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Literature Review

Control of Contamination of Tissue Plant Cultures During in Vitro Clonal Micropropagation

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Abstract: Plant micropropagation is currently an important method for modern large-scale propagation of valuable plant crops. Among the risks limiting the success of this method is the contamination of cultured plants. Conventional surface sterilization methods are not always effective against internal infection of plant tissues, and secondary contamination of tissue cultures during replication is also a major problem. This causes scientific laboratories to lose valuable experimental material and commercial enterprises to incur significant losses. The aim of this review is to analyze and demonstrate the causes and tools for controlling contaminating microflora during in vitro plant propagation in light of recent research. This review considers and analyzes the main contaminants of plant cultures and examines the effect of saprophytic and endophytic contaminants on plant tissue culture, showing that not only the plant species but also the plant variety may have different sensitivities to sterilizing agents and exposure duration. Many studies in this direction have been conducted worldwide, the results of which are often narrowly specific for particular plant cultures. This review analyzes the main approaches to address the contamination of different plant crops during clonal micropropagation. The review is aimed at reducing losses of plant crops through the use of different sterilization methods and the involvement of new-generation sterilizing agents in the process.

Keywords: Contaminants, Disinfectants, Microorganisms, Plant Tissue Culture, Sterilization

Introduction

Clonal micropropagation of plants is an important method in modern plant breeding. Currently, it is a method of large-scale multiplication of plant crops based on the technology of in vitro cultivation, which has become the basis for obtaining valuable plant material due to the ability to work on increasing the volume of plant material in a short time and in a relatively limited space, regardless of climatic and seasonal conditions (Abdalla *et al.*, 2022). This method has undeniable advantages over traditional methods of vegetative propagation (Iacuzzi *et al.*, 2023). For example, in France, 94% of all floral production is obtained by this method. About 100 commercial factories in the USA produce planting material by clonal micropropagation, and 5 of these commercial factories have a capacity of 15-20 million plants per year. In Western Europe, there are 248 commercial laboratories with a total annual production of 212 million plants per year.

Micropropagation is a valuable method for solving many seed production problems, e.g., for the multiplication of most horticultural and ornamental crops and other plants with high heterozygosity and clonal micropropagation can be used to quickly obtain virus-free planting material (Podwyszyńska *et al.*, 2022). For example, in potato seed production, it is the basis for the production of primary seeds (Zhang *et al*., 2022; Singh *et al.*, 2015; Azad *et al*., 2020). Micropropagation is also widely used to obtain bioactive compounds of plant origin (Espinosa-Leal *et al.*, 2018; Chandran *et al.*, 2020; Hasnain *et al.*, 2022; Ahmadpoor *et al.*, 2022).

Advances in plant biotechnology offer new opportunities for selection, collection, propagation, and short-term and long-term conservation of plant diversity

using in vitro cultivation techniques (Mukherjee *et al.*, 2018; Thakur *et al.*, 2021). Significant progress has been made in the conservation of endangered, rare agricultural, ornamental, medicinal and forest species, especially unorthodox seed and vegetatively propagated plants of temperate and tropical origin (Limera *et al.*, 2017; Pe *et al.*, 2020; Dhiman *et al.*, 2020; Mantovska *et al.*, 2021).

The in vitro cellular system is a convenient model for studying the complex mechanisms of proliferation, cell differentiation, histogenesis, organogenesis, somatic embryogenesis and whole organism regeneration from isolated cells with totipotency. Based on cell technologies, cell selection, genetic and cell engineering methods have been developed (Cüce and Sökmen, 2017; Anikina *et al.*, 2021; Hossain *et al.*, 2021; Karki *et al.*, 2021).

The method of clonal propagation includes obtaining an initial sterile regenerant plant of a particular cultivar that is free of infection by phytopathogens and further replicating this sterile culture under in vitro conditions to the required volumes associated with the cultivation target (Podwyszyńska *et al.*, 2022). The replication stage includes a long period of time during which cuttings and passages of explants to newly prepared nutrient media are carried out. During this period, there is a high risk of contaminating microorganisms developing in the sterile vessels in which the plants are grown, which, by actively multiplying, can negate all previous efforts to grow plant crops (Abdalla *et al.*, 2022).

The main prerequisite for the successful production and cultivation of plant cell cultures is the sterilization of plant objects, which consists of destroying fungal and bacterial spores present on the plant without damaging the internal tissues (Gammoudi *et al.*, 2022). Compounds containing active chlorine (sodium hypochlorite, calcium hypochlorite, chloramine), mercury preparations (Mercuric Chloride, Diacid) and oxidizing agents (Hydrogen Peroxide, Potassium Permanganate), ethyl alcohol, less frequently concentrated sulfuric acid, silver nitrate and antibiotics are used for the sterilization of plant tissues (Meng *et al.*, 2014; Putri *et al.*, 2019; Gerszberg and Grzegorczyk-karolak, 2019; Mohamad Puad *et al.*, 2022).

The problem of improving the efficiency of plant tissue sterilization when working with in vitro plant tissue culture remains relevant (Dagne *et al.*, 2023). However, many studies have been conducted in this direction, including Orlikowska *et al.* (2017), Singh (2018); Hesami *et al.* (2019); Izarra (2020); Gammoudi *et al.* (2022); Babu *et al.* (2022); Kidasi *et al.* (2023) and others. According to these authors, the choice of sterilizing agent and the duration of exposure depends on the characteristics of the explant. The more delicate the plant tissue, the lower the concentration of sterilizing agent should be in order to maintain its viability. In this case, not only the plant species but also the cultivar may have differences in sensitivity to sterilizing agents and exposure time (Anikina *et al.*, 2020; Baharuddin *et al.*, 2023; Carriel *et al.*, 2023). But equally important is the problem of controlling secondary contamination when contamination occurs during the mass multiplication of plant crops in vitro (Babu *et al.*, 2022). This causes the loss of valuable experimental plant material in research laboratories, as well as significant losses to commercial enterprises (Okoroafor, 2022). The high importance of this problem has led to attempts to solve it, including using artificial neural network-based models (Hesami *et al.*, 2019; Gammoudi *et al.*, 2022).

The aim of this review was to investigate the proposed methods for sterilization of plant tissues during in vitro culture introduction, types of contaminating microbial cultures and approaches to prevent and control secondary contamination during cultivation.

Traditional Methods of Plant Tissue Sterilization

The condition of the mother plant is one of the factors that determine the success of obtaining an aseptic culture as a source of explants. Mother plants in the field are more infected with spores and fungal pathogens. The mother plant must be pre-cultivated before creating a stock collection to produce aseptic explants. For example, the sprouts of sweet potato (*Ipomoea batatas* (L.) Lam) from tubes were transplanted into the soil and watered with Dimanin (2 ml/L) before being initiated into in vitro culture to eliminate latent bacteria (Izarra *et al.*, 2020). The mother plants of Aglaonema were previously maintained without watering for 2 months to reduce the accumulation of pathogenic flora. Water stress can inhibit the growth of fungi in the vascular tissue of plants (Chen and Yeh, 2007). An effective sterilization method in plant in vitro culture is a method that can eliminate bacterial and fungal contaminants while still producing regenerative explants. Therefore, a case-specific sterilization protocol according to the contamination involved (Saporta *et al.*, 2017; Anikina and Abiyeva, 2022). The sterilization methods applied for different species are given in Table (1).

There are different types of microorganisms that exhibit varying resistance to sterilizing agents. Mose (2015) indicated that as pollutants exhibiting persistent resistance to disinfectants based on hypochlorite, the following microorganisms should be noted: *Staphylococcus aureus, Streptococcus enterococcus, Pseudomonas aeruginosa, Clostridium difficile, Salmonella, E. coli, Acinetobacter baumannii and Mycobacterium tuberculosis.*

Table 1: Sterilization method applied in different plant species

Plants	Treatment	Reference
Solanum tuberosum L.	0.1% $HgCl_2$ solution with gentle shaking for 2–8 min	Azad *et al.* (2020)
Fabiana imbricata	0.1% solution of $HgCl_2$ for 1 min	Halkoglu *et al.* (2019)
Allium cepa L.	70% ethanol for 90 sec 20% NaClO for 5 min	Keighobadi *et al.* (2020)
Vaccinium uliginosum L.	70% ethanol for 1 min 3% NaClO for 15 min	Cüce and Sökmen (2017)
Hordeum vulgare	20% NaClO for 15 min three following treatments with distilled water for 5 min each treatment	Hazrati *et al.* (2019)
Malus domestica	70% ethanol 2 sec 0.1–0.2% HgCl for 15 min	Magyar-Tábori *et al*. (2011)
Mimosa pudica L.	70% ethanol 1 min 0.1% HgCl for 8 min	Hassan *et al.* (1970)
Humulus lupulus L.	70% ethanol for 5 min 30% NaClO for 20 min	Iacuzzi *et al.* (2023)
Vitis vinifera	1.62% NaOCl for 14 min	Dagne *et al.* (2023).
Ocimum basilica, Withania somnifera Rauvolfia tetraphylla	10 min wash in 3.15% calcium hypochlorite, followed by a 15 min wash in 10% sodium hydrogen carbonate and 2 min wash in 1% sodium azide	Waheeda and Shyam (2017)
Solanum tuberosum L.	UVC irradiation for 5 min, followed by 1% Sodium hypochlorite (NaOCl) for 10 min	Gangopadhyay *et al.* (2017)
Melia azedarach L.	benomyl (2 g/l) for 2 h and then 7% H_2O_2 for 10 min and NaOCl 2% for 12 min	Ahmadpoor *et al.* (2022)
Different plants	0.1% mercury chloride ($HgCl_2$). Further, it was dipped in 70% ethanol for 30 seconds. Then, it's dipped in antibiotic solution Bavistin for 10 min	Dangariya *et al.* (2020)
Fragaria ana nassa	200 mg/L AgNPs solution for 20 min	Tung *et al.* (2021)
Psidium friedrichsthalianum	5 mg/L AgNPs as a permanent bilayer	Andújar *et al.* (2020)
Limonium sinuatum (L.)	200 mg/L AgNPs for 20 min	Cuong *et al.* (2023)

Furthermore, Mukherjee *et al.* (2018) used a more complex sterilization technique. Nodal segments of *Ramie (Boehmeria nivea)*, 8-10 cm in length, were excised and kept in an ice-cold antioxidant solution containing 0.15% citric acid and 0.1% ascorbic acid for 20 min. The explants were washed thoroughly under running water for 5 min. Under aseptic conditions, they were snap-dipped for 10 sec in 70% ethyl alcohol, followed by immersion in 1 (N) H_2SO_4 for 1 min and quick dipping in 0.5 (M) Na2CO3 solution. Then, they were washed in distilled autoclaved water 4-5 times and dipped in 2.0% NaClO with 0.1% Tween 20 for 30 min and subsequently washed with distilled autoclaved water 7-8 times.

According to the results obtained by Zhang *et al.* (2022), among the six sterilization options for stem explants of Castanopsis hystrix, the best result was obtained with a combination of 1% (vol) benzalkonium bromide for 2-4 min and 0.1% (wt) of sublimate containing 2-3 drops of Tween 80 for 1-4 min. The survival rate of explants was up to 75%, and the contamination rates were the lowest. These studies have proven that multi-stage disinfection can significantly reduce the contamination of explants. This is consistent with the results of other researchers (Kuppusamy *et al.*, 2019).

The main limitation of the effectiveness of tissue sterilization during in vitro culture is the sensitivity of plant tissues to the toxic effects of sterilizing agents, as well as the accumulation of internal infection (Gammoudi *et al.,* 2022; Agbadje *et al.*, 2021; Anikina and Abiyeva, 2022).

The success of plant tissue sterilization is influenced by many factors, such as explant type, temperature, pH of the medium, types of contaminants, level of contamination, type and dosage of biocidal agents and exposure time. All this determines the complexity and non-linearity of this dynamic system. At the same time, it opens up opportunities for modelling and optimization of this system with the help of artificial intelligence. According to Dagne *et al.* (2023), the use of artificial intelligence-based simulation processes and optimization algorithms provides more accurate and optimal in vitro disinfection conditions compared to traditional optimization methods. In a study by Pepe *et al.* (2021), a high level of prediction (R^2>0.91) was obtained using a Generalized Regression Neural Network (GRNN). At the same time, the authors used a Genetic Algorithm (GA) to determine the optimal type and dosage of disinfectants and exposure time to determine the most effective method of contamination reduction. As a result of the optimization, a cannabis sterilization regimen was determined that should have used 4.6% sodium hypochlorite along with 0.008% hydrogen peroxide for 16.81 min. A validation experiment confirmed that this protocol provided complete clearance of contamination (Pepe *et al.*, 2021). The great prospects for the use of artificial neural network-based models in clonal micropropagation techniques are also confirmed by Hesami *et al.* (2019; 2021); Gammoudi *et al.* (2022); Dagne *et al.* (2023).

Use of Disinfectants and Nanoparticles in Culture Media

The use of disinfectants, antiseptics and nanoparticles in culture media has been proposed to address microflora contamination problems in in-vitro coding (Permadi *et al.,* 2023). In a study by Weber *et al.* (2015), the introduction of a 5% NaOCl solution into the medium was suggested to reduce the cost of potato clonal micropropagation. According to the author, this suppressed microbial development without inhibiting plant growth (Weber *et al.,* 2015).

Brondani *et al.* (2013) demonstrated that concentrations of 0.001-0.003% available chlorine in the culture medium, even without autoclaving, contributed to the suppression of contaminant microflora and the formation of nodal segments of *Eucalyptus benthamii* in vitro was the same as when a traditionally prepared nutrient medium was used. Other researchers have also concluded that the use of active chlorine during cultivation is an effective method of suppressing contamination and has significant potential for cost reduction in plant culture systems (Teixeira *et al.,* 2006; Tiwari *et al.,* 2012; Peiris *et al.,* 2012; Luna *et al.,* 2013).

The addition of active chlorine to the medium maintains the stability of heat-sensitive substances such as growth regulators and vitamins (Peiris *et al.,* 2012).

Positive results have also been obtained when using nanoparticles against microbial contamination in plant tissue cultures (Andújar *et al.,* 2020; Nazir *et al.,* 2024).

Some of the most utilized types of nanoparticles with antimicrobial properties include metal nanoparticles (e.g., Au, Ag, Pt, Pd, Ni, Cu, Se, Zn, Fe) (Alphandéry, 2020). Silver nanoparticles, according to Solís-Sandí *et al.* (2023), are of the greatest interest as a biocidal agent for biological systems. Their small size contributes to their high biocompatibility due to internalization through cell membranes (Algotiml *et al.,* 2022). Silver nanoparticles have been found to inhibit the growth of many bacteria, including multidrug-resistant bacteria and also possess antifungal and antioxidant properties (Khalil *et al.,* 2021). This is due to the ability of nanoparticles to bind to enzymes and proteins vital for microorganisms and their effect on cellular respiration, membrane permeability, replication and transcription (Bruna *et al.,* 2021).).

The antimicrobial activity of metallic nanoparticles against bacteria, fungi and even viruses has been proven many times and is actively used in various fields of human activity, including medicine (Makvandi *et al.,* 2020). At a concentration of 200 mg/L, zinc nanoparticles and zinc oxide nanoparticles exhibit antibacterial properties and no antagonistic effect in plant tissue cultures (Helaly *et al.,* 2014). Similar results were obtained for silver nanoparticles (AgNPs), which, at concentrations ranging from 20 to 100 mg/L, inhibited bacterial contamination in *Valeriana officinalis* tissue cultures (Abdi *et al.,* 2008). (AgNPs and ZnO NPs) in the control of exo- and endophytic contaminants in banana tissue culture.

The positive effect of silver nanoparticles on deterring contaminant microflora in *Rosa hybrida L.* multiplication was reported by Shaafi *et al.* (2022).

Interest in the use of nanoparticles in nutrient media continues to grow, and methods to enhance the activity of nanoparticles in media are already being developed. For example, Gimenez-Ingalaturre *et al.* (2022) proposed adding surfactants like Tween 80 and Triton X100 to the nutrient media to prevent aggregation of silver nanoparticles, which will increase the stability of nanoparticles.

Compared to conventional biocidal agents, it has been observed that nanoparticles for controlling contaminants in plant tissue culture have undeniable advantages (Andújar *et al.,* 2020; Cuong *et al.,* 2023).

Nazir *et al*. (2024) demonstrated the advantages of Zinc Oxide (ZnO) and silver (Ag) nanoparticles compared to conventional biocides used to prevent contamination, such as ethanol, NaOCl, $H2O_2$ and $HgCl_2$ in banana culture. The studies of Andújar *et al*. (2020) demonstrated the efficacy of the biocidal action of AgNPs as a liquid layer on a semi-solid nutrient medium for in vitro contamination control on *Psidium friedrichsthalianum* culture, the number of contaminated plants decreased by 30%, while the authors observed an increase in leaf area by 560% and multiplication rate by 180%.

Cuong *et al.* (2023) also found not only a higher disinfectant effect compared to the traditional biocide $HgCl_2$ but also a better effect on shoot growth than explants treated with 1000 mg/L $HgCl_2$ for 5 min. When using a nutrient medium supplemented with 1.0 mg/L AgNPs, a positive effect of nanoparticles on the development of *Limonium sinuatum (L.)* plants was observed, and the number of shoots increased more than 2-fold.

There are some controversies regarding the use of nanoparticles for controlling microbial contaminants in plant tissue culture. In addition to their proven efficacy in suppressing contaminants (Kim *et al.,* 2011; Singh *et al.,* 2015), the results indicate the cytotoxicity of metal nanoparticles towards explants (Dakal *et al.,* 2016; Xiong *et al.,* 2022). Liao *et al*. (2019) also pointed out the high risks associated with the cytotoxicity of metal nanoparticles, particularly silver nanoparticles, on living cells. Additionally, the widespread introduction of metal nanoparticle preparations into the production cycle will increase environmental risks, which is unacceptable (Beddow *et al.,* 2017; Tlili *et al.,* 2017). The great interest in AgNP preparations is of concern to the world community because the expansion of the use of silver nanoparticles will increase their entry into the aquatic natural environment, where they are used to form already toxic Ag ions. At present, there is no information on the

long-term safety and subsequent adverse effects of nanoparticles for humans. On the contrary, toxic effects of AgNPs on human cell cultures in vitro have been identified (Jiang *et al.,* 2018; Liao *et al.,* 2019).

Numerous studies have established that long-term oral and inhalation exposure to silver can cause chronic microvascular diseases of the skin and eyes in humans (Miyayama *et al.,* 2016; Li *et al.,* 2018). In vivo animal studies have revealed the toxic effects of AgNPs in rodents by accumulation in their liver, spleen and lungs (Liao *et al.,* 2019). There are also no long-term studies on the effect of nanoparticles on the genotype of plant organisms, in particular on self-clonal variability, which is inadmissible when obtaining certain varietal material. This still limits the use of nanoparticles for the control of microbial contaminants in plant tissue culture.

Conclusion

Due to the great importance of the microtonal propagation method for solving crop production problems, there is a need to reduce the risk factors limiting the success of this method. Contamination of plant tissue cultures undoubtedly belongs to such factors. The main limitation of the success of tissue sterilization when introduced into culture in vitro is the sensitivity of plant tissues to the toxic effects of sterilizing agents, as well as the accumulation of internal infection in the tissues. Contamination problems are often associated with the fact that standard sterilization methods are not effective enough to obtain aseptic explants. Chemical sterilization methods have undergone significant changes in the development process from traditional treatments with one of the preparations containing active chlorine (Sodium hypochlorite, calcium hypochlorite, chloramine) or a mercury preparation (Mercuric chloride, diacid) or oxidants (Hydrogen peroxide, potassium permanganate) or ethyl alcohol to combined methods in which several methods are used sequentially with biocidal agents. Multi-stage sterilization using various biocidal agents has proven its advantage in overcoming endogenous infections when introducing plant tissues into the culture. Good prospects are opening up with the use of artificial neural network-based models in the development of effective sterilization protocols for tissue cultures. This makes it possible to develop more accurate and optimal sterilization protocols for a specific crop in specific growing conditions. Another approach to combating contaminants in tissue culture is the use of antiseptic drugs and nanoparticles in culture media, which has significant potential to reduce costs in plant cultivation systems. Thus, the concentration of 0.001-0.003% of available chlorine in the culture medium, even without autoclaving, contributes to the suppression of contaminant microflora while plant development is not suppressed.

Wide opportunities are opening up with the use of nanotechnology to solve the problem of combating contamination. Compared to traditional biocidal preparations, nanoparticles have undeniable advantages for the control of contaminants in plant tissue culture. For example, silver nanoparticles are of great interest as a biocidal agent for biological systems. Their small size contributes to their high biocompatibility due to internalization through cell membranes; they effectively inhibit the growth of many bacteria, including multidrug-resistant bacteria, as well as have antifungal and antioxidant properties. In addition, they have a positive effect on plant development. However, there are high risks associated with the cytotoxicity of metal nanoparticles. There are also no long-term studies on the effect of nanoparticles on the genotype of a plant organism, in particular on self-clonal variability, which is unacceptable with clonal micro-multiplication of varieties. In addition, the widespread introduction of drugs with metal nanoparticles into the production cycle will lead to an increase in environmental risks. In this regard, this area requires further long-term research, but it seems to be the most promising.

Antibiotics have become the most widespread in the fight against secondary infection and the liberation of tissues from entophytic microflora. When using antibiotics to treat in vitro infection, the limiting factor is the thermal stability of antibiotics and low effectiveness against all types of pollutants. In addition, they are toxic directly to explants since they damage the protein-synthesizing apparatus of cells and inhibit the formation of phytohormones, which leads to a decrease in the survival, growth and development of plants.

The development of reliable, universal sterilization methods is of utmost importance; it is necessary to prevent secondary contamination and increase the effectiveness of plant micro-propagation methods *in vitro*.

The development of standard effective protocols for the sterilization of multifunctional action for various tissue cultures will help to increase the profitability of production cycles for the reproduction of economically important plant crops, increase their survival rate and increase the volume of planting material for further successful cultivation and production of valuable products for humanity.

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Author's Contributions

Irina Anikina: Authored the initial draft of the manuscript.

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Dora Dayu Rahma Turista, Zhursinkul Tokbergenova: Critically reviewed and provided substantial edits to the manuscript.

Ethics

The corresponding author acknowledges that other authors have reviewed and approved this manuscript; no ethical issues are involved.

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