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Identification and Prevention of Microbial Contamination in Tissue Culture of *Catharanthus roseus* - An Important Medicinal Herb

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Abstract

The explants of two varities rosea and alba of *Catharanthus roseus* used for *in vitro* propagation and found to be more than 50% of the cultures became contaminated. The most common bacterial contaminants were *Bacillus licheniformis, Micrococcus, Panibacillus* and fungal contaminants were *Fusarium, Alternaria, Cladosporium* and *Aspergillus*. Combinations of different antibiotics (Penicillin, norfloxacin, tobramycin, gatifloxacin, ofloxacin) and fungicides (Bavastin, captan, fluconazole and trichoderma) were used to control the growth of the contaminants. Gatifloxacin and ofloxacin inhibited 100% growth of bacteria whereas, bavastin and captan appeared to be the most effective fungicides. Combination of gatifloxacin, ofloxacin with bavastin and captan inhibited the growth of contaminants at their minimum phytotoxic concentration (MIC). The observed minimum phytotoxic concentration (MPC) of ofloxacin, gatifloxacin, bavastin and captan was 15, 9, 6 and 5% at their respective MIC. More than 90% of the cultures responded for callus formation in the combination of gatifloxacin (4%) + bavastin (1%). While the combination of gatifloxacin and captan was highly toxic that reduces the growth of the culture.

Introduction

The demands for herbal plant products ever increasing in national and global drug markets because of the higher plants are rich sources of natural products that are being used as pharmaceutical products, agrochemicals, fragrance ingredients, food additives pesticides etc. (Philipson 1990).

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Two important varieties *viz.,* rosea and alba of *Catharanthus roseus* (Family : Apocynaceae) are commonly called Sadabahar, distributed in tropical and subtropical parts of the world. It is indigenous to Madagaskar, hence it is called as Madagascar periwinkle. *Catharanthus roseus* contains more than 130 alkaloids. It holds a unique place in allopathic formulations that are responsible for its therapeutic utilities.

It is an aphrodisiac herb with immunomodulatory, antidiabetic, antioxidant, antistress, antimicrobial, antiaging, anti-tumorous, and anti-inflammatory activities, respectively (Kaul et al. 2013, Senbagalakshmi et al. 2017, Yadav et al. 2018). The root contains ajmalicine, which is antidiabetic whereas, the aerial parts exhibited most prominently with flavonoids, phenolics, tannins, tri-terpenoids, catharanthine etc (Pereira et al. 2010). Two important compounds such as vinblastine and vincristine are anticancerous. Unfortunately, the level of these phytocompounds was found to be very low. The status and medicinal utility of this herb and their demand can be fulfilled by using micro propagation techniques under *in vitro* techniques for *ex situ* conservation (Desai et al. 2018). The major threats under *in vitro* conditions are to remove the contamination. Therefore, it is necessary to minimize contamination through antimicrobial compounds for the reduction of *in vitro* culture mortality rate. Considering this important issue, the objectives of this work were to identify the microbial contaminants and determination of minimum phytotoxic concentration (MPC) of antibiotics and fungicides.

Materials and Methods

Inflorescence axis (Inf. axis), leaves, shoot apex, node and internode of the variety rosea and alba and Inf. axis *Chlorophytum borivilianum* (*C. borivilianum*; Safed musli) was used as explant for micropropagation simultaneously. Sterilization of the media was done at 121°C for 30 min at 15 psi. Whereas, sterilization of explants was made by washing with double distilled water followed by 70% ethanol (v/v) for 2 min and 0.1% $HgCl_2$ (w/v) for 1 min. Micropropagation was done in the plant tissue culture laboratory at 22 ± 2°C temperature under 16 hrs light and 8 hrs dark photoperiod. More than 50% of tubes was found to be contaminated during culture initiation and establishment stage (Stage I) of in-vitro culture. 15 to 30 days old infected *in vitro* cultures of *Catharanthus roseus* (*C. roseus*) and *C. borivilianum* was used to isolate bacterial and fungal contaminants. The bacterial isolates were cultured at 37 ± 1°C for 24 hrs on nutrient agar (NA) medium. Whereas, fungal isolates were grown on potato dextrose agar (PDA) at 24 ± 2°C for 24 to 72 hrs. The purified bacterial isolates were identified using Bergey's Manual of Bacteriology (Holt et al. 1994) and authenticated by microbial type culture collection and Gene Bank (MTCC), Indian Institute of Toxicology Research, Lucknow, India. The fungal isolates were identified on cultural characters described by various workers (Gilman 1957, Nelson et al. 1982, Barnett and Hunter et al. 1998). Similar types of infectants were

isolated from *C. roseus* and *C. borivilianum* was categorized in group I and the rest was placed in group II.

Antibiotics such as penicillin (PNC) 10 mcg, norfloxacin (NFC) 10 mcg, tobramycin (TBC) 10 mcg, gatifloxacin (GFC) 5 mcg and ofloxacin (OFC) 5 mcg were prepared and serially diluted in all the culture tubes ranged from 1 to 10% at 1% interval. 0.5 to 5% with the interval of 0.5% solutions of fungicides i.e., bavastin (BVN), captan (CPT), fluconazole (FCZ) and trichoderma (TCD) were prepared, filtered, sterilized and added in the media just before plating. Group I was selected for the test as the infecting pathogens inhibited the growth and development of *C. roseus*. Due to their anticancerous, antidiabetic and other important medicinal properties *C. roseus* 'rosea' and 'alba' cultivars were selected and carried forward for *in vitro* propagation.

Initially, culture sensitivity test (C/S test) of bacteria was done by antibiotic disc sensitivity method or Kirby-Baure method (Claus 1995) by using antibiotics discs of PNC, NFC, TBC, GFC, and OFC. C/S test of fungal isolates was performed by poisoned food technique by using fungicide discs of BVN, CPT, FCZ and TCD (Borum and Sinclair 1965), respectively. The inhibition zones were measured in mm scale. The efficacy of fungicide was expressed as cent per cent inhibition of mycelial growth over the control. Minimum inhibitory concentration (MIC) of antimicrobial compounds was determined as the lowest concentration at which the growth of bacteria and fungi became inhibited.

Minimum Phytotoxic Concentration (MPC) is the concentration of antimicrobial compounds which kill microbes without affecting the growth of *in vitro* cultures. To determine MPC approximately 54 treatment combinations (antibiotic + fungicide) were added in MS with BAP (0.1 - 0.5 mg/l), Kn (0.1 - 0.5 mg/l), IAA (0.1 - 0.5 mg/l)], Kn (10%), sucrose (3%) and agar (0.8%). Approximately 10 tubes for each treatment were inoculated with Inf. axis, leaves, shoot apex, nodes and internodes incubated at 16 hrs light and 8 hrs dark photo period at 24 ± 2°C for Stage-I. Data for culture survival was recorded for 45 days. Culture survival (> 70%) was marked as non-phytotoxic, (> 60%) moderately phytotoxic and (< 50%) as phytotoxic.

Results and Discussions

During this study a number of bacteria and fungi were found to be associated with the *in vitro* contamination. On the basis of morphology and biochemical characteristics, isolated bacteria were compared with standard characters of Bergeys Manual of bacteriology and the isolated bacteria were found to be *Bacillus licheniformis*, *Micrococcus* and *Paenibacillus* whereas, the isolated fungi were found to be *Fusarium*, *Aspergillus*, *Alternaria* and *Cladosporium sphaerospermum,* respectively (Fig. 1a). The isolates were authenticated by MTCC and Gene Bank, IITR, Luck now, India that is presented in Tables (1 - 3). Preliminary experiments on the C/S test of the antibiotics showed that *B. licheniformis*, *Micrococcus* and *Paenibacillus* were susceptible to GFC and OFC at varied MIC where OFC was found to be most effective. All the isolated fungi were sensitive to BVN, CPT,

FCZ and TCD the only difference being their MIC. From the observations, it was evident that the species of *Cladosporium* and *Aspergillus* are more sensitive to BVN than *Alternaria* and *Fusarium.* BVN and CPT proved to be the most effective fungicides giving no growth to all the fungal isolates at 1 to 2%. The experiment revealed that the combination of two antibiotics (GFC and OFC) and two fungicides (BVN and CPT) are found to be most effective *in vitro* contamination. MPC values were recorded as 15, 9, 6 and 5% for OFC, GFC, BVN and CPT, respectively. Treatment of GFC(4%) + BVN (1%) and OFC (9%) + BVN (1.5%) was found to be more effective against the microbial growth (Fig. 1b).

Table 1. MIC and MPC of effective antibiotics.

Sl. no.	Isolates (Bacteria)	Antibiotics Name/inhibition (%) / MIC (%)	MPC (%)
1	*Bacillus licheniformis*	GFC/100/7: OFC/100/15	GFC (4 - 9)
2	*Micrococcus* sp.	GFC/100/9: OFC/100/10	OFC(10 - 15)
3	*Paenibacillus* sp.	GFC/100/4: OFC/100/10	

Table 2. MIC and MPC of effective fungicides on culture survival.

Sl. no.	Isolates (Fungi)	Fungicides Name/inhibition (%)/MIC (%)	MPC (%)
1	*Fusarium* sp.	BVN/100/4: CPT/100/5 FCZ/100/2: TCD/100/2	BVN (4 - 6) CPT (4 - 5)
2	*Aspergillus* sp.	BVN/100/6: CPT/100/4 FCZ/100/2: TCD/100/1	
3	*Cladosporium sphaerospermum*	BVN/100/5: CPT/100/6 FCZ/100/4: TCD/100/2	
4	*Alternaria* sp.	BVN/100/4: CPT/100/5 FCZ/100/2: TCD/100/2	

The effect of antibiotics and fungicides on both microbes and plants are crucial for the elimination of contaminants and recovery of healthy plants. To prevent microbial contamination of *in vitro* plants growing throughout the culture condition, incorporation of a chemical compound into the culture medium at a concentration that effectively reduces or prevents the growth of microbes without adversely affecting cultures is essential. Researchers Streated the surface-sterilized explants before inoculation where they achieved cent per cent elimination of *Aspergillus, Fusarium, Alternaria, Penicillium, Rhizopus* and *Cylendrocarpon* by utilizing benomyl (100 mg/dm^3) + nystatin (100 mg/dm^3) treatment in *Lilium candidum* culture (Altan et al. 2010, Chai et al. 2010, Jena and Samal

2010, Kumar et al. 2019). Axillary explant of *Centella asiatica* was cultured in MS fortified by BVN with and without growth regulators the result indicated that BVN not only promoted regeneration frequency but also increased shoot proliferation. This shoot

Table 3. Effect of the treatments on phytotoxicity and culture survivability.

Phytotoxic (< 50%)		Moderate phytotoxic (> 60%)		Non-phytotoxic (> 70%)	
Treatments	Culture survival (%)	Treatments	Culture survival (%)	Treatments	Culture survival (%)
G-4% + B-2.5%	66	G-3% + B-1%	74	G-3% + B-1.5%	88
G-4% + C-2%	60	G-3% + C-1.5%	72	**G-4% + B-1%**	90
O-8 % + C-2.5%	59	G-3% + C-2%	68	G-4% + B-1.5%	86
O-8% + C-2%	56	G-4% + C-1.5%	67	G-5% + B-1%	80
		G-5% + C-1.5%	67	O-8% + B-1.5%	75
		G-5% + C-2%	65	**O-9% + B-1%**	90
		O-8% + B-1%	72	O-9% + B-1.5%	85
		O-8% + C-2%	68	O-8% + C-1.5%	81
		O-9% + C-2%	66	O-9% + C-1.5%	88
		O-10% + B-1%	67		
		O-10% + B-1.5%	66		

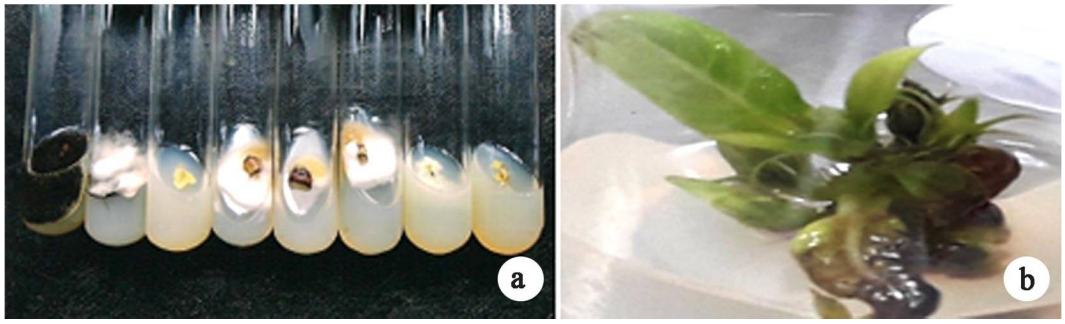

Fig. 1. *In vitro* propagation of *C. roseus* 'rosea' and 'alba' explants. (a) Bacterial and fungal contaminants in culture. (b) Shooting after 45 days of treatment GFC (4%) + BVN (1%) in MS fortified with BAP, NAA, IAA and Kn.

regeneration promoting activity of BVN was due to an increase in the biosynthesis of endogenous cytokinin within the culture explants. Since BVN is a broad spectrum fungicide it also eliminates fungi contaminating cultures (Panathula et al. 2014). Similar observations were made in several other medicinal plants which were discussed in the light of stronger cytokinin like activity of BVN that have the resemblance of its molecular structure with kinetin, adenine and cytokinin. Moreover, it is least toxic to the plant cells

and has a beneficial effect on the physiology of the plant (Habiba et al. 2002, Garcia et al. 2003). Generally, bacteria and fungi are dominant contaminants of *in vitro* cultures. The most common bacterial contaminants encountered are species of *Klebsiella, Erwinia, Staphylococcus, Pseudomonas, Bacillus* and *B. licheniformis* while fungi include species of *Aspergillus, Fusarium, Alternaria* and *Cladosporium* infecting from external sources (Leifart et al. 2001). The endogenous contaminants are species of *Cellulomonas, Corynebacterium, Klebsiella, Pseudomonas*, *Bacillus megaterium* and *Rhodotorula slooffiae* (Nagy et al. 2005). Recently scientist gave evidence for the presence of mycorhizal fungi and endophytes i.e. *Aspergillus, Alternaria, Fusarium, Cladosporium sphaerospermum, Alternaria alternata, Botryosphaeria dothidea, Botrytis cinerea, Cercospora* sp., *Colletotrichum gloeosporioides, Fusarium graminearum, Sphaeropsis sapinea, Valsa sordida, and Phytophthora cinnamomi* whereas, afew bacteria such as *Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes, Enterococcus facealis, Corynebacterium* sp.*, Pseudomonas aeruginosa, Escherichia coli, Candida albicans* (Kaul et al. 2013, Sreekanth et al. 2017, Dong-Hui et al. 2018, Geethanjali et al. 2019) have also been reported.

All the contaminants appeared during Stage-I. The airborne spore former microbes are present indoors, thriving even under nutrient-deficient conditions. Scientific studies revealed that such contaminant or microbes may enter the cultures during inoculation ordering the experimental activity. Some other contaminants such as *Paenibacillus* and *Cladosporium* are the endophytes they also infect the culture and destroy the cultures. To remove such contaminants alternative scientific approach would be required to investigate so that the culture contamination could be prevented.

Antibiotics inhibit the bacterial growth by killing action by cell wall lysis and by inhibiting the synthesis of DNA, RNA and protein. The microbes respond according to their nature and specificity at different MIC of antimicrobial compounds. Except for PNC, all the antibiotics used in the present study are broad-spectrum but the only difference lies in their mode of action. NFC and OFC belong to quinolones that kill bacteria by preventing its DNA from unwinding and duplicating by topoisomerase II. GFC has a superior antibacterial spectrum due to good aqueous solubility and better penetration which kills microbes regardless of its metabolic state. Moreover, the presence of methoxy side chain at C-8 position increases the bactericidal action as well as its ability to inhibit the growth of mutants and also reduces the quinolone associated phytotoxicity. Hence, GFC is advantageous in the bacterial eradication of infecting pathogens (Gradelski et al. 2002). GFC, TBC and NFC are used against Gm^{+ve} and Gm^{-ve} aerobic and anaerobic bacteria. Researchers also reported that GFC exhibited more potency than ciprofloxacin and levofloxacin. On the integral ribosome, TBC has a low and very high number of primary and secondary selective binding sites, respectively (Shailja et al. 2004). The 6-amino function determines the non-selective binding and the kanosamine ring seems to be determining moiety for recognition (Kotra et al. 2000). NFC is well tolerated without severe adverse effect and the *in vitro* antimicrobial activity diminishes by acidic pH and high concentration of Mg^{2+} in the medium. NFC kills microbes by

inhibition of DNA gyrase which adversely affects the relaxation of super coiled DNA and promotes its breakage (Sharma et al. 2008). PNC is a narrow-spectrum antibiotic but when used with fungicide it becomes part of broad-spectrum therapy. It is less toxic which kills susceptible bacteria by inhibiting the protein. Different classes of drugs target fungal plasma membrane, biosynthesis of sterol and ß-glucan. Earlier it has been reported that those drugs which target β-glucan biosynthesis have low side effects (http://www.pharminfo.com; WHO 2014). Both BVC and CPT are cheap, have low toxicity but differs in their mode of action i.e., BVN inhibits the development of germ tube appressoria and mycelium while CPT blocks the ability to produce energy (Nagy et al. 2005). The foregoing discussion establishes the higher efficacy of OFC, GFC, BVN and CPT over other antimicrobial compounds used in this investigation. Investigators commented that MIC of an antimicrobial compound may or may not be phytotoxic for callus induction, callus proliferation and plant regeneration i.e. the effect may be potentiated, synergistic, additive or antagonistic. Combinations of antimicrobial compounds may be advantageous where synergistic action occurs but at times may be phytotoxic and its repeated use may lead to resistance (Andrew 2001). Therefore, it is essential to determine the MIC before treatment. The observations made in this investigation may be due to the additive or synergistic effect of antimicrobial compounds as they were used in various combinations as advocated by previous authors.

Investigation concluded that the combination of GFC (4%) + BVN (1%) and OFC (9%) + BVN (1.5%) appeared to be the best as 90% contamination-free cultures were initiated exhibiting their additive or synergistic effect. GFC (4 - 9%), OFC (10 - 15%), BVN (4 - 6%) and CPT (4 - 6%) were found to be most susceptible. For reproducibility of the present findings, *Catharanthus roseus* "rosea" and "alba" cultures should be reinitiated with the recommended treatment condition and culture mortality should be evaluated. The findings will prove a milestone for beginners working in the field of tissue culture of medicinal plants and secondary metabolite production.

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