THE FUNGAL AND BACTERIAL CONTAMINATIONS IN PLANT TISSUE CULTURE GROWTH MEDIA

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Abstract:

The contamination in tissue culture can be originated mainly from two sources, where one through carryover of the microorganisms present in the tissues or the surface of explants and second being by the faulty procedures which we take up in the laboratory. Fungal and bacterial contaminations cause major problems which makes it harder to deal with media of the plant tissue cultures. Vessels used for tissue culture are generally loose fitted in order to allow gaseous exchange with external environment, this is how the fungal or bacterial contamination takes place. Usually fungal contamination is the sign of mites or thrips infection in the culture media. *Escherichia coli, Micrococcus* sp., *Pseudomonas fluorescens* and such species cause bacterial contaminations. *Aspergillus niger, Yeast, Penicillium species, etc,*.were identified as fungal contaminants. This paper mainly focused on the fungal and bacterial contaminations on the culture growth media in the plant tissue culture laboratory.

Key words:

Contamination, external environment, culture media, spores, fungus, bacteria, faulty procedures, antibiotic discs, sterilization, zone of inhibition.

Introduction:

Plant tissue culture could be defined as growing of plant cells or the tissues away from the parent plant on an artificial media *in vitro*. It is a renowned method in the study of plant metabolism, genetics of plant, morphogenesis and the physiological conditions of the plant and in the genetic transformation of the plants, in the removal of plant pathogens, in the preservation of the important plant species in small space, and in the multiplication of plant tissues and cells *in vitro*. However, aseptic conditions are usually suggested, many plant cultures do not stay aseptic *in vitro* and contamination with micro-organisms is observed to be the most prominent reason for

losses of the plants during *in vitro* cultures. The organisms known as contaminants in plant-tissue cultures involves viruses, yeasts, bacteria, fungi, thrips and mites. Contamination with bacteria is assumed to be the most dangerous and has been described extensively in the literature [1],[2]. Few publications described fungal and yeast contaminants and their effect on plantlets grown *in vitro*. Mites and thrips which are found in the tissue cultures, usually do not harm the plants directly [3],[4]. A broad range of microorganisms like the filamentous fungi, bacteria, yeasts, and viroids and viruses and micro-arthropods such as vectors have also known to be the reason for contamination in plant tissue cultue [5],[6]. Contaminants may be imported with the explants during changes in workplace or laboratory or they can also be by the vectors [7],[8]. The contaminants may even express themselves either immediately or sometimes may remain latent for longer period of time [9]. This often makes it quite tough to identify the source of contamination [10]. Commercial micro propagation laboratories, usually report that the constant bacterial and fungal contamination arising in the media is a concerning issue [11],[12]. Lack of surface sterilization process produces aseptic cultures, this is a problem especially with the woody plants. Isolated meristems and the explants from the stock plants grown under controlled or aseptic environmental conditions has been used to retain or extract aseptic cultures from some plant species [13],[14]. Contamination is always not seen at the culture initial stages but some internal contaminants become highlighted only at the later subculture stages and are difficult to eradicate at the time. Detection at an early stage can help in selecting microbial free cultures [15],[16]. Bacterial contamination has been a big problem in plant tissue culture. Even though, some achievements have been marked in past using antibiotics often the antibiotics resist bacterial growth at the initial stages but, they do not provide a major solution to the problem [17]. Continuous antibiotic use can also be expensive, particularly at the commercial level. Moreover, antibiotics that are most effective against bacteria are most toxic to plant material [18]. It has been noticed that such bacteria are majorly members of the common plant surface related and plant pathogenic genera such as *Erwinia*, *Pseudomonas*, *Agrobacterium* spp and *Xanthomonas* [2],[19]. Antibiotic and other treatments might be required to remove persistent microbial contamination, but the type and the quantity of antibiotics and the time period of treatment useful for various plant tissue cultures differs and therefore they have to be checked carefully before use. The contamination in hazelnut was found to be caused by bacteria internally when they were grown in the laboratory [18]. Contaminants were apparent at culture initial

stages or became evident after several subcultures. Some plants were lost when bacteria overgrew them but few were survived and grew in the presence of the bacteria [15],[20],[21]. Bacterial and fungal contaminations were very commonly noticed after the incubation of cells or tissues, especially the one taken from the older trees [9],[22],[23]. *Taxus* spp is associated with microflora which includes more than 300 fungal and bacterial species and the latter of which involves a taxol-producing *Micrococcus* spp provided limited information on the contaminants and their control by antibacterial and antifungal agents in *in vitro* cultures of *Taxus brevifolia* without any detailed information regarding that of the control measures [16],[23]. The cause of the contaminated cultures are usually difficult to determine. Bacteria which are infect the plant cultures may appear from the explants, laboratory environments, mites, operators, and thrips or the need of sterilization techniques [24]. Bacteria are associated with plants as epiphytes or the endophytes [15],[25]. Explants which are from the field grown plants, diseased specimens or from the plant parts which are situated near to the soil or below the soil surface may be difficult or impossible to disinfect due to the presence of both endophytic and epiphytic microbes [26],[27].

Methodology:

Preventing microbial contamination in plant tissue culture is crucial. Bacterial contaminants are very difficult to detect because they remain within the plant tissue [24],[29]. There are some following procedures used to detect and prevent the contaminations in plant tissue culture growth media.

1. Collection of the explants

2. Surface sterilization of explants

3. Identification and isolation of selected bacterial isolates

4. Purification of bacteria

5. Identification by use of morphological and biochemical characters

6. Culture and sensitivity (CS) test of the selected bacterial isolates

7. Control of contamination

Collection of the explants: Varieties of plant materials were used in different research articles. Before collecting the explants, there are some factors that have to be considered as follows

1. Quality of the plant (healthy versus diseased)

2 .Physiological state of the explants

3. Size and location of the explants

4. Plant genotype

5. Season in which explant is obtained

6. Orientation of the explant in the medium.

After considering these factors the plant taken for *In vitro* culture should be trimmed with clean knife or cutter and from this explant can be obtained [3]. Surface sterilization of the explants should be treated with different sterilants, it is used in inappropriate concentration and with different exposure. The most common ones are mercuric chloride (HgCl2), sodium hypochlorite, 70% ethyl alcohol. The surface sterilized explants should be inoculated onto the media. The media that are used are nutrient agar, MS media (murashige and skoog), Difco bacto agar. To isolate an endogenous bacteria the explant should be inoculated onto the MS medium, after the inoculation the developed bacterial contaminants should be transferred to the nutrient agar medium [27],[28],[30]. According to the literature, bacterial contaminants were present in media introduced into the nutrient broth with sterile inoculation loop and this bacteria were grown in incubator shaker at around 26 ± 2 °C and 200 r/min until the medium was visibly turbid. The suspension was then streaked in nutrient agar (NA; NB with 2% m/v DifcoBacto agar) continued in a sterile 80 mm diameter culture plate to isolate single colonies. It is also helpful in checking the purity of isolates. Each plate was incubated for 48–72 hrs at 26 ± 2˚C for growth of isolated colonies [13],[31],[32]. Purification of bacteria was done by the technique called as serial dilution technique and this technique was only considered when the colonies are morphologically dissimilar to identify. Purified bacteria were observed under microscope with proper staining [17],[33]. For the morphological characterization the bacteria were grown onto the nutrient agar medium can be visualized like size, shape, margin, colour, opacity and elevation by doing the biochemical tests. We can identify bacterial species by differentiating them on the basis of biochemical activities [34]. Each of tests in biochemical activities either there is a positive

result indicated by + sign and negative result indicated by – sign. For identification, characterized bacterial strains were compared with the standard strains of Bergey's manual [27]. For Culture and sensitivity (CS) test for the selected bacterial isolates were done by Kirby-bauer method and the medium used was Mueller-Hinton. In this method seven antibiotic discs were used such as ampicillin, cefradine, chloramphenicol, gentamicin, vancomycine, tetracycline, doxycycline were placed after the inoculation of test organisms [34]. The inoculated plates were incubated at 37˚C for 24 hrs. The zone of inhibition was measured around the discs terms of millimetre (mm) [3],[29]. Susceptibilities of the bacterial isolates to different antibiotics were monitored using Biodisc-12™ filter discs impregnate with known concentrations of twelve different antibiotic by the top agar method [35]. The effectiveness of each antibiotic against each isolate was measured using the diameter of the clearing zone around each antibiotic filter disc. After incubation of the cultures at 26 ± 2˚C for 24 hrs in dark, the 12 antibiotics were used like ampicillin, co-trimoxazole, cefotaxime, piperacillin, chloramphenicol, ciprofloxacin, ceftizoxime, tetracycline, ofloxacin, gentamicin, amikacin, pefloxacin [35],[36]. For preventing microbial growth in culture media some chemical agents were added such as methyl chloro isothiazolin, methylisothiazolinone, magnesium chloride and magnesium nitrate will helps in reducing or preventing the microbial growth in media and further helps in plant growth development. A chemical agent also contains potassium sorbate or sodium benzoate, or both [24],[36].

Precautions to be taken against culture contamination:

Fungal pathogens and mycoplasma contaminations leave us fewer visual clues which can be of severe damage to the culture if it’s left unchecked. It is always be a nightmare to deal with the contaminations. Hence prevention is always the best way rather than scrambling to eliminate.We could suggest a few ways in which we can keep our experiments clean, healthy and free from contaminations.

- Use of PPE (personal protection equipment)
- Usage of hood properly
- Incubator must be clean
- Sanitize
- Usage of plant preservative mixture (PPM)

- Cell exposure to a minimum
- Stay organized

Discussion:

To effectively control the bacterial and fungal contaminations in the plant tissue culture media, the non selective industrial biocide PPM can be used. The presence of isothiazolinone in the PPM shows little phytotoxicity at the levels recommended by the manufacture. The most effective control to prevent the water borne and air borne fungal by the use of PPM but which is seen effective only for 24hrs. It is also seen that 10 mg/l natamycin suppressed almost most of the filamentous fungi without the growth of plant tissue culture in *Arabidopsis thaliana* and *Oryza sativa* being affected. It is suggested that natamycin is a very safe and effective fungicide and the 10 mg/l is strongly recommended in *Arabidopsis thaliana* and *Oryza sativa* tissue culture.

Conclusion:

In the plant cell culture, the biological contaminations originate from mainly two sources, one from the tissue used to begin the culture and second is taken from the laboratory environment. Environmental organisms and the plant pathogens are usually the contaminants transferred in the plant material or on the plant material. Laboratory contamination is caused by plant associated microbes, environmental germs and human associated bacteria, yeasts and micro arthropods. Most of the microbial contaminants are revealed when they grow on plant tissue culture media but some of them may be latent (i.e. suppressed) or subliminal. Early detection and at least the partial identification of the contaminants is a prerequired for the control and eradication of laboratory contaminants. Sometimes may not be possible to control fast growing microorganisms before they crowd the culture but latent, or slow growing microorganisms, can be detected and treated early with antibiotics. To avoid contamination, use PPM™ to sterilize the tissue culture media and follow the correct protocols. The most essential step in the prevention is maintain the lab and lab equipment clean, using a disinfectant on the working surfaces and making sure that the disinfectant could eliminate the potential bacterial spores, cleaning your work surfaces often, maintaining a procedure for sterilization and lab instruments (incubators, water baths, and laminar air flow) remains clean. Many other minute areas should receive attention and

cleanliness around the laboratory as well, make sure to clean every where especially the areas that could be a potential hiding places for microbial spores. Hence it is important to know the essence of cleanliness in the plant tissue culture lab and it is surrounding to avoid all possible sources of contaminants for a better yield. The most serious problem in the plant tissue culture technique is contamination and the remedy for this issue is prevention.

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