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CONVENTIONAL METHODS OF CONTROLLING MICROBIAL CONTAMINANTS IN MERISTEMATIC TISSUE CULTURES: A REVIEW

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Abstract
Microbial contaminants in meristematic tissue cultures remain a big problem in the quest to grow plants *in vitro* in the laboratory prior to commercial scale roll out. The ubiquitous nature and the ability to compete favourably with explants for the same nutrient in the growth medium make these contaminants a serious threat in meristematic tissue cultures. The common microbial contaminants frequently reported in *in vitro* meristematic tissue cultures are endophytes such as bacteria, fungi and sometimes viruses. Most of the epiphytic microbes are usually removed by surface sterilization but the endophytes may persist to contaminate the culture. Endophytic microorganisms; usually bacteria, actinomycetes and fungi colonize almost every plant species. This review therefore focuses on the present conventional methods of controlling microbial contaminants in meristematic tissue cultures from the list of relevant available articles.

Keywords: Conventional Control Methods, Microbial Contaminants, Meristematic Tissue Cultures

Introduction

Meristem is a zone of active cell divisions, situated in the growing tip of stems and roots of plants (Faccioli, 2011). Meristematic tissue culture refers to the growth and multiplication of meristematic tissues on a defined sterile solid or liquid media under aseptic and controlled environment (Chinnappan, 2018). This technique is being widely used for large-scale multiplication, improvement, selections, enhanced stress or pest resistance, production of pathogenic free plants and somatic hybridizations (Odutayo *et al.,* 2007). The commercial technology of meristematic tissue culture is mainly by micropropagation, in which rapid proliferation is achieved from tiny meristematic tissues (IAEA, 2002). Microbes especially, viruses travel through the plant vascular system, but are absent in the meristem. Moreover the cell-to-cell movement of the microbes, such as viruses through plasmodesmata cannot keep up with the growth and elongation of the apical-tip (Faccioli, 2011). The high metabolic activity of meristematic cells, usually accompanied by elevated endogenous auxin content in shoot apices may also inhibit growth of endophytes and viral replication (Popescu *et al.,* 2013). Thus, the meristem is highly protected from infection (Panattoni *et al.,* 2013). Based on this finding, meristematic tissue culture has been extensively used to eliminate viruses and endophytes. Endophytic microorganisms usually bacteria, actinomycetes and fungi colonize almost every plant species (Porras-Alfaro and Bayman, 2011). Infection caused by endophytes can be asymptomatic but may become symptomatic if the immunity of the plant is compromised. Most of these epiphytic microbes are usually removed by surface sterilization but the endophytes may persist to contaminate the meristematic tissue culture (Hallmann *et al*., 2007). The culture of meristems or alternatively small shoot tips, in combination with enhanced cell division *in* vitro, chemotherapy and thermal pre-treatment, allows the elimination of microbes like viruses in plants propagated from vegetative parts. The recovery of pathogen-free clones from source-infected plants through the use of meristem tissue culture techniques is based on the fact that pathogen concentration is not uniform throughout the infected plant (Panattoni *et al.,* 2013); since most of the differentiated vascular tissues are far away from the meristems, the vascular elements of the primordium leaves are incipient and are not yet in contact with the principal part of the stem's vascular system (Faccioli, 2011). Despite this fact, microbial contamination still persist during meristem culture since the mechanism by which explants are freed of microbial pathogens, especially viruses is well understood (Popescu *et al.,* 2013, Gambino *et al*., 2016). Conventional methods such as chemotherapy and thermotherapy have been developed and widely used in controlling microbial contaminants in meristematic tissue culture compared to non-conventional methods like cryotherapy, incorporation of disinfectants, antiseptics and nano particles in culture medium, among

others, because they are easily be adapted, commercialized and posed little or no side effects to the explant.

Conventional Control Methods of Microbial Contaminants in Meristematic Tissue Culture

Chemotherapy

Bacteria: Use of antibiotics in culture medium

Bacteria species such as *Pseudomonas, staphylococcus, corynebacterium, Bacillus, Agrobacterium, Propionibacterium, Proteus,* among others (Chinnappan, 2018), are contaminants medium used in meristematic tissue as they compete favourably with the explant (Izarra *et al.,* 2020). Antibiotics like tetracycline, streptomycin, vancomycin, rifampicin, gentamycin, cefotaxime etc are therefore incorporated in the culture medium to inhibit the growth of these bacterial contaminants (Wakil and Mbah, 2012). These antibiotics can be used in combinations to achieve better results. A novel method that has been 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 (Carey *et al.,* 2015). Contamination in *Gauda angustifolia* Kunth, detected as endophytes has been treated by the administration of kanamycin and streptomycin sulphate, where kanamycin at a concentration of 10µg/ml exhibited best results with no phytotoxicity (Nadha *et al.,* 2012). Table 1 shows selected antibiotics popularly used to control bacteria in plant tissue culture and their mode of action.

Table 1: Selected antibiotics popularly used to control bacteria in plant tissue culture and their mode of action

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

Source: Leelavathy (2016)

Fungi: Use of fungicides in culture medium

Fungal species such as *Penicillium*, *Curvularia*, *Cladosporium*, *Aspergillus*, *Acremonium, Fusarium, Alternari*, *Rhizopus*, *Trichoderma, among others* (Omamor *et al.,* 2007, Cobrado and Fernandez, 2016) are known to contaminate meristamatic tissue culture as endophytes. These fungal as endophytes are isolated and used for the production of phytochemicals which possess anti-cancerous, anti-neoplastic, anti-depressant and other medicinally important compounds such as taxol (Shweta *et al.,* 2010). They may on the other hand, pose threat to plants under *in-vitro* conditions. The growth of most endophytes can be controlled by the use of systemic fungicides. The commonly used fungicide in culture media is Bavistin (50% carbendazim). Bavistin at a range of 150-300mg/L incorporated into media showed significant reduction in fungal contamination (Panathula *et al.,* 2014). It has anti-mitotic and anti-neoplastic 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 (Preethi *et al.,* 2011). The use of other fungicides such as ProClin®300, mancozeb and thiabendazoles were reported in controlling the contamination of yeast in apple cultures (Nagy *et al.,* 2005).

Viruses: Use of antiviral agents in culture medium

Anti-viral compounds are useful in the control of plant viral diseases. Chemical compounds such as ribavirin (RBV) (virazole), azidothymidine, and 2-thiouracil (Chauhan *et al.,* 2019), and some antiviral drugs such as inosine monophosphate dehydrogenase (IMPDH) inhibitors, S-adenosylhomocysteine hydrolase inhibitors, and neuraminidase (NA) inhibitors are generally used in plant chemotherapy (Panattoni *et al.,* 2013). Prior to meristem tip culture, chemotherapy was to completely eliminate Lily symptomless virus (Chinestra *et al.,* 2015). Although these chemical compounds exhibited some disadvantages as they all have different modes of action and are not effective *ex vitro* even at higher concentration (DeClercq, 2005).

Thermotherapy

Plant thermotherapy aims at achieving a cellular environment which is progressively less adequate for virus vitality (Pennazio, 2005). For instance, the effects of heat treatment on the functionality of viral movement proteins produce less restriction of infected tissues (Mink *et al.*, 2008). During thermotherapy, the plants are first grown at high temperature (38-42^{o}C) for 4-6 weeks. Under tropical or subtropical conditions, this can be accomplished simply by installing a small compartment of a glasshouse equipped with a roof vent on one end and an exhaust fan on the opposite end, both temperature-regulated (Panattoni *et al.,* 2013). This approach removes the excess heat and provides a constant high temperature daytime treatment. In climates with temperate conditions, the same effect can be achieved by placing fluorescent lights including ballasts and/or heat producing incandescent lamps in the necessary minimum distance to avoid damage over the

plants to be treated in a dark box just large enough to include the plants (Mink *et al.*, 2008). Such a system has been used for virus elimination in sweet potato (Schmidt *et al.,* 2002). After thermotherapy, 0.2 –0.4mm explants are preferentially cultured singly in test tubes. If the explant is too big, it likely has a vascular system that may contain microbial contaminants including viruses. The plants thus obtained are multiplied, and re-indexed. A better strategy is to culture 2-5mm long explants for 4-5 weeks, maintain the *in vitro* grown plant at high temperature for 4-5 weeks, and excise 0.2-0.4mm or even longer explants to initiate subcultures. This procedure avoids *in-vivo* contamination problems and gives high survival and multiplication rate. In potato, *in-vitro* cultures were established from several millimeter long shoot tips and axillary buds after *in-vitro* thermotherapy to free potato from all contaminant viruses (Schmidt *et al.,* 2002). By using this method, it was possible to eradicate viruses A, Y and X from potato varieties in one single step (Ahloowalia, 2002). A method called multiple lateral shoot technique of *in vitro* elimination of three common viruses, X, Y and S of potato, has also been reported (IAEA, 2002). In this method, a stem with at least five nodes is treated with ribavirin in liquid medium, and cultured for 5 days at room temperature. The stems are further subjected to thermotherapy at 32-35^0 C for 25 days after which, apical buds are taken from the lateral shoots and cultured on solid medium. The growing plantlets are then checked for virus infection with ELISA. The differing ability of the movement of viral particles in plant tissues often influences the choice of elimination treatment. Thermotherapy is the more effective control procedure against viruses that are characterized by parenchymatic localization, compared to meristem culture technique which is more suitable for phloematic viruses that are limited to vascular tissues and rarely found in parts of the plant where differentiated tissues are absent (Panattoni *et al.,* 2013). However, up to now, differences in localization of phloem and parenchymatic viruses in the host tissue have not fully explained their different susceptibilities to thermotherapy elimination (Panattoni and Triolo, 2010). Developments over the last 20 years in research aimed at investigating the metabolic processes involved in defense mechanisms of plants have suggested an interpretation of the heat treatment effects according to new metabolic "pathways" triggered by the natural antiviral response produced by the infected plant, with particular reference to Virus-Induced Gene Silencing (VIGS) induced by the presence of viral RNA in infected plants (Panattoni *et al.,* 2013). RNA silencing was described as such an effective defense as to constitute an immunity mechanism at the genomic level. Over the course of investigations on infected plants (Voinnet, 2011), a correlation between VIGS and the thermal regimes to which a plant is subjected has emerged. Studies including treatments with temperatures (30°C) lower than those set out in standard thermotherapy protocols (36°C), revealed that gene silencing and thermal treatments stimulate recovery in infected plants (Qu *et al*., 2005). Moreover, in a research conducted by Szittya *et al*. (2003), *Nicotiana benthamiana* plants infected with *Cymbidium ringspot virus* were exposed to different thermal regimes between 15°C and 27°C. For each heat treatment, concentrations of short interfering RNA(SiRNA) were determined with elevated concentration of SiRNA observed at 27°C and undetectable at 15°C. Also, an increasing SiRNA concentration gradient, starting from 21°C, was observed in reference to the treatments. In relation to the differential spread of viral particles observed in the temperature range tested, the authors identified a hyper activity of the system of temperature-dependent gene silencing, as a mechanism of antiviral protection of the plant. Virus-Induced Gene Silencing was defined by these authors (reference) as a defense system that operates ineffectively at low temperatures, therefore increasing the plant's susceptibility to virus infections that do not encounter virus blocking gene systems. In contrast, increased heat stress induces an increase in the host defense system's capacity by creating a barrier to infection (reference). In their work, and in order to determine the influence of temperature on virus silencing in *Geminivirus* (ssDNA), Chellapan *et al*. (2005) applied heat treatment (25-30°C) on cassava (*Manihot esculenta*) and tobacco (*Nicotiana benthamiana*) plants infected by Cassava mosaic disease. The authors also confirmed the close relationship between temperature and VIGS.

Non-conventional Control Methods of Microbial Contaminants in Meristematic Tissue Culture

Cryotherapy

Researchers use saline solutions containing crushed ice at a temperature of −18° to −24°C for the treatment of human tumors (breast, cervical, and skin), which also help in decreasing pain (Chauhan *et al.,* 2019). This idea has been applied in plant virus control. In plant cryotherapy, pathogens such as viruses, phytoplasma, and bacteria are exposed to low temperature (−196°C) for a prolonged time which successfully eradicates virus complexes resulting in virus-free plants with high frequency as compared to meristem tip culture (Lal *et al.,* 2015). It does not allow the occurrence of thermally directed metabolic reactions in viral particles. It has been found that three Closteroviridae viruses who cause leafroll disease in grapevine were eradicated by vitrification using dehydrating material based cryotherapy of buds of contaminated clones (Pathirana *et al.,* 2015). Advantages of cryotherapy include treatment of large numbers of plantlets at the same time, and the technique is applicable independent of shoot tip size. A major disadvantage is the large consumption of certain gases like Argon and Nitrogen (Littrup *et al.,* 2006).

Use of disinfectants, antiseptics and nano particles

Apart from the use of thermotherapy, chemotherapy and cryotherapy in the control of contaminants in culture media, such agents as disinfectants, antiseptics and nano particles are also incorporated into the plant tissue culture media to control contaminants. For instance, the use of 5-10ppm of NaOCl in *in-vitro* propagation of

potato to control pathogenic contaminants was demonstrated by Weber *et al.* (2015). Disinfection of contaminated culture media using active chlorine at 0.001% and 0.005% gave similar results to that of the conventional methods (Brondani *et al.,* 2013). Incorporation of active chlorine into the media maintains the stability of heat liable compounds such as Vitamin B and growth regulators (Peiris *et al.,* 2012). 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) (Luna *et al.,* 2013). These compounds inhibit major enzymes produced in the microbial metabolic and energy production pathways (Williams, 2007). Nanoparticles have been engineered to eradicate the prevalence of microbial contaminants in plant tissue cultures. Zinc nanoparticles and zinc oxide nanoparticles have been shown to possess antimicrobial properties and no antagonistic activity at a concentration of 200mg/L in plant tissue cultures e.g. banana cultures (Helaly *et al.,* 2014). Similarly silver nanoparticles (AgNPs) were also reported to have the potential to reduce bacterial contamination at a range of 20-100mg/L in tissue cultures of *Valeriana officinalis* (Abdi *et al.,* 2008). The mechanism of action of nano particles especially silver nanoparticles in the control of microbial contaminants in meristamatic tissues culture include; adhesion to microbial cell membranes (Habash *et al.,* 2014), penetration inside the cells (Singh *et al.,* 2015), reduce oxygen species and free radical generation, induced cellular toxicity and oxidative stress, modulation of microbial signal transduction pathways, among others (Kim *et al.,* 2011). However, silver nanoparticles exposure induces cytotoxicity, genotoxicity, and inflammatory response in explants in a cell-type dependent manner. This has raised concerns regarding use of AgNPs in the control of microbial contaminants in meristamatic tissues culture (Dakal *et al.,* 2016).

Conclusion

Epiphytic bacteria, fungi and sometimes viruses are common microbial contaminants frequently reported in *in vitro* meristematic tissue cultures. Most of these epiphytic microbes are usually removed by surface sterilization, but the endophytic bacteria, fungi and viruses may persist to contaminate the meristematic tissue culture. Conventional control methods such as chemotherapy and thermotherapy used in meristematic culture media have been acknowledged by several scholars to reduce both epiphytic and endophytic microbial contaminants in meristematic tissue culture at significant levels and are widely applied over non-conventional methods like cryotherapy, application of disinfectants, antiseptics and nano particles due to ease of adaption, highly commercialized and posed little or no side effects to the explant.

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