JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2016. Vol. **10**(3), p. 2145-2152

Curbing the Menace of Contamination in Plant Tissue Culture

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(Received: 10 April 2016; accepted: 25 May 2016)

Regeneration of multiple individual plantlets from a single cell or explant is one of the main aims of plant tissue culture. This field has a wide scope for production of pharmaceutical metabolites through suspensions, production of plantlets from plants which does not set seeds, production of viral free plants etc. The productions systems are brought down by the event of microbial contamination that is generally fungal, bacterial and viral in origin, which occurs at different levels. Controlling these check points brings out a reduction in the microbial hazards. Similarly following a sterile and strict protocol at different stages reduces the percentage of accidental contamination, which starts from the process of selection of explants to the stage before hardening. Traditional techniques like proper sterilization of explants and equipment, to the latest measures such as incorporation of biocide and nanoparticles have reduced the effects caused by the microbial pollutants. Proper knowledge about the mechanism of action and the concentration of the antimicrobial compounds are necessary as higher dosage could lead to deleterious effects to the plantlets grown under *in vitro* conditions. Optimized control measures are mandatory to reduce the contamination as well as the cost of production, which are vital to improve the standards of plant tissue culture industries.

Keywords: Contamination, checkpoints, microbial hazards, biocide, nanoparticles, plant tissue culture.

Plant tissue culture is a field of biotechnology where, quite a large number of plantlets are produced from a single cell. This makes use of less sophisticated methods for commercial production of plantlets in large numbers. Introduction of molecular techniques in this field has improved its scope and applications. Plant tissue culture plays a vital role in production of plants which takes many years to flower and also where the seeds remain dormant. The thrust areas include the production of plantlets where vegetative propagation is cumbersome, the production of secondary metabolites from cell suspensions and for conduction of new trials of mutation induction through plant cell lines.

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The production of disease free homogenous plants is the main characteristic feature of plant tissue culture. Contamination is caused by the exogenous and endogenous microbes. Possibilities of contamination occur mainly due to two factors. It can be from the explants where, the microbes have the potency to remain latent for a period of time and due to improper handling of the operator[1]. Proper identification and knowledge about the microbes are essential to eradicate them from the explants and aseptic handling measures have to be standardized. Several alterations have been followed for the reduction of contamination and cost for economic production of plantlets. Microbial contamination occurring at various levels has been discussed as checkpoints.

Check point – Mother stock

Mother plants or stocks infested with

mites, nits, insects and microbes should not be chosen for deriving explants. Juvenile plants are the best source of explants as the level of microbial load is very much reduced in them and these tissues would be free from viral infections[2]. Treatment of mother plants with bactericides, fungicides and insecticides may decrease the effect of contamination. Amendment of pesticides reduces significant losses caused by insect pests and other biological agents[3]. Apart from these methods following integrated pest management can also reduce the burden of losses caused by the chemicals to the plants and to the environment as being followed by the Food and Agricultural Organization (FAO) in Asia, Near East and West Africa and in few other places[4].

Check point - Explants

The sterilization protocol for explant varies with different groups of plants. The chemicals used and the treatment time also varies with the nature of the explant. Commercial detergents such as Extran or Tween20 (HiMedia) at a concentration of 1-5% has been recommended as these help in dislodging the microbial spores from the pubescent plants[5,6,7]. The commonly used sterilants are 0.1% mercuric chloride, 70% ethanol and 30% Chlorax [6,7,8]. Treatment of explants with fungicides such as 1mg/ml concentration of Bavistin (50% carbendazim), 0.2 % (w/v) of benomyl and antibiotics such as 1mg/ml concentration of streptomycin and chloramphenicol for half an hour have played a significant role in reduction of microbial contamination[9,10,11]. Addition of silver nitrate as surface sterilant has been studied, where the compound had a very minimal inhibitory effect on the *in vitro* cultures[12]. The latest technology that has been incorporated to reduce the deleterious effects caused by microbes is the use of nanoparticles. Silver nanoparticles have been used as a disinfectant[5]. Similar use of the same has been reported in surface sterilization of rootstock cultures of hybrid almond at different concentrations, as silver generally possess antimicrobial properties [10,13].

Check point - Equipment

Glasswares and other equipment are autoclaved at 121ºC for 15 psi for 15-20 minutes in laboratories and industries. Chemical treatment of the glasswares is also carried out with sodium hypochlorite (NaOCl). A concentration of 0.002% NaOCl was used for disinfecting non autoclavable vessels for micropropagation of banana, but was not efficient for Agrobacterial cell cultures[14]. Similarly 50ppm of NaOCl was used to clean deli containers which had significant cost reduction as the need of autoclaving had been replaced with the use of NaOCl[15].

Check point - Sterilization of the culture media

For preparation of sterile media, the chemicals should be of standard grade. Use of deionized water avoids the interaction of ions between the water and the chemicals. Once prepared, the culture media are autoclaved. Heat liable compounds such as antibiotics, growth regulators and supplements are sterilized through syringe filters of 0.22µm size and then incorporated into the sterile media. A period of 4-6 days is provided as incubation time to check the microbial growth on the sterile media. Contamination occurring even after practicing these strict protocols can be reduced by incorporation of the following into the media.

1. Use of antibiotic
2. Use of fungicide
3. Use of other chemicals
4. Use of nanoparticles

Use of antibiotic or incorporation of any other chemical compound into the media is a necessity only when the contamination is endogenous. Table.1 depicts the management of contamination in diverse plant groups by the use of biochemical and serological methods. The plant system has a lot of microbial flora associated with it that occurs exogenously and endogenously. The endogenously occurring microbes are commonly referred to as endophytes, remain intact within the plant tissues form the collection date, during the initiation protocols and even during subcultures. These microfloras are less susceptible to any disinfection protocols[16]. These endophytes have impact on plants and provide tolerance against biotic and abiotic stresses in field conditions but these are undesirable as they compete for nutrients when cultured under *in vitro* conditions[17].

Use of antibiotics

Antibiotics which are commonly used in plant tissue culture are tetracycline, streptomycin, vancomycin, rifampicin, gentamycin, cefotaxime etc. These antibiotics are also used in combinations[22]. A novel method that has been

Table 1. Methods of elimination of contamination in plant tissue culture

Plant culture	Contaminant	Detection	Treatment	Significance
Boat orchid *(Cymbidium* Sw.) **Protocorm**	*Odontoglossum* Ringspot Virus	ELISA	Ribavirin (VIRAZOLE®) at 35ppm along with media	Early and reliable indexing of *in vitro* grown plantlets to eradicate virus[26]
Apple *(Malus domestica)* **Axillary bud**	*Rhodotorula slooffiae*	Morphological Physiological, Biochemical characteristic and 26S rDNA sequencing	Mancozeb (15mg/L) thiabendazoles (40mg/L), silver nitrate (588µM) and Silveret77(0.01%)	Increase in decontamination was observed when compounds were added into the media[27]
Valerian *(Valeriana . officinalis* L*)* **Node**	*Xanthomonas* sp.	Special laboratory methods	Nano silver solutions at 25, 50 and 100mg/L were used before and after sterilization	Nano silver had no detrimental effects on the cultured plantlets[5]
Grape vine *(Vitis vinifera)* **Embryo**	GRSPaV, GLRaV1, GVA, GLRaV3	RT-PCR and ELISA	-	Somatic embryogenesis was efficient against phloem limited grapevine virus[28]
Madonna lily *(Lilium candidum* L.) **Node**	*Fusarium, Alternaria, Rhizopus, Cylindrocarpon and Aspergillus sp.*	Slide culture techniques	Benomyl with Nystatin ($100mgl^{-1}$) after surface sterilization	Effective sterilization using chemotherapeutic substances provided higher percentage of decontamination[29]
Bamboo *(Phyllostachys* sp. and *Fargesia* sp. **Node**	Unidentified bacteria	Physiological and 16S rDNA gene sequencing	-	15 out of 18 species of bacterial endophytes were reported for the first time[30]
Lamba *(Curculigo latifolia* Dryand) **Rhizome**	Unidentified bacteria	Gram staining	Chloramphenicol, steptomycin and Bavistin(0.1%)	Pretreatment for 9 hours significantly reduced contamination[9]
Yerba mate *(Ilex para guareinsis* **Node**	*Stenotrophomonas maltophilia*	16S rDNA gene analyses, analytical profile index and biochemical tests	0.75ml/L of Delcide™ TG and other isothiazolone biocides	Bactericidal activity in transpiration stream was reported using isothiazolone biocides[31]
Almond hybrid *(Prunus amygdalus* × *P. persica)* **Node**	Unidentified bacteria and fungi	Visual assessment of contamination	Nano silver solution at 100ppm as immersion solution and along with media	Optimum concentration of 100ppm, was observed to contain microbe growth[10]
Banana *(Musa sapientum* L.) **Sucker**	Unidentified bacteria and fungi	Biochemical and gram staining and lactophenol cotton blue staining	Zinc and zinc oxide nanoparticles(100 mg/L)	Reduction in contaminations were observed at 200mg/L without affecting the physiological and morphological processes[32]
Carrot *(Daucus carota* L.) **Protoplast**	Unidentified bacteria	Antibiotic sensitivity screening	â- lactam antibiotics	Inhibited the biosynthesis of peptidoglycan[24]
Bamboo	*Pantoea sp*	Antibiotic	Streptomycin and	Kanamycin had best

(Guadua angustifolia Kunth) **Node**		sensitivity screening and 16S rRNA gene sequencing	kanamycin(10µg/ml) along with media for ten days	resultants but streptomycin resulted in yellow and stunted plantlets[7]
Mosses *(Ceratodon purpureus* and *Physcomitrella patens)* **Protonema**	*Paenibacillus taichungensis, P. humicus, Bacillus megaterium*	16S rRNA gene sequencing	Vancomycin at 50µg/ml in embedded agar block	Contamination reduced gradually, as the tissues were in constant contact with antibiotic[23]

Table 2. Mode of action of few antibiotics against bacteria

S.No	Antibiotic	Mechanism of Action	Reference
1	Vancomycin	Inhibits synthesis of peptidoglycan in cell membrane	18
2	Cefotaxime	Inhibits bacterial cell wall synthesis	19
3	Rifampicin	Inhibits bacterial RNA polymerase	20
4	Tetracycline	Prevents the attachment of aminoacyl rRNA to ribosomal acceptor (A) site	21
5	Streptomycin	Prevents the initiation of protein synthesis	7
6	Kanamycin	Inhibits translocation during protein synthesis	7

reported for eliminating bacterial contamination in *in vitro* propagation of Moss protonema is by the agar embedding system where, antibiotics were added to the agar and embedded on to protonema, thereby reducing the microbial growth due to the continuous contact between the tissues and antibiotics[23]. Contamination in *Gauda angustifolia* Kunth, detected as endophytes have been treated by administration of kanamycin and streptomycin sulphate, where kanamycin at a concentration of 10µg/ml exhibited best results with no phytotoxicity[7]. Similarly incorporation of 80µM/L of kanamycin in *Centella asiatica* L., culture medium induced shoot growth and also significantly reduced the bacterial contamination[11]. ²-lactam antibiotics such as cefotaxime, carbecillin and timentin were exploited in protoplast cultures of carrot at a range of 100-500mg/L[24]. Proper knowledge about the mode of action and the minimum inhibitory concentration level of antibiotics is a prerequisite as malformations and retarded growth of the *in vitro* grown cultures results if the dosage of the antibiotics is increased[25]. The role and mode of action of few antibiotics has been discussed in Table 2.

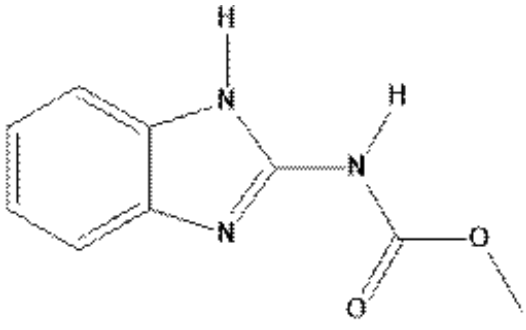

Fig. 1. Bavistin

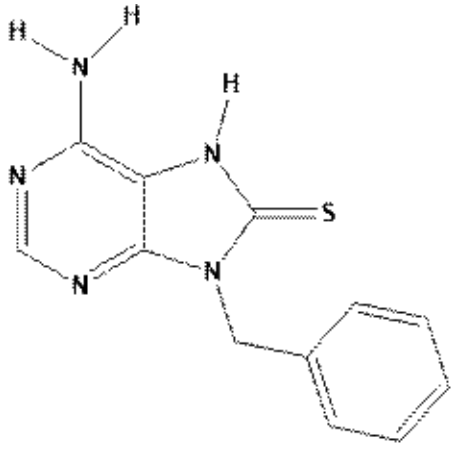

Fig. 2. Benzyl aminopurine

Usage of fungicides in the culture media

Like bacterial, fungal contamination are also predominantly found in plant tissues as endophytes. These fungal endophytes are isolated and used for the production of phytochemicals which possess anticancerous, antineoplastic, antidepressant and other medicinally important compounds such as taxol[33, 34]. These endophytes pose threat to plants under *in vitro* conditions. Their growth can be controlled by use of systemic fungicides. The commonly used fungicide in culture media is Bavistin (50% carbendazim). Bavistin at a range of 150-300 mg/L incorporated into media showed significant reduction in fungal contamination[11]. It has antimitotic and antineoplastic activities in fungal cells. It has a similar structure to that of cytokinin (adenine derivatives). Bavistin has been reported to cause shoot proliferation in *Stevia rebaudiana* cultures [35]. Similarly, Dhingani *et al.,* have reported that Bavistin had significant potential to inhibit root rot diseases under field conditions in *Cicer arietinum* L. caused by *Macrophomina phaseolina* (Tassi) Goid[36]. Figure 1, 2 and 3 brings out a comparison between Bavistin and two cytokinins and also depicts the similarity in the structure among the adenine derivatives.

The use of other fungicides such as ProClin®300, mancozeb, thiabendazoles were reported in controlling the contamination of yeast in apple cultures[27]. Mancozeb used in a commercial fungicide formulation termed EmCarb (63% mancozeb and 27% carbendazim) had successful results when incorporated at a concentration of 0.5% (w/v) in culture media grown with *Asparagus adscendens* Roxb[2].

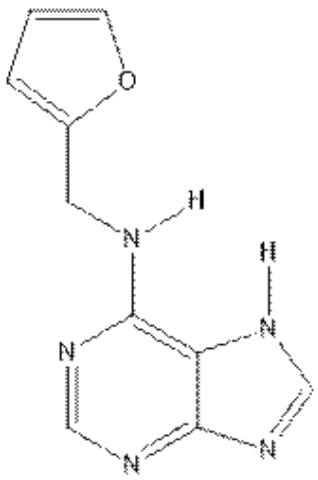

Fig. 3. Kinetin

Use of other chemicals

Apart from these antimicrobial compounds, other disinfectants and chemicals are also incorporated into the culture media. *In vitro* propagation of potato was carried out in a media containing 5-10 ppm of NaOCl[15]. Disinfection of contaminants using active chlorine at 0.001% and 0.005% gave similar results to that of the conventional methods[37]. Incorporation of active chlorine into the media maintains the stability of heat liable compounds such as Vitamin B and growth regulators[38]. Biocides such as Plant Preservative Mixtures™ have also been supplemented into the culture media, as it contains a combination of methylisothiazolone (MIT) and chloromethylisothiazolone (CMIT)[31]. These compounds inhibit major enzymes produced in the microbial metabolic and energy production pathways[39]. *Ilex paraguariensis* grown with Delcide™ at a concentration of 75µl/L in culture medium resulted in 100% clean cultures without any sign of growth malformations[31].

Use of nanoparticles

Nanoparticles have been engineered to eradicate the prevalence of contaminants. Zinc nanoparticles and zinc oxide nanoparticles have been found to possess antimicrobial properties. They have also been found to show no antagonistic activity at a concentration of 200mg/L in banana cultures[32]. Similarly silver nanoparticles were also reported to have the potential to reduce bacterial contamination at a range of 20-100mg/L in tissue cultures of *Valeriana officinalis*[5]. The root stock cultures of an almond hybrid, immersed in a solution containing silver nanoparticles at a concentration of 100mg/L and then cultured in a media containing 150 mg/L of silver nanoparticles were found to have a reduced effect of contamination[10].

Check point - Sub culture and storage

This is the major step in the process of plant tissue culture as it produces multiple plantlets. Leifert *et al.,* have reported that, during sub culturing process contamination occurs due to unsterile air, improper sterilization of the equipment and aseptic handling by the operators[40]. Once the sub culturing has been completed the

media containing vessels need to be properly sealed, labelled and stored in clean environment with sufficient environmental factors[1]. Fumigation of culture rooms with solutions of potassium permanganate and formaldehyde at weekly intervals were carried out for the mass propagation of banana [41]. Culture rooms housing the bioreactors for the production of Artemisinin through hairy root cultures were also given similar treatment for reducing the microbial load in the culture rooms[42]. Sterility also needs to be maintained when the plantlets are subjected to primary hardening. Ammonium bicarbonate has been used as a fumigating agent for controlling wilt disease caused by a soil borne fungi, *Fusarium*, which has detrimental effects on major horticultural and food crops[43]. Some of the other soil fumigants under use are chloropicrin, dimethyl disulfide, Telone C35 and methyl bromide[44,45].

CONCLUSION

Microbial hazards cause drastic economic losses in the plant tissue culture industries. Improper handling and non-sterile environment will increase the rate of contamination. Each explant is very precious as they form multiple plantlets. During contamination, competition for the nutrient rich media occurs between plants and microbes leading to the death of plantlets as they are fragile when grown in a controlled environment. This review tries to give an insight into the different levels at which contamination occur and also provides solutions to these problems.

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