodal explants (mature and immature) were obtained from axillary branch of mature field grown tree for axillary shoot proliferation. The part of leaf explants including leaf base with petiole, leaf lamina with midrib, leaf lamina without midrib and leaf apex with midrib were taken from second leaf of axillary branch for indirect organogenesis. Hypocotyl explants were obtained from green house grown 25 days old seedlings. Hypocotyl were decapitated below cotyledonary leaves and above the root and divided into one centimeter segments under aseptic condition. Hypocotyl were designated as H_1, H_2 and H_3 segments (H_1 being nearer to cotyledons while H_3 being nearer to root and H_2 being in between both the segments) for direct organogenesis.

2.3 Explant Surface Sterilization and Inoculation

Isolated explants were cleaned under running tap water for about 20 to 25 min. Explants were then thoroughly washed with 0.1% Tween-20 solution

(Loba chemie) followed by 2-3 wash of distilled water uniformly. Various treatments, differing in time duration of the disinfectants (70% Alcohol, Carbendazim, Cefotaxime, Kanamycin, Sodium hypochloride and 0.1% $HgCl_2$) used under laminar air flow, were experimented for establishment of the axenic cultures for axillary shoot proliferation using nodal and shoot tip (Table 1) for indirect organogenesis using leaf explants (Table 2) and for direct organogenesis using hypocotyl explants (Table 3). Later the explants were given a fresh cut under laminar air flow and inoculated on Murashige and Skoog (MS) media enriched with 2% sucrose (Qualigens, USA). Nodal and hypocotyl explants were maintained at 25 ± 2°C and subjected to a photoperiod of 16 h, provided by cool white fluorescent tubes (36 W; Phillips, India) having a light intensity of 36.8 µmol m^{-2} s^{-1} , followed by the dark period of 8 h. Leaf explants were kept under dark condition for indirect organogenesis.

2.4 Observations

Percentage contamination was recorded after seven days of inoculation as the total number of explants contaminated out of total number of explants inoculated and expressed in terms of percentage. For the percentage of survived material, the number of dried explants were

Table 1. Effect of different sterilizing agents in reducing contaminants (Age - shoot tip and node)

Treatment	Sterilization agents	Conc.	Shoot -tip	Soft node	Hard node
		(mgl^{-1} or %)	Time (min)	Time (min)	Time (min)
T_1	Carbendazim	500	6	8	10
	Cefotaxime	250	8	10	12
	Kanamycin	250	8	10	12
	$HgCl_2$	0.10%	2	3	5
T_2	Carbendazim	1000	10	12	15
	Cefotaxime	500	8	10	12
	Kanamycin	500	8	10	12
	$HgCl_2$	0.10%	5	8	10
T_3	Carbendazim	1000	12	15	20
	Cefotaxime	500	10	12	15
	Kanamycin	500	8	10	12
	$HgCl_2$	0.10%	8	10	12
T_4	Allite	1000	12	15	20
	Cefotaxime	500	10	12	15
	Kanamycin	500	8	10	12
	$HgCl_2$	0.10%	8	10	12
T_5	Carbendazim	2000	15	18	20
	Cefotaxime	1000	10	12	15
	Kanamycin	500	8	10	12
	$HgCl_2$	0.10%	8	10	12
T_6	Carbendazim	2000	8	10	12
	Cefotaxime	1000	10	12	15
	Kanamycin	500	8	10	12
	$HgCl_2$	0.10%	12	15	18
T_7	Alcohol	80.00%	10 sec	10 sec	10 sec
	Carbendazim	2000	8	10	12
	Cefotaxime	1000	10	12	15
	Kanamycin	500	8	10	12
	Sodium hypochloride	2%	10	15	20
T_8	Alcohol	70%	10 sec	10 sec	10 sec
	Carbendazim	2000	15	18	20
	Cefotaxime	1000	10	12	15
	Kanamycin	500	8	10	12
	Sodium hypochloride	2%	5	8	10
	$HgCl_2$	0.10%	6	7	8

Table 2. Efficiency of different sterilizing agents in reducing contaminants (Age - young and mature leaf)

Treatment	Sterilization agents	Conc.	Young leaf	Mature leaf
		(mgl^{-1} or %)	Time (min)	Time (min)
T_1	Carbendazim	500	8	10
	Cefotaxime	500	8	10
	Kanamycin	500	8	10
	$HgCl_2$	0.10%	2	3
T_2	Carbendazim	1000	12	15
	Cefotaxime	500	8	10
	Kanamycin	500	8	10
	$HgCl_2$	0.10%	2	3

Table 3. Surface sterilization treatments to reduce contamination from age – hypocotyls

Treatment	Sterilization agents	Conc.	Hypocotyl segments
		(mgl^{-1} or %)	Time (min)
T_1	$HgCl_2$	0.10%	3
T_2	$HgCl_2$	0.10%	4
T_3	Bavistin	500	5
	Cefotaxime	200	5
	$HgCl_2$	0.10%	5

recorded and remaining explants were expressed in percentage against the total number of explants inoculated. The various parameters recorded were analyzed using CRD (Completely Randomized Design) statistical design [6].

2.5 Disk Diffusion Assay for Antibiotic Activity

The bacterial colonies were obtained from culture media contaminated with endophytic bacteria. Bacterial colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of a nutrient broth (Himedia). The broth culture is incubated at 35°C for 24 hours. A broth culture growth was mixed with a single molten agar layer [10], added to a thin agar layer which was spread over a solid base agar layer. Twelve antibiotic disks with concentration of 30 microgram diffused on agar plates and incubated for 24 hours. The zone of inhibitions were measured using measuring scale.

2.6 Anatomical Studies

Percentage contamination was recorded after seven days of inoculation as the total number of explants contaminated out of total number of explants inoculated and expressed in terms of percentage. For the percentage of survived material, the number of dried explants were recorded and remaining explants were expressed in percentage against the total number of explants inoculated.

2.7 Identification of Effective Antibiotic in Medium

Antibiotic identified as most effective antibiotic in disc diffusion antibiotic sensitivity test or the agar diffusion test [11] was incorporated in culture medium to control the growth of endophytic bacteria. Antibiotic was added to medium at specific concentration with varying time durations in days. After particular time duration, explants were transferred to without antibiotic medium.

3. RESULTS

3.1 Surface Sterilization of Nodal and Shoot Tip Explants Obtained from Mature Tree

Fungal growth from nodal explants and endophytic bacterial contamination from newly sprouted seeds are shown in Fig. 1. Pretreatment of explants using tween 20 with thoroughly washing with distilled water resulted in higher survival of explants because tweens are a series of non-ionic surfactants and tween 20 makes the surface of plant wet and repel the air therefore making the treatment effective. Tween 20 is also non-toxic and inert as it is an ester.

Surface sterilization of different explants were obtained using various disinfectants. The experiment was based on eight different combinations of antibiotics, fungicides, alcohol and $HgCl_2$ and were used sequentially to eliminate surface contaminants completely. Inwana et al. [12] reported the shoots and petioles of *Annona muricata* seedlings sterilized under running tap water for two minutes and disinfected in 10% (w/v) $HgCl_2$ with Tween-20 for 10 min. Among various treatments, treatment consisting of pre-incubation with 70% alcohol for 10 sec followed by carbendazim (2000 ppm), 1000 ppm cefotaxime, 500 ppm kanamycin, 2% sodium hypochloride and 0.1% $HgCl_2$ (Fig. 2) sequentially resulted in complete elimination of surface contaminants from shoot tip, soft node and hard node obtained from field grown mature tree but after 2-3 subcultures, all the shoots showed endophytic bacterial contamination which was latent in the explants, despite all the precautions taken during manual subculture. The sprouted shoots slowly turned yellow and brown, although some part of the tissue continued to form new shoot buds, within two weeks all the tissues turned necrotic. It become impossible to continue cultures any longer. This problem is very much similar to one that was the report by Thomas et al. [13] found that apparently clean stocks of banana harbored viable but non-culturable bacteria initially which was expressed after repeated sub culture and became culturable.

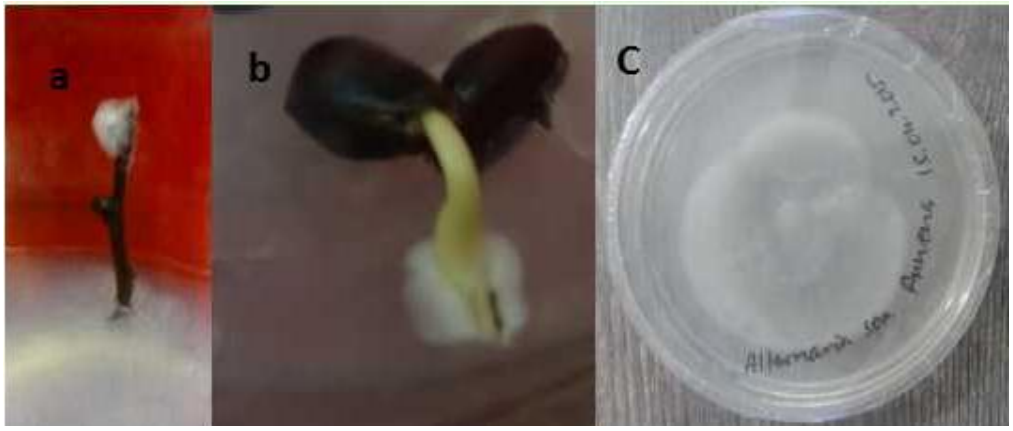

Fig. 1. Fungal contamination of mature node [a] endogenous bacterial contamination [b] and identification of fungal species *Alternaria* sp found prominant in fungal contamination while *in vitro* culturing media composition of *Annona squamosa* [c]

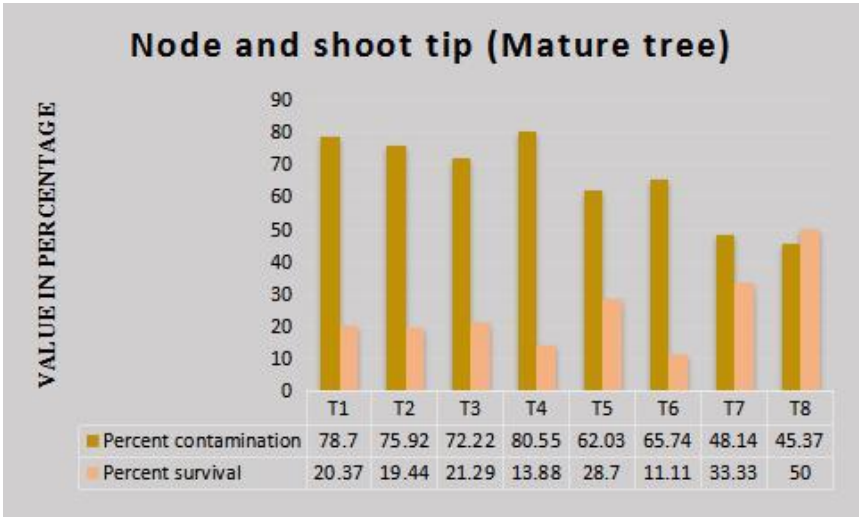

Fig. 2. Effect of sequential application of surface sterilization agents on age - shoot tip and nodal explants

3.2 Surface Sterilization of Leaf Explants

In the present study, optimal elimination of contaminants (77.38%) (Fig. 3) from leaf explants were obtained using sequential surface sterilization (1000 ppm carbendazim, 500 ppm Cefotaxime, 500 ppm Kanamycin and 0.1% $HgCl_2$). It may be due to very low bioload of microorganisms which might have been gradually decreased during different sequential steps employed for surface sterilization while moderate survivability of explants might have been due to longer sterilization period. Irrespective of various reasons, the treatment based on carbendazim were found to be less harsh to the explants and showed elimination of fungal contamination.

3.3 Surface Sterilization of Hypocotyl Segments

Mercuric chloride ($HgCl_2$) 0.1% with various time duration were tried to find out its effectiveness in controlling surface contamination and improved health of hypocotyl segments. The least contamination (13.88 %) was obtained when explants were treated with 200 ppm cefotaxime and 500 ppm carbendazim along with 0.1% $HgCl_2$ for 5 min with best survival percentage (83.33%) (Fig. 4). These results are contradictory to the reports of Nagori et al., [14] wherein 0.1% $HgCl_2$ treatment alone was given for 5 min for hypocotyl segments. In our case sequential surface sterilization along with $HgCl_2$ was found effective because plant genotype and environment may influence the surface sterilization protocol. $HgCl_2$ acts through the action on protein sulfhydryl groups and disruption of enzyme functions of the microorganisms. Dipre et al. [15] reported disinfectant treatments for hypocotyl, epicotyl, plumule and radicle of *Annona muricata* consisting of a 30 minutes incubation in a mixture of Agrimicin-100® (streptomycin 18.7%, oxytetracycline 2%) and Bravo500SC® (500 g/l chlorothalonil). After this, the explants were incubated for 20 minutes in a 0.7% mercuric chloride solution. *Annona squamosa* hypocotyl segments impregnate endophytic bacterial contamination which expressed itself after 2-3 subcultures in the medium. Initially, these bacteria did not hinder formation and development of new shoots, but later they affect the growth of newly developed shoots.

3.4 Sensitivity Test Using Antibiotics in the Bacteriological Medium to Control Endophytic Bacteria

A sensitivity test was performed using 12 antibiotic against endophytic bacterial contamination, among which gentamicin (Himedia) was found highly effective (Fig. 5) while tetracycline, rifampicin, kanamycin, cefotaxime, streptinomycin S^{100}, streptomycin S^{25} and chloramphenicol were found moderately

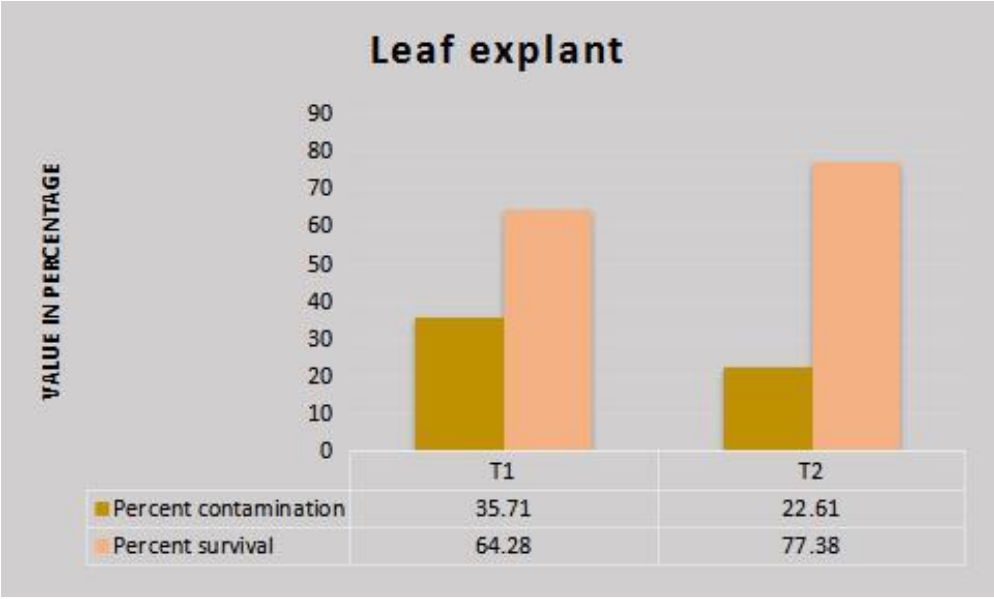

Fig. 3. Effect of sequential application of surface sterilization agents on leaf explants age

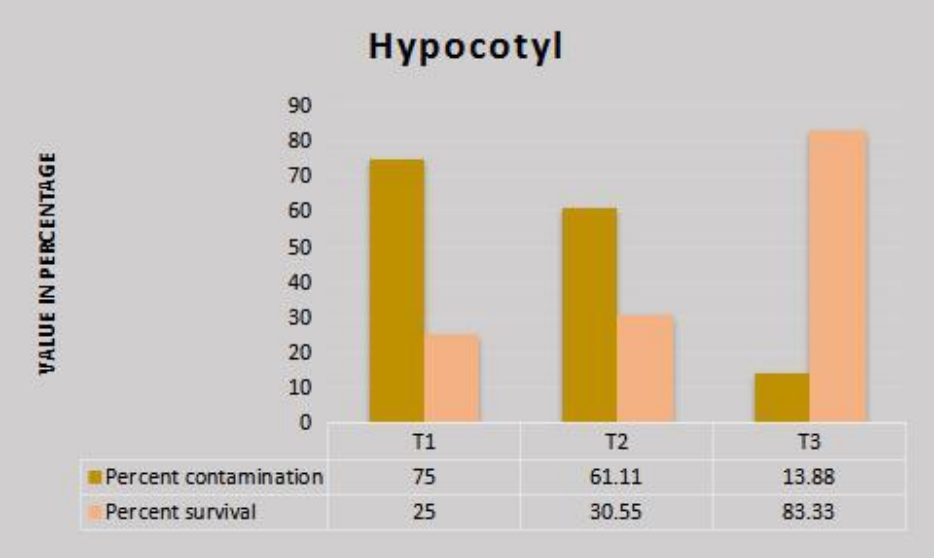

Fig. 4. Effect of sequential application of surface sterilization agents on hypocotyl explants age

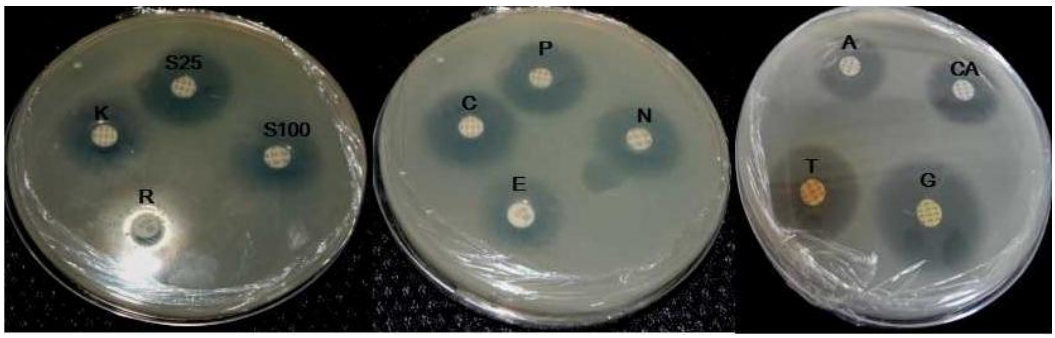

Fig. 5. Comparative effect of antibiotics on endophytic bacterial culture from sugar apple
[Tetracycline (T), Rifampicin (R), Kanamycin (K), Cefotaxime (C), Gentamicin (G), Streptinomycin (S100), Streptomycin (S25), Erythromycin (E), Nystatin (N), Ampicillin (A), Carbenicillin (CA), PolymyxinB (P)]

Table 4. Antibiogramme of different antibiotics showing sensitivity of endophytic bacterial contamination in sugar apple culture age

No.	Name of antibiotics (Himedia)	Sensitivity response	Diameter of the ring (cm)
1	Tetracycline (T)	Moderately sensitive (++)	2.5
2	Rifampicin (R)	Moderately sensitive (++)	2.5
3	Kanamycin (K)	Moderately sensitive (++)	2.3
4	Cefotaxime (C)	Moderately sensitive (++)	1.5
5	Gentamicin (G)	Highly sensitive (+++)	3
6	Streptinomycin (S100)	Moderately sensitive (++)	2.3
7	Streptomycin (S25)	Moderately sensitive (++)	2.5
8	Erythromycin (E)	Least sensitive (+)	1.1
9	Nystatin (N)	Least sensitive (+)	1.2
10	Ampicillin (A)	Least sensitive (+)	1
11	Carbenicillin (CA)	Least sesitive (+)	1
12	Polymyxin B (P)	Least sesitive (+)	1.5

Table 5. Effect of commonly used plant antibiotic on contaminated age cultures of sugar apple

No.	Antibiotic used in medium	Concentration (mol/l)	Removal of bacteria	Toxicity	Health status of the shoots
1	Gentamicin	48	++	-	Green and healthy
2		96	+++	-	Green, healthy & growing shoots
3		144	+++	+	Yellowish green shoots
4		192	+++	++	Shoot necrosis

(+++) Highly effective, (++) Moderately effective, (+) Least effective

Table 6. Effect of gentamicin at varying duration on contaminated age cultures of sugar apple

No.	Antibiotic used in medium	Conc. (mol/l)	Duration (days)	Removal of bacteria	Toxicity	Health status of the shoots
1	Gentamicin	96	10	++	-	Green and healthy
2			15	+++	-	Green, healthy & growing shoots
3			20	+++	+	Yellowish green shoots

(+++) Highly effective, (++) Moderately effective, (+) Least effective

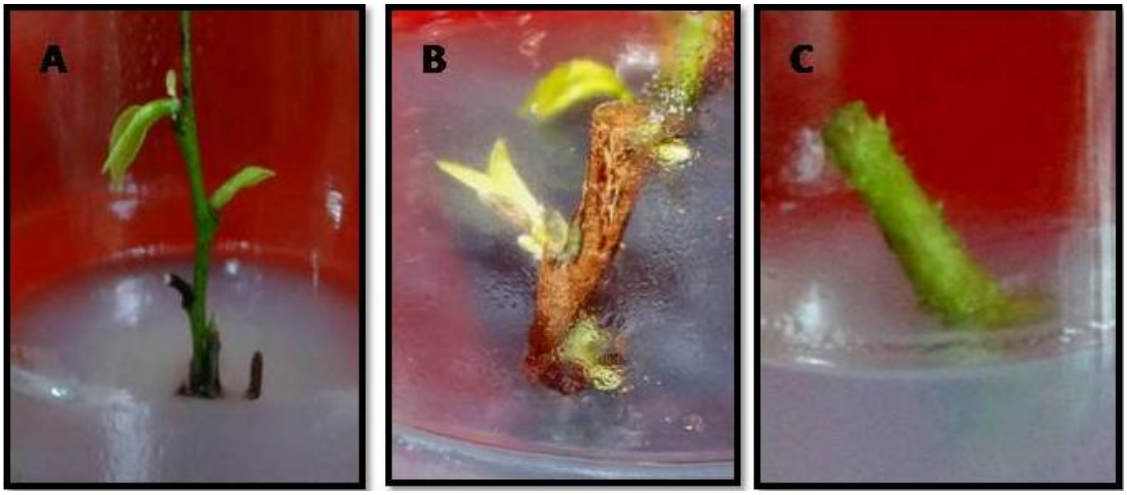

Fig. 6. Healthy sprouting from shoot tip [a], mature node [b] and hypocotyl [c] explants after complete removal of surface and endophytic bacterial and fungal contamination media composition

effective (Table 4). Gentamicin was used in the shooting media at different concentrations (Table. 5). It was found that antibiotic used in the medium was able to control the bacterial contamination completely in the first cycle of 15 days itself at higher concentration of 96 mol/l and within two cycles when used at lower concentration of 48 mol/l. However all the concentrations of antibiotics could control bacterial contamination but did not support plant growth. Only gentamicin, an aminoglycoside at 96 mol/l was effective and did not adversely affect sprouting of shoots for one cycle of 15 days (Fig. 6) (Table. 6). In second cycle, explants were transferred in medium without antibiotic because the use of higher concentration during subsequent cycles inhibited sprouting of shoots.

4. DISCUSSION

Surface sterilization is important step in *invitro* culturing of any tissue or cell. For commercial regeneration protocol from plant tissue, axenic culture is required therefore surface sterilization along with removal of endophytic bacterial contamination is necessary. There are only few reports on *in vitro* culturing of sugar apple because of major problem with endophytic

bacterial contamination. Many fungicides have been used, among which carbendazim used as systemic fungicide. It contains methyl-3-benzimidazol carbamate and acts on fungal cell replication [16]. There are several reports of kanamycin and cefotaxime as a selective agent and sterilant in tissue culture [17]. Kanamycin fall under the aminoglycosidase group of antibiotics with cause changes in metabolism *viz*., cell permeability, transport and inhibition of protein synthesis and misreading of the genetic code of bacteria [18] therefore kanamycin is less harmful to plant cells. Bacterial cell wall synthesis is inhibited by cefotaxime by binding to one or more of the penicillin binding proteins Which inhibit the final step of peptidoglycan synthesis in bacterial cell walls [19]. Gentamicin irreversibly bind to 30s subunit of the bacterial ribosome and inhibit protein synthesis [20]. Other antibiotics like ampicillin and antifungal agents like benomyl had also been used to control contamination in *Annonaceae* [21]. Sekhar et al., [22] isolated and identified shoot tip associated endophytic bacteria from banana cv. Grand naine which were retain after surface sterilization. In *Jatropha curcas* L. the problem of endophytic bacterial contamination had been identified and resolved, where aseptic cultures remained green and regenerative with the addition of growth hormones as well as antibiotics in the medium up to 45 days of incubation without any sub culture [23]. Rifampicin has been reported very effective at 100 mgl^{-1} concentration in medium while polymyxin B and tetracyclin have found less effective and toxic to the explants of *A. squamosa* [24]. The use of antibiotics to control the growth of contaminants in the medium was earlier reported by Pollock et al, [25] and fungicides by shields et al., [26]. They also reported that exposure of explants with 95% alcohol for 30 seconds was effective against contamination. Here we demonstrated the use of antimicrobial compounds for the successful surface sterilization along with removal of endophytic bacterial contamination with highest survival rate.

5. CONCLUSION

In the present research, sequential surface sterilization along with elimination of endophytic bacterial contamination were carried out without effecting plant growth. 200 ppm cefotaxime and 500 ppm carbendazim along with 0.1% $HgCl_2$ are the best treatment to obtain axenic culture of hypocotyl explants. Alcohol treatment, carbendazim at 2000 ppm concentration followed by 1000 ppm cefotaxime, 500 ppm kanamycin, 2% sodium hypochloride and 0.1% $HgCl_2$ sequentially removed maximum surface contaminants of shoot tip and nodal segments. For young leaves, treatment consisting of 1000 ppm carbendazim, 500 ppm cefotaxime, 500 ppm kanamycin and 0.1% $HgCl_2$ were identified best treatment for surface sterilization. To eliminate endophytic bacterial contamination from explants without effecting plant growth, gentamicin was found effective while added to medium in the first cycle of 15 days at higher concentration of 96 mol/l.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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