Scientific Papers. Series B, Horticulture. Vol. LXX, No. 1, 2026
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

MICROBIAL CONTAMINATION IN PLANT TISSUE CULTURE - A REVIEW

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Abstract

Microbial contamination remains one of the most important biological limitations affecting plant tissue cultures used in both horticultural research and commercial micropropagation. Despite continuous improvements in sterilization protocols and aseptic techniques, contamination continues to compromise culture establishment, multiplication efficiency and plant quality. The present review critically synthesizes classic and recent literature addressing the sources of microbial contamination, the types of microorganisms involved, and their biological and physiological impact on in vitro explants. Particular emphasis is placed on latent endophytic microorganisms and secondary contamination arising during repeated subcultures, which challenge the traditional concepts of surface sterilization and absolute axenic culture. During the comparison, the effectiveness, limitations, and applicability of conventional sterilization methods and emerging integrated contamination control strategies are highlighted. In conclusion, the review emphasizes a conceptual shift from complete microbial elimination to case-specific, biologically informed contamination management and discusses future research perspectives aimed at improving the reliability and sustainability of horticultural micropropagation.

Key words*: plant tissue culture, in vitro micropropagation, microbial contamination, endophytic bacteria, fungal contamination, aseptic cultures.*

INTRODUCTION

Plant tissue culture (micropropagation) has revolutionized modern agriculture, horticulture, and forestry, enabling the rapid large-scale multiplication of disease-free plants, preserving endangered species, and facilitating genetic manipulation (George et al., 2008; Rout et al., 2021; Yapa and Bandalaralage, 2023). The success of this technology is based on the ability to keep explants (cells, tissues or organs) in an artificial, strictly controlled and aseptic environment. However, biological contamination remains a pervasive threat and a major challenge, often compromising months of research and generating substantial financial losses in commercial laboratories (Cassells, 2012; Leifert and Cassells, 2001).

The inherent vulnerability of *in vitro* cultures derives from the very nature of the culture media used. The basal medium formulated by Murashige and Skoog (1962), used almost universally in various variants, is extremely rich in inorganic salts, sources of carbon (sucrose) and vitamins. This high availability of nutrients, combined with optimal incubation temperatures (usually 22-25°C) and high humidity in culture vessels, creates an ideal environment not only for plant cells, but also for the explosive proliferation of heterotrophic microorganisms (Murashige and Skoog, 1962; Permadi et al., 2023).

Historically, efforts have focused on obtaining completely axenic cultures (an *in vitro* culture containing only the intended plant tissue and no detectable contaminating microorganisms). Contaminants have been regarded exclusively as pathogens or "vitropathogens" (environmental microorganisms that become pathogenic under *in vitro* conditions) (Cassells, 2001). However, research in recent decades has highlighted a much more complex reality. As Herman (2017) points out, the concept of completely sterile plant culture is often an illusion, with many plants hosting dormant endophytic microbial communities that can survive rigorous surface

sterilization procedures. This paradigm shift has led to a clear distinction between harmful contaminants (which cause necrosis, hyperhydricity, or explant death) and neutral or even beneficial endophytes, which can promote plant growth and stress resistance in the *ex vitro* acclimatization phase (Orlikowska et al., 2017; Quambusch et al., 2014).

This review article aims to provide a detailed and up-to-date analysis of the phenomenon of in vitro contamination. The types of contaminants, sources of infection, physiological impact on plants, as well as the evolution of detection methods and eradication strategies will be explored, integrating the classic reference literature with the latest discoveries in the field of plant biotechnology.

MATERIALS AND METHODS

In order to carry out this scientific review, a rigorous literature selection methodology was adopted, inspired by the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. The search was carried out in the main academic databases: Web of Science, Scopus, PubMed, Google Scholar and the MDPI platform. Combinations of keywords such as: "plant tissue culture contamination", "*in vitro* microbial hazards", "endophytic bacteria micropropagation", "virus elimination tissue culture", "mycoplasma detection in vitro", and "sterilization protocols explants" were used.

The selection criteria aimed to include the fundamental works, internationally recognized, developed by the pioneers of the field (e.g. Cassells A.C., Leifert C., Herman E.B., Murashige T., Skoog F., Reed B.M., Viss P.R.). To ensure the timeliness of the information, special attention was paid to articles published between 2015 and 2025 in journals with a high impact factor (Q1-Q2), especially from the MDPI portfolio (Plants, Horticulturae, Agronomy, International Journal of Molecular Sciences), as well as from prestigious journals such as Plant Cell, Tissue and Organ Culture, Acta Horticulturae and Scientia Horticulturae.

Studies that did not present verifiable data or that did not specifically focus on the plant *in vitro* environment were excluded. In total, more than 35 scientific articles, book chapters and reviews were selected and analyzed in depth, the data being synthesized to cover all aspects of contamination management: from etiology and diagnosis, to treatment and biotechnological perspectives.

RESULTS AND DISCUSSIONS

Types of contaminants

Biological contamination in plant tissue culture is a heterogeneous phenomenon, involving a diversity of organisms that can be classified according to their biological nature and how they interact with the host plant. Leifert and Cassells (2001) classified microbial hazards into bacteria, fungi (including yeasts), viruses, viroids, mycoplasmas, and microarthropods.

1. Bacteria are the most common and persistent form of contamination *in vitro*. They can be divided into two major categories: epiphytes (which colonize the surface of the explant) and endophytes (which reside in the intercellular spaces or vascular system of the plant) (Orlikowska et al., 2017). While epiphytic bacteria can usually be eliminated by standard surface sterilization protocols, endophytic bacteria are protected inside the tissues and can manifest weeks or months after culture initiation (Reed et al., 1995). Commonly isolated bacterial genera include *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Lactobacillus*, *Agrobacterium*, and *Enterobacter* (Leifert et al., 1989). A particular case is *Agrobacterium tumefaciens*, used as a vector in genetic transformation, whose incomplete post-transformation elimination is a major problem, requiring specific control methods (Viss et al., 1991).

Quambusch et al. (2014) identified diverse endophytic bacterial communities in cultures of *Prunus avium* (cherry), demonstrating that difficult-to-propagate genotypes harbored endophytic populations dominated by *Pseudomonas* spp. and *Curtobacterium* spp. - distinctly different from those present in easily propagated genotypes. In *Rubus idaeus* (raspberry) cultures, Trzewik et al. (2025) isolated and identified three persistent bacterial contaminants via DNA sequencing: *Pseudomonas* sp. (Gram-negative, cultivar 'Polka'), *Curtobacterium* sp. (Gram-positive, cultivar 'Norna'), and *Luteibacter* sp. (Gram-negative, cultivar 'Polana'). The researchers

noted that these nitrogen-fixing bacteria did not cause immediate visible symptoms but significantly impaired survival during cold storage (4°C): contaminated cultures became necrotic within 4-6 months, whereas decontaminated cultures survived.

For *Fragaria* × *ananassa* (strawberry), Reed et al. (1995) demonstrated the presence of latent bacteria in 40-60% of explants tested via indexing on multiple bacteriological media, with *Xanthomonas fragariae* frequently isolated from apparently healthy runners.

2. Fungi and yeasts. Fungal contamination is usually more noticeable and develops faster than bacterial contamination. Fungal spores are ubiquitous in laboratory air and on the surface of donor plants. Genera such as *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and *Cladosporium* are frequently reported (Permadi et al., 2023). Fungi form mycelial structures (hyphae) that can quickly cover the explant and culture medium, suffocating the plant and depleting nutrients. Yeasts (e.g., *Candida*, *Rhodotorula*) often manifest as mucous colonies, similar to bacterial ones, and can ferment sugars in the environment, drastically altering pH (Leifert and Cassells, 2001).

In *Rosa* sp. cultures, *Botrytis cinerea* and *Fusarium* spp. constitute the dominant fungal contaminants during culture initiation from field buds, particularly during the rainy season (Balo, 2023). Micropropagation studies of *Fragaria* × *ananassa* report fungal contamination with *Botrytis* spp. and *Alternaria* spp. in 10-25% of explants initiated during warm months; sterilization with 1% sodium hypochlorite for 10 minutes significantly reduces this rate (Anikina et al., 2025).

In *Prunus persica* (peach) and *Prunus avium*, contamination with *Pseudomonas syringae* pv. *syringae* is frequent; Brison et al. (1997) reported that treatment with 250 mg/L cefotaxime reduced bacterial contamination to under 5%.

3. Viruses and Viroids. Unlike bacteria and fungi, viruses and viroids do not grow on the culture medium, but multiply exclusively inside plant cells. Their presence does not cause turbidity of the environment, which makes them invisible to a routine macroscopic inspection. Their passage into the *in vitro* culture is done systemically, with the explant taken from an infected mother plant. Although meristem culture is the standard technique for eradicating viruses, success is not 100% guaranteed, and viruses such as Plum pox virus (in *Prunus* species) or Yam mosaic virus often require combination therapies (thermo- or cryotherapy) to be completely eliminated (Brison et al., 1997; Elansary et al., 2024).

The Plum pox virus (PPV, "Sharka") severely affects micropropagation cultures of *Prunus* species - peach (*Prunus persica*), apricot (*Prunus armeniaca*), and plum (*Prunus domestica*). Szabó et al. (2024) synthesized literature on virus elimination from *Prunus* spp. and found that meristem culture alone has a highly variable efficacy (20-80%), depending on the species and viral load, whereas the combination of thermotherapy (37°C, 4-6 weeks) + meristem culture increases efficacy to 60-95%. Brison et al. (1997) demonstrated that cryotherapy on a *Prunus* rootstock experimentally infected with PPV eliminated the virus completely; the cryopreserved meristems regenerated healthy plants confirmed by ELISA and RT-PCR.

4. Mycoplasmas (Phytoplasmas). Phytoplasmas are bacteria without a cell wall, necessarily parasites, which colonize the phloem of plants. Due to their extremely small size and membrane flexibility, they can pass through standard microbiological filters (0.22 μm or 0.45 μm) used for cold sterilization of liquid media or thermolabile supplements (Nikfarjam and Farzaneh, 2012). Mycoplasma contamination is insidious, causing abnormalities of growth, yellowing and abnormal proliferation of shoots ("witch broom" symptoms), being extremely difficult to eradicate with conventional antibiotics (Carrillo-Ávila et al., 2023).

5. Microarthropods (mites and thrips). Although they are not microorganisms, microarthropods ("vitro pests") are a major biological vector for cross-contamination in growth chambers. Mites and thrips may enter culture vessels through inadequate ventilation systems or imperfect closures, acting as mechanical vectors of fungal spores and bacteria and facilitating cross-contamination among *in vitro* cultures (Cassells, 2012; Leifert and Cassells, 2001).

Table 1. Summary of the main types of contaminants in plant tissue culture

Contaminant Type	Common Examples	Primary Source	Manifestation / Symptoms	Eradication Difficulty	References
Epiphytic bacteria	*Pseudomonas* spp., *Bacillus* spp., occasionally *Staphylococcus* spp. (handling-related contamination)	Explant surface, water, air, laboratory environment, operator contact	Turbidity in medium; mucilage/biofilm at explant base; growth inhibition; reduced regeneration	Moderate (surface sterilization reduces contamination, efficiency depends on explant type, mkicrobial load and protocol)	Leifert et al. (1989); Reed et al. (1995); Orlikowska et al. (2017)
Endophytic bacteria	*Enterobacter* spp., *Curtobacterium* spp., *Luteibacter* spp., occasionally *Agrobacterium* spp. *Xanthomonas fragariae*	Internal tissues of mother plants (*Prunus, Rubus, Musa*), latent infections in stock plants	Delayed contamination (weeks–months); internal necrosis; growth inhibition; culture decline or failure	Very high (systemic localization makes elimination difficult; antibiotics may suppress but rarely eliminate; phytotoxicity is common)	Quambusch et al. (2014); Viss et al. (1991); Cassells (2012); Trzewik et al. (2025)
Fungi	*Aspergillus* spp., *Fusarium* spp., *Botrytis cinerea*, *Penicillium* spp. *Pseudomonas syringae*	Laboratory air, personnel, greenhouse-derived explants, contaminated vessels	Mycelial growth (white, green, black); sporulation; tissue browning and necrosis; incidence varies depending on species and culture conditions	Moderate to high (fungal spores are highly resistant; thermotherapy and aseptic techniques may reduce contamination depending on pathogen and conditions)	Permadi et al. (2023); Anikina et al. (2025); Balo (2023); Cassells (2012)
Viruses	*Plum pox virus* (PPV), Grapevine leafroll-associated viruses (GLRaVs), *Chrysanthemum virus B* (CVB)	Systemically infected mother plants	Foliar mosaic, chlorosis, deformation; often asymptomatic in *in vitro* cultures; detected via RT-PCR or ELISA-based methods	High (meristem culture and thermotherapy may reduce virus presence; success is variable and virus-dependent)	Brison et al. (1997); Szabó et al. (2024); Elansary et al. (2024); Gao et al. (2023)
Phytoplasmas	*Candidatus Phytoplasma vitis*, *Candidatus Phytoplasma solani*	Infected mother plants and vegetative propagation material	Abnormal shoot proliferation; stunting; chlorosis; witches' broom;	Very high (phytoplasmas are phloem-limited and difficult to eliminate; antibiotic treatments may temporarily suppress infection but do not ensure eradication)	Nikfarjam and Farzaneh (2012); Carrillo-Ávila et al. (2023); Cassells (2012)
Microarthropods	*Rhizoglyphus* spp. (mites), *Frankliniella* spp. (thrips)	Contaminated plant material (explants), greenhouse-derived material, laboratory entry through ventilation or inadequate sealing	Mechanical damage to explants; tissue collapse; facilitation of secondary fungal and bacterial contamination; culture loss	Moderate (prevention through insect-proofing, sanitation, quarantine of plant material, and regular inspection)	Cassells (2012); Leifert and Cassells (2001)

Sources of Contamination

Accurately identifying the source of contamination is the critical first step in implementing an effective quality assurance (HACCP) system in micropropagation laboratories (Cassells, 2012). Sources are often multiple and deeply interconnected.

1. The Initial Explant (Mother Plant)

Plant material collected from natural environments (fields, greenhouses) is heavily colonized by a multitude of microorganisms. Explants derived from plant parts close to the soil (roots, rhizomes, basal stems) exhibit a significantly higher degree of contamination compared to apical buds. Furthermore, the health status of the mother plant dictates the presence of systemic pathogens (viruses, endophytic bacteria). Irrigating stock plants with unfiltered tap water can introduce resistant

bacteria that will subsequently manifest in culture (Snyman et al., 2011).

2. Culture Medium and Water

Incorrect preparation of the culture medium is a frequent source of failure. Insufficient autoclaving (insufficient sterilization time or temperatures below 121°C) fails to destroy bacterial endospores (e.g., *Bacillus* spp.) or thermoresistant fungal spores. Additionally, the use of contaminated distilled or deionized water, stored improperly, can introduce microorganisms that form highly resistant biofilms on the walls of storage vessels (Karpinski et al., 2021).

Endospores of *Bacillus subtilis*, frequently present in laboratory dust and on work surfaces, easily survive standard autoclaving temperatures if the sterilization cycle falls below 121°C/15 min. Microbiological analyses of distilled water stored under standard laboratory conditions have detected biofilms of *Pseudomonas* spp. and *Sphingomonas* spp. after just 48-72 hours, underscoring the critical importance of using water immediately after preparation or storing it under strictly sterile conditions (Leifert & Cassells, 2001).

3. Laboratory Environment and Air

Laboratory air acts as a major vector for fungal spores and bacteria-carrying dust particles. Poorly maintained ventilation systems, the absence of HEPA filters at the room level, or air currents created by personnel movement can introduce contaminants directly into culture vessels during subculturing. Laminar flow hoods, if not tested and certified periodically, can suffer breaches in the sterile air flow, allowing contaminated outside air to penetrate the workspace (Balo, 2023). Cassells (2012) strongly recommends testing laminar flow hoods every 6-12 months and replacing HEPA filters when filtration efficiency drops below 99.97% for 0.3 µm particles.

4. Personnel and Instruments

Human error and deficient aseptic techniques represent major causes of secondary contamination (during subculturing). The operator's hands, breath, hair, and clothing are rich sources of bacteria (e.g., *Staphylococcus*) and yeasts. Instruments (scalpels, forceps) that are inadequately sterilized between manipulations (via flaming or glass bead sterilizers at 250°C) directly transfer contaminants from one explant to another (Leifert & Cassells, 2001). Studies cited by Reed et al. (1995) indicate that a single subculture performed with lax aseptic techniques can cross-contaminate 40-60% of the additional cultures processed in the same work session.

Effects of Contamination on Plant Development

The presence of microorganisms in culture vessels has, in the vast majority of cases, detrimental effects on explant development, often resulting in culture loss. These effects can be direct (pathogenesis) or indirect (environmental modification).

1. Nutrient Competition**:** Microorganisms possess a multiplication rate exponentially higher than that of plant cells. They rapidly consume the sucrose, mineral salts, and vitamins from the Murashige & Skoog medium, depriving the explant of the resources necessary for growth. This leads to developmental stagnation and the absence of shoot or root formation (Permadi et al., 2023). A single bacterial colony of 10^6-10^8 cells/mL can completely deplete the sucrose from a standard MS vessel within 24-72 hours (Leifert & Cassells, 2001).

2. pH Modification and Phytotoxicity**:** Microbial metabolism generates excretion products that can drastically alter the pH of the culture medium (often lowering it to toxic acidic levels, pH 3-4). Furthermore, many fungi and bacteria secrete phytotoxins, pectolytic enzymes, or phenols that cause severe necrosis of plant tissues, a phenomenon frequently observed as a lethal browning of the explant and surrounding medium (Wijerathna-Yapa et al., 2023). In *Musa* spp., lethal browning - where bacterial contaminants and phenolic oxidation act synergistically - can lead to explant death within 7-14 days of culture initiation (Permadi et al., 2023).

3. Latent Effects and Somaclonal Variations: Latent endophytic bacteria may not immediately kill the plant *in vitro*, but they induce chronic stress. This stress can trigger epigenetic or genetic modifications, significantly increasing the rate of somaclonal variations, which is entirely unacceptable in commercial propagation where genetic fidelity (cloning) is paramount (Volk et al., 2022). Moreover, these

latent infections can erupt violently during the *ex vitro* acclimatization phase, when the plant is transferred to soil and humidity drops, leading to massive greenhouse mortality. Trzewik et al. (2025) experimentally demonstrated that raspberry (*Rubus idaeus*) plantlets contaminated with *Luteibacter* sp. and *Curtobacterium* sp. died almost entirely (90-100% necrosis) during dark storage at 4°C for 4-6 months, whereas decontaminated plantlets survived normally.

Opportunities and Beneficial Effects

Although microbial contamination has traditionally been associated with negative effects on *in vitro* cultures, research conducted over the last two decades has demonstrated that certain endophytic microorganisms may exert beneficial effects on plant development. Within the classical paradigm, Leifert and Cassells (2001) advocated the complete elimination of microorganisms from plant cultures, arguing that the risks associated with contamination, including phytotoxicity, reduced regenerative capacity, and the occurrence of somaclonal variation, outweigh any potential benefits. However, an alternative perspective subsequently emerged, suggesting that the effects of contaminants largely depend on the identity and functional role of the microorganisms involved.

An important contribution to this shift in perspective was provided by Cassells and Doyle (2004), who introduced the concept of "controlled biotized culture". The authors proposed that certain endophytic microorganisms may contribute to maintaining plant physiological status and enhancing survival during ex vitro transfer, indicating that complete eradication of the associated microbiota may not always represent the most advantageous strategy. This hypothesis was further supported by the findings of Quambusch et al. (2014), who demonstrated that the success of in vitro propagation in *Prunus avium* is influenced by the composition of endophytic microbial communities. Genotypes that were difficult to propagate were associated with the predominance of potentially phytotoxic bacteria belonging to the genus *Pseudomonas*, whereas highly propagable genotypes harbored communities dominated by beneficial bacteria such as *Methylobacterium* and *Sphingomonas*. These observations suggest that the mere presence of microorganisms is not necessarily detrimental and that their effects on plant development depend on the composition and functionality of the associated microbiota.

This paradigm shift was further reinforced by Herman (2017), who highlighted the role of certain endophytic bacteria as Plant Growth-Promoting Bacteria (PGPB). These microorganisms can enhance plant development through the production of phytohormones such as auxins and cytokinins, biological nitrogen fixation, and the induction of systemic resistance against pathogens. In this context, species belonging to genera such as *Bacillus* and *Pseudomonas* may exert positive effects on plant growth and adaptation, transforming what is conventionally regarded as contamination into a mutualistic interaction with beneficial outcomes.

Collectively, the available evidence indicates that the effects of microbial contaminants on plant development cannot be assessed solely on the basis of their presence or absence. In many cases, microorganisms associated with in vitro cultures may contribute to plant growth, acclimatization, and stress tolerance. Therefore, rather than adopting a strategy focused on the indiscriminate eradication of all contaminants, a more appropriate approach involves the selective management of microbial communities based on accurate taxonomic identification and functional assessment of the microorganisms involved in the plant–microbiota system.

Detection and Diagnosis

Early and accurate detection of contaminants is vital to prevent their spread throughout the laboratory. Methods range from simple visual observations to highly precise molecular techniques.

1. Visual Inspection and Indexing on Bacteriological Media

The simplest method is visual monitoring of turbidity in liquid media or the appearance of mucilaginous halos/mycelia on solid media. However, this method is ineffective for slow-growing bacteria or endophytes. For these, "culture plating" is used. Viss et al. (1991) and Reed et al. (1995) developed methods in which the base of the explant is scraped or sectioned

and inoculated onto specific bacteriological media (e.g., Medium 523, Nutrient Agar, Sabouraud Dextrose Agar). If microbial growth is observed on these media after several days of incubation at 28-30°C, the plant line in question is considered contaminated and is discarded.

2. Molecular Methods (PCR and RT-qPCR)

For pathogens that cannot be cultured on artificial media (viruses, viroids, phytoplasmas) or for the precise identification of bacterial species, molecular techniques are the gold standard. Polymerase chain reaction (PCR) using primers specific to the 16S rRNA region allows for the detection and identification of endophytic bacteria even at very low concentrations (Dangariya et al., 2020). For RNA viruses (e.g., Plum pox virus), RT-PCR (Reverse Transcription PCR) or RT-qPCR (quantitative) is used to assess the viral load of explants before and after eradication treatments (Elansary et al., 2024). These methods are rapid and highly sensitive but require expensive equipment and qualified personnel.

3. ELISA (Enzyme-Linked Immunosorbent Assay)

Serological tests such as ELISA are widely used for the initial screening of mother plants for common viruses. Although less sensitive than PCR, ELISA tests are less expensive and allow for the simultaneous processing of a large number of samples, making them ideal for commercial micropropagation laboratories.

Prevention and Control Strategies

Contamination management must be proactive, based on prevention, rather than reactive. Once a culture is visibly contaminated, eradication is extremely difficult, costly, and frequently associated with the phytotoxic effects of antimicrobial treatments.

1. Aseptic Techniques and Laboratory Management

The foundation of prevention is strict adherence to aseptic techniques. This includes the use of protective equipment (lab coats, gloves, masks), regular disinfection of work surfaces with 70% alcohol or chlorine-based solutions, and periodic maintenance of laminar flow hoods. Implementing a unidirectional workflow system in the laboratory (from clean zones to dirty zones) drastically reduces the risk of cross-contamination (Balo, 2023).

2. Explant Sterilization

Surface disinfection of initial explants is achieved using chemical agents such as sodium hypochlorite (NaOCl), calcium hypochlorite, mercuric chloride ($HgCl_2$), or hydrogen peroxide, often combined with a surfactant (e.g., Tween 20, 0.1%) to ensure penetration into tissue micro-crevices. Exposure time and concentration must be meticulously optimized for each individual species to balance microbial destruction with plant tissue survival (Trzewik et al., 2025).

Reference quantitative data: 1-2% sodium hypochlorite applied for 15-20 minutes - the most widely used protocol for easily sterilized herbaceous species - reduces epiphytic bacterial contamination by 85-95%, but rarely eliminates endophytes (Reed et al., 1995). For woody species, more aggressive protocols are required: 3-5% NaOCl with Tween 20, followed by 70% ethanol for 30 seconds. In *Rosa* sp., researchers reported that immersing explants in 1% NaOCl for 5 minutes plus 70% alcohol for 30 seconds reduced contamination to 10% compared to 100% in the unsterilized control (Balo, 2023). Hydrogen peroxide (H_2O_2) 3-10% for 10-15 minutes represents a less phytotoxic alternative for sensitive tissues, with an efficiency of 75-85% in reducing epiphytic bacteria.

A frequently reported issue in the literature is the complete lack of a universal sterilization protocol. Trzewik et al. (2025) showed that sodium hypochlorite, even at extreme concentrations (0.1-60%) applied via vacuum infiltration, failed to eliminate endogenous bacteria from *Rubus idaeus* cultures; only $HgCl_2$ 0.05-0.1% (highly toxic) proved completely effective. These results directly conflict with general guidelines promoting NaOCl as a first-line method for all species.

3. Use of Antibiotics and Antifungals

When endophytic contamination persists, researchers resort to incorporating antibiotics (e.g., cefotaxime, kanamycin, rifampicin, streptomycin) or antifungals into the culture medium. Nadha et al. (2012) demonstrated the efficacy of kanamycin (10 µg/mL) in eliminating bacteria from bamboo (*Guadua angustifolia*) cultures. Typical concentrations used in practice are: cefotaxime 250-500 mg/L, rifampicin 50-200 mg/L, streptomycin 10-50 mg/L, and kanamycin 5-20 mg/L (Reed et al.,

1995; Trzewik et al., 2025). Broad-spectrum commercial products, such as PPM™ (Plant Preservative Mixture), are often used prophylactically at 1-2 mL/L, although their efficacy varies considerably between species and bacterial strains. Comparative studies have shown that cefotaxime at 250 mg/L reduces bacterial contamination by 70-85% without significant phytotoxic effects in *Prunus*, *Malus*, and *Pyrus* spp., whereas concentrations >500 mg/L can severely inhibit regeneration in sensitive genotypes (Brison et al., 1997; Viss et al., 1991).

Trzewik et al. (2025) comparatively evaluated the efficacy of rifampicin (50-200 mg/L) versus PPM™ (0.2-4%) in raspberry cultures and found that rifampicin was effective against *Pseudomonas* sp. and *Curtobacterium* sp. without significant phytotoxicity, but failed to completely eliminate *Luteibacter* sp., which reappeared after 2-4 subcultures on antibiotic-free medium. PPM™ failed to produce inhibition zones for any tested contaminant and did not stop bacterial growth, refuting the commercial promises of the product in this specific context.

Leifert and Cassells (2001) explicitly warned that the repeated and prolonged use of antibiotics presents three major risks: (1) Phytotoxicity - broad-spectrum antibiotics, especially aminoglycosides (kanamycin, gentamicin) and rifampicin, inhibit protein synthesis in chloroplasts and mitochondria (organelles of prokaryotic origin), causing chlorosis, reduced photosynthetic rates, and foliar necrosis; (2) Microbial Resistance - suboptimal antibiotic treatment can select for resistant bacterial strains, making them exponentially harder to control later; (3) Infection Masking - bacteria can become latent under antibiotic pressure but are not eliminated, reappearing violently upon antibiotic withdrawal. Reed et al. (1995) confirmed this risk, reporting that 30-40% of mint cultures treated with antibiotics and appearing clean presented detectable bacteria via indexing after 2-3 subcultures without antibiotics.

4. Eradication of viruses: Thermotherapy and Cryotherapy

To obtain virus-free plants, meristem culture (taking the 0.1-0.5 mm apical dome, which is often virus-free due to the lack of vascular connections) is combined with other therapies. Thermotherapy involves exposing plants *in vitro* to high temperatures (35-38°C) for several weeks to inhibit viral replication. Cryotherapy, a more modern technique, involves immersing meristems in liquid nitrogen (-196°C). Virus-infected cells, having a higher water content (larger vacuoles), are destroyed by ice crystals, while dense meristematic cells survive, regenerating a healthy plant (Brison et al., 1997; Elansary et al., 2024).

The analysis of the scientific literature regarding *in vitro* contamination reveals not only an accumulation of knowledge but also a series of tensions, contradictions, and gaps that demand a highly critical approach. A profound understanding of the field requires evaluating the methodological limitations of existing studies and unresolved debates.

1. Paradigm Evolution: From Total Eradication to Microbiome Management

The literature reveals a constant tension in tissue culture: the desire to obtain completely sterile cultures versus the biological reality of intimate plant-microbe associations. The traditional approach, aiming for total eradication through aggressive biocides and antibiotics, is reaching its limits. As demonstrated by Trzewik et al. (2025) and Leifert and Cassells (2001), bacteria can form biofilms inside culture vessels or hide in deep intercellular spaces, becoming inaccessible to surface sterilants and developing antibiotic resistance.

Examining the literature of the last 30 years, a clear evolution emerges: (1) The Eradication Paradigm (1980s-2000s): Cassells (1991) and Leifert et al. (1989) defined contamination as a one-dimensional threat. The tools - biocides, antibiotics - aimed for "pure axenic culture." (2) The Nuanced Paradigm (2000s–2010s): Herman (2017) reformulated the concept, recognizing that "completely sterile culture is often an illusion" and that some endophytes are inevitable and sometimes beneficial. (3) The Microbiome Paradigm (post-2010): Quambusch et al. (2014) and Orlikowska et al. (2017) applied microbial ecology methods (16S rRNA metagenomics) to characterize complex endophytic communities and correlate them with *in vitro* performance, paving the way for "deliberate biotization" - controlled inoculation with beneficial endophytes.

2. Critical Evaluation of Methodologies

A critical analysis highlights several systematic methodological limitations. First, small sample sizes: many studies use n<50 explants per treatment, limiting statistical power. For example, Leifert et al. (1994) used batches of 20-30 explants, insufficient to detect subtle differences between protocols. Second, lack of biological replication: most studies use a single genotype or cultivar, making it impossible to extrapolate results to the species level. Trzewik et al. (2025) is a notable exception, testing three different *Rubus idaeus* cultivars and demonstrating that antibiotic response varies significantly between genotypes. Third, the gap between laboratory and industrial scale: academic studies are conducted under strictly controlled conditions that do not reflect the variability of a commercial laboratory processing tens of thousands of explants daily. Cassells (2012) estimates that the efficacy of published protocols drops by 20-40% when implemented in real commercial settings.

3. Research Gaps

Several areas remain insufficiently studied. The impact of latent contaminants on the quality of acclimatized plantlets remains poorly documented quantitatively. Furthermore, systematic comparative studies are lacking: few studies compare the efficacy of detection methods (PCR vs. ELISA vs. bacteriological indexing) on the same crop under identical conditions. Molecular mechanisms by which latent contaminants affect somatic embryogenesis and organogenesis are poorly understood; transcriptomic and metabolomic studies elucidating plant-microbe interactions *in vitro* are rare. Finally, there is a glaring lack of international standardization: no ISO standards or harmonized guidelines exist regarding sterilization protocols, antibiotic concentrations, or mandatory detection methods for certifying propagation material.

4. Contradictions and Debates in the Literature

The critical analysis reveals significant contradictions that reflect fundamental differences in approaches and interpretations of the same biological phenomena.

Contradiction 1: Antibiotic Use. Supported by Leifert and Cassells (2001) as essential for controlling endophytic bacteria, but heavily criticized by Reed et al. (1995) and Orlikowska et al. (2017) due to severe phytotoxicity and resistance selection. Trzewik et al. (2025) showed that rifampicin eliminates bacteria temporarily, but they reappear after subculturing, suggesting a bacteriostatic effect. There is no consensus on optimal treatment duration or safe concentrations.

Contradiction 2: Endophytic Bacteria. Cassells and Doyle (2004) and Quambusch et al. (2014) argue that endophytes can be beneficial (producing phytohormones). In direct contrast, Leifert et al. (1994) advocate for total eradication, reporting 30-50% mortality in contaminated plantlets during *ex vitro* acclimatization compared to <10% in sterile plantlets. The impact of endophytes depends critically on the bacterial species, the host plant, and acclimatization conditions.

Contradiction 3: Sterilization Protocols. George et al. (2008) recommend 1-3% NaOCl for woody species, while Anikina et al. (2025) indicate that 0.5-1% is sufficient for herbaceous species and higher concentrations cause necrosis. Trzewik et al. (2025) demonstrated that even 60% NaOCl failed to eliminate endogenous bacteria in raspberry, contradicting general guidelines promoting NaOCl as a universal method.

Future Perspectives

The future of *in vitro* contamination management leans heavily towards advanced technological and ecological solutions. The application of nanotechnology, particularly silver nanoparticles (AgNPs) or zinc oxide, shows enormous potential as antimicrobial agents in culture media, exhibiting low plant toxicity at optimal concentrations and minimal risk of generating microbial resistance. Studies on *Chrysanthemum morifolium* have shown that silver, copper, and gold nanocolloids at 10-50 mg/L can drastically reduce fungal and bacterial contamination without negatively affecting plantlet regeneration (Eisa et al., 2022). However, long-term ecological safety data remain insufficient.

Artificial Intelligence (AI) and Machine Learning are beginning to be applied to model and optimize culture media composition and predict contamination risks based on historical laboratory data (Bhattacharya & Bhattacharya, 2023). Computer vision systems can detect

incipient bacterial or fungal colonies with sensitivity superior to the human eye, allowing early isolation of affected vessels.
Furthermore, gene editing (CRISPR/ Cas9) offers the prospect of creating plant lines intrinsically resistant to specific viruses, potentially eliminating the need for laborious thermo- or cryotherapy procedures. Concurrently, research will increasingly focus on *in vitro* biotization - the controlled inoculation of sterile explants with synthetic beneficial microbial consortia (PGPB) to create robust plants capable of resisting pathogenic contaminants independently (Soumare et al., 2021).

CONCLUSIONS

In vitro contamination remains a limitation of plant tissue culture, representing a constant barrier to efficiency and profitability. Bacteria, fungi, viruses, and mycoplasmas can infiltrate cultures through the initial explant, handling errors, or defective equipment, with massive economic consequences: Leifert and Waites (1994) estimated that between 15% and 40% of commercial micropropagation cultures are lost annually due to contamination.
The species-specific examples analyzed in this review - from endophytic bacterial contamination in *Prunus avium* and *Rubus idaeus*, to viral contamination in *Prunus persica* and *Vitis vinifera*, to the synergistic challenges of browning and contamination in *Musa* spp. - demonstrate unequivocally that there is no universal solution. Each species, and often each cultivar, dictates a specific prevention and eradication protocol, adapted to the nature of the explant and the microbiological profile of the mother plant.
Effective management requires a holistic, integrated approach based on HACCP principles: rigorous selection of mother plants, impeccable aseptic techniques, optimized sterilization, and constant monitoring via visual and molecular methods (PCR). While antibiotics and antifungals remain useful tools - with cefotaxime (250-500 mg/L) and rifampicin (50-200 mg/L) demonstrating documented efficacy - their use must be strictly limited due to the severe risks of phytotoxicity and resistance.
The future belongs to ultra-sensitive detection methods (qPCR, next-generation sequencing), the judicious use of nanomaterials, and, fundamentally, a paradigm shift that integrates the beneficial role of the endophytic microbiome into the ecology of *in vitro* systems. The contradictions within the literature analyzed in this review underscore the urgent need for comparative, multi-species studies with standardized methodologies to generate universally applicable, evidence-based practical guidelines for plant biotechnology laboratories.

ACKNOWLEDGEMENTS

The authors would like to thank all colleagues who contributed to the successful completion of this study. The authors also acknowledge the support provided by their institution and research facilities.

USE OF GENERATIVE ARTIFICIAL INTELLIGENCE (GenAI)

The authors declare the use of Generative Artificial Intelligence (GenAI) tools in the preparation of this manuscript. GenAI was used exclusively for language editing and text refinement in the Introduction and Conclusions sections. All scientific content, data analysis, and interpretations were developed and verified by the authors.

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