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REVIEW

Shifting the paradigm in *Vanilla odorata* micropropagation: *Trichoderma* spp. as a strategic agent for biocontrol and biostimulation

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ABSTRACT

Vanilla (*Vanilla* spp., Orchidaceae) is one of the most economically valuable tropical crops, yet commercial production relies almost exclusively on *V. planifolia*, creating genetic vulnerability to vascular diseases, particularly *Fusarium oxysporum* f. sp. *vanillae* (Fov). *Vanilla odorata* represents a strategic genetic resource, but propagation faces critical bottlenecks: Low multiplication efficiency, fungal susceptibility, and > 60% acclimatization mortality. This review synthesizes evidence (2010-2025) on *Trichoderma* spp. as dual-function agents in micropropagation: Biocontrol and plant growth promotion (PGP). Analysis of 14 studies shows selected strains (*T. harzianum*, *T. virens*, *T. asperellum*) inhibit Fov by > 80% via mycoparasitism/antibiosis, increasing *ex vitro* survival 1.6-fold and biomass 3.6-fold *vs*. controls. Mechanisms include nutrient solubilization, auxin production, and induced systemic resistance (ISR) activation. Crucially, unlike conventional chemical disinfection (ethanol, hypochlorite, antibiotics), which often fails against endophytic contaminants and risks explant damage; biotization enhances viability while reducing reliance on synthetic inputs. Though strain-specific and requiring standardization, its potential economic benefit lies not in direct cost-cutting, but in mitigating seedling loss and acclimatization mortality, a costly phase. Integration with temporary immersion systems or Ag nanoparticles offers a sustainable, scalable protocol. This marks a paradigm shift from absolute asepsis toward managed beneficial microbiota, addressing key technical-practical challenges in tropical crop biotechnology. Implementing biotized protocols is essential for valorizing *V. odorata* germplasm and ensuring long-term sustainability of natural vanilla production.

Key words: Biological control, endophyte, *Fusarium oxysporum*, induced systemic resistance, micropropagation, mycoparasitism, plant growth-promoting fungi, tissue culture, *Trichoderma*, *Vanilla odorata*.

INTRODUCTION

The genus *Vanilla* (Orchidaceae) occupies a unique position in tropical agriculture as the only orchid industrially cultivated for human consumption. Natural vanillin, extracted primarily from cured pods of *Vanilla planifolia* Andrews, commands premium prices in global markets (400-600 USD kg^{-1}) due to production complexity involving manual pollination and labor-intensive curing processes (Adame-García et al., 2015). However, the global vanilla industry rests on a perilously narrow genetic base. Intensive clonal propagation over centuries has resulted in

genetic uniformity that, while ensuring product consistency, has left the crop vulnerable to evolving biotic pressures, particularly *Fusarium oxysporum* f. sp. *vanillae* (Fov), the causal agent of root and stem rot (RSR) responsible for 30%-90% yield losses worldwide (Koyyappurath et al., 2015).

In this vulnerability scenario, *V. odorata* C. Presl emerges as a strategic resource. Distributed from Mexico to southern tropical America, this species is characterized by robustness, distinctive aromatic profiles, and, crucially, potential sources of disease resistance genes (Warner et al., 2023). Conservation and utilization of *V. odorata* is not merely a botanical curiosity but also an agricultural security imperative for the natural flavoring industry. Hybridization and genetic improvement programs require availability of healthy, vigorous germplasm, placing efficient *V. odorata* propagation at the center of current biotechnological research.

In vitro micropropagation or tissue culture revolutionized vanilla agronomy, enabling exponential production of pathogen-free clones in reduced spaces and controlled timeframes. Recent protocols, such as those developed by Warner et al. (2023) specifically for *V. odorata*, have achieved optimization of direct and indirect regeneration using growth regulators such as 6-benzylaminopurine (BAP) and indolebutyric acid (IBA), achieving shoot induction rates exceeding 75%. Nevertheless, micropropagation faces two critical challenges threatening its economic viability: (i) Endogenous contamination- despite rigorous surface sterilization protocols, endophytic microorganisms (bacteria and fungi living inside tissues) can remain latent and emerge in advanced culture stages, destroying entire batches (Ayele and Tefera, 2018); and (ii) acclimatization mortality- transfer of plantlets from the aseptic, heterotrophic, high-humidity laboratory environment (in vitro) to the autotrophic, variable greenhouse conditions (*ex vitro*) generates severe physiological stress, with mortality rates exceeding 60% in vanilla (Gutiérrez-Miceli et al., 2008).

Among causal agents of losses, Fov is the most devastating pathogen worldwide. This soil-inhabiting fungus invades the plant's vascular system, causing RSR and plant death. Its capacity to produce resistance structures (chlamydospores) allows survival in soil for decades, making chemical eradication ecologically disastrous (Adame-García et al., 2015). Recent molecular studies reveal that vanilla plants harbor a complex of *Fusarium* species (*F. solani*, *F. concentricum*) behaving as asymptomatic endophytes in mother material. Upon in vitro introduction, tissue cutting stress and medium sugar richness break fungal latency, shifting from endophytic to aggressive necrotrophic phases (Koyyappurath et al., 2015).

The conventional strategy of combating contaminants through surface sterilization (sodium hypochlorite, ethanol, mercuric chloride) has proven insufficient and unsustainable due to: (i) Inefficacy against endophytes—surface disinfectants do not reach fungi lodged in vascular systems or deep intercellular spaces; (ii) phytotoxicity—potent agents damage plant tissues, causing necrosis and reduced regenerative capacity; and (iii) ecological vacuum—creating an axenic environment eliminates beneficial microbiota, allowing any surviving pathogen to proliferate exponentially without competition (Soumare et al., 2021).

This scenario demands a paradigm shift: Instead of seeking absence of microbial life, controlled presence of beneficial life must be sought. The genus *Trichoderma* positions itself as the cornerstone of this new "biotization" approach—filamentous soil fungi that are formidable pathogen antagonists and establish opportunistic symbiosis with plants, enhancing their physiology (Zehra et al., 2017).

The hypothesis is that strategic inoculation of selected *Trichoderma* spp. strains in *V. odorata* micropropagation systems will simultaneously achieve: (i) > 70% inhibition of *Fusarium oxysporum* f. sp. *vanillae* mycelial growth through mycoparasitism and antibiosis; (ii) significant increase (≥ 1.5-fold) in *ex vitro* survival rates via induction of systemic resistance (ISR); and (iii) enhanced vegetative growth (≥ 2-fold increase in root biomass) through auxin production and nutrient solubilization, compared to non-inoculated controls.

The objective is synthesize and critically analyze current evidence (2010-2025) on the multifunctional role of *Trichoderma* spp. as biological control agent (BCA) and plant growth-promoting fungus (PGPF) in *V. odorata* micropropagation, integrating biocontrol mechanisms against *Fusarium*, biostimulation effects during acclimatization, and emerging technologies (temporary immersion systems [TIS], silver nanoparticles [AgNPs]) into a comprehensive, economically viable, and ecologically sustainable biotization protocol for commercial vanilla propagation.

TECHNOLOGICAL ADVANCES IN *V. ODORATA* MICROPROPAGATION

Regeneration protocols and bioreactors

Vanilla odorata micropropagation has recently overcome important technical barriers. Warner et al. (2023) established reproducible protocols for direct and indirect regeneration, demonstrating that shoot induction from nodal segments requires species-specific hormonal balance distinct from *V. planifolia*, optimizing with 1.0-2.0 mg L^{-1} BAP combined with 0.5 mg L^{-1} IBA, achieving shoot rates exceeding 75%. This level of specificity underscores the importance of adjusting protocols to genetics of each species within the genus.

Parallel to this, the industry has transitioned from traditional solid media to TIS such as recipient for automated temporary immersion (RITA, Vitropic, Saint-Mathieu-de-Tréviers, France) or temporary immersion bioreactors (BIT, Vitropic). Ramírez-Mosqueda and Iglesias-Andreu (2016) demonstrated that TIS significantly increase multiplication rate (2.8-fold) and fresh biomass (3.2-fold) in *Vanilla* compared to solid agar media. Intermittent contact with liquid medium improves nutrient availability and facilitates gas exchange, reducing physiological disorders such as hyperhydricity and decreasing operational costs by reducing need for frequent manual subcultures. However, greater nutrient availability in TIS presents a double-edged sword: Any microbial contaminant, such as *Fusarium*, proliferates with devastating speed in these liquid systems (generation time < 8 h in TIS *vs*. > 24 h in agar), making sanity control more critical than ever.

Fusarium infection complexity

Root and stem rot pathology in *Vanilla* is complex. Although *F. oxysporum* f. sp. *vanillae* (Fov) is the primary agent, recent research using molecular tools (internal transcribed spacer [ITS] sequencing, random amplified polymorphic DNA [RAPD] markers) has revealed that vanilla plants, both cultivated and wild (*V. odorata*), harbor a complex of *Fusarium* species, including *F. solani* and *F. concentricum*, which can act as opportunistic or secondary pathogens (Adame-García et al., 2015). A detailed molecular study of isolates in Colombia and Mexico confirmed pathogenic variability of these strains, with virulence indices ranging from moderate (disease severity index, DSI = 2.5) to severe (DSI = 4.8 on 0-5 scale). Alarming for micropropagation is that these fungi can behave as asymptomatic endophytes in mother material. Upon introducing explants in vitro, stress from cutting and richness of sugars in medium (typically 30 g L^{-1} sucrose) break fungal latency, shifting from endophytic to aggressive necrotrophic phases (Koyyappurath et al., 2015).

Failure of the sterility paradigm

The conventional strategy of combating contaminants through surface sterilization has proven insufficient. Use of 0.1% mercuric chloride ($HgCl_2$) or 2%-4% sodium hypochlorite (NaOCl) for 10-20 min achieves 85%-95% surface disinfection but does not reach endophytes. Moreover, potent agents cause necrosis in 15%-30% of explants and reduce regeneration capacity by 20%-40% (Ayele and Tefera, 2018). Creating an axenic environment eliminates beneficial microbiota, creating an ecological vacuum where any surviving pathogen faces no competition, allowing exponential expansion. This scenario demands controlled presence of beneficial microbial life—the biotization approach.

Genetic resource value and microbial interaction gaps in *Vanilla odorata*

While *V. odorata* represents a critical genetic resource for vanilla diversification — supported by ISSR-based genetic variability in Ecuadorian populations (Vasquez Arriaga, 2024) — its specific receptivity to *Trichoderma* spp. remains unproven. Unlike mycorrhizal fungi (e.g., *Rhizoctonia*/*Ceratobasidium*), *Trichoderma* does not form obligate symbioses; its interactions are opportunistic and strain-dependent (Cano, 2011). No comparative studies exist evaluating whether *V. odorata* exhibits greater colonization efficiency or physiological response to *Trichoderma* than *V. planifolia*. Although endophytic fungi have been isolated from vanilla roots (Jaramillo Aguayo and Salazar Orellana, 2025), successful germination or bio-stimulation via *Trichoderma* has not been

demonstrated in vitro. Thus, any claim of superior affinity is speculative. We therefore frame *Trichoderma* application in *V. odorata* as an experimental strategy — not a trait inherent to the species — pending validation under standardized micropropagation conditions.

TRICHODERMA SPP. AS MULTIFACETED AGENT FOR HEALTH AND VIGOR

The efficacy of *Trichoderma* against *Fusarium* results from three principal mechanisms of biocontrol mechanisms against *Fusarium* (Figure 1).

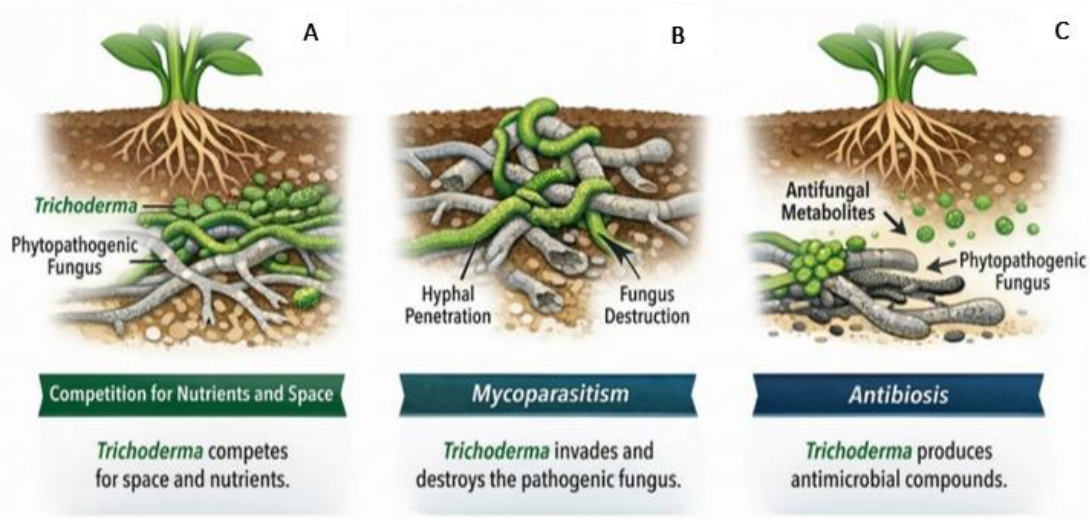

Figure 1. Tripartite biocontrol mechanism of *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *vanillae* in *Vanilla odorata* micropropagation systems. The conceptual scheme was developed based on published descriptions of *Trichoderma* biocontrol mechanisms, including competition for nutrients and space, mycoparasitism, and antibiosis (Zehra et al., 2017). The figure was edited by the authors for academic presentation. AI-generated illustration created by the authors using Google NotebookLM on 21 January 2026. A) Competitive exclusion. Rapid mycelial growth (4.5-9.0 mm d^{-1}); Fe sequestration via siderophores (log K = 30-32); physical substrate monopolization. B) Necrotrophic mycoparasitism. Chemotactic detection; appressoria formation and hyphal coiling; secretion of cell wall-degrading enzymes: Endo-chitinases (EC 3.2.1.14), β-1,3-glucanases (EC 3.2.1.39); cytoplasm leakage and cell death. C) Antibiosis. Non-volatile metabolites: Peptaibols (alamethicin), gliotoxin, viridian. Diffusion radius: 15-25 mm. Volatile organic compounds (VOCs): 6-Pentyl-alpha-pyrone (6-PP), sesquiterpenes (trichodiene). Effective concentration: 50-200 mg L^{-1}. Conidial germination inhibition: 60%-90%.

Competition for nutrients and space (competitive exclusion)

Trichoderma possesses extremely rapid mycelial growth rate (4.5-9.0 mm d^{-1}), superior to *Fusarium* (2.5-5.0 mm d^{-1}). In culture medium, it colonizes substrate before *Fusarium*, monopolizing physical space. At the chemical level, *Trichoderma* secretes high-affinity siderophores (desferrioxamines, ferrichromes) with Fe-binding constants (log K = 30-32) that sequester ferric Fe (Fe^{3+}) from medium, making it inaccessible to *Fusarium*. Since Fe is essential for spore germination and fungal pathogenesis, this nutritional deprivation effectively inhibits pathogen development without requiring direct contact (Zehra et al., 2017).

Necrotrophic mycoparasitism

This is direct and sophisticated attack. *Trichoderma* chemotactically detects *Fusarium*, grows toward it, and adheres to its hyphae via specialized structures (appressoria) that coil around the victim. Subsequently, it secretes a cocktail of cell wall-degrading enzymes (CWDEs), mainly endo-chitinases (EC 3.2.1.14) and β-1,3-glucanases (EC 3.2.1.39). These enzymes degrade *Fusarium* cell wall (composed of 20%-30% chitin and 50%-60% glucans), causing cytoplasm leakage and cell death. Molecular studies using quantitative PCR (qPCR) confirm that presence of *Fusarium* cell walls induces 5.2-8.7-fold upregulation of chitinolytic genes (*ech42*, *nag1*) in *Trichoderma* spp. within 6-12 h of contact (Zehra et al., 2017).

Antibiosis and volatile metabolites

Trichoderma is a biofactory of secondary metabolites. It produces non-volatile antibiotic compounds (peptaibols such as alamethicin, gliotoxin, viridian) that permeate agar medium (diffusion radius 15-25 mm) and inhibit protein synthesis in pathogen. Additionally, it releases volatile organic compounds (VOCs) such as pyrones (6-pentyl-alpha-pyrone, 6-PP) and sesquiterpenes (trichodiene). In confined in vitro culture flask environment, these gases accumulate (concentrations 50-200 mg L^{-1}) and exert potent fungistatic effect, inhibiting *Fusarium* conidial germination by 60%-90% even at distance (5-10 cm), thus protecting non-colonized aerial plant parts (Adame-García et al., 2015).

Induction of systemic resistance

Root colonization of *Vanilla* by *Trichoderma* triggers immune response known as induced systemic resistance (ISR). Unlike systemic acquired resistance (SAR) mediated by salicylic acid (SA) and typical of pathogenic attacks, ISR by *Trichoderma* is mediated by jasmonic acid (JA) and ethylene (ET) pathways. This "priming" or molecular priming process pre-activates vanilla defense system. When *Fusarium* attempts attack, plant responds faster (response time reduced from 72-96 h to 12-24 h) and with greater intensity, accumulating phytoalexins (scopoletin, capsidiol), controlled reactive oxygen species (ROS) such as H_2O_2, and reinforcing cell walls with lignin and callose (Zehra et al., 2017). Transcriptomic studies in *V. planifolia* using RNA-seq have shown 2.5-4.8-fold upregulation of defense genes (pathogenesis-related proteins PR-1, PR-2, PR-5; phenylalanine ammonia-lyase PAL; lipoxygenase LOX) in *Trichoderma*-treated plants compared to non-treated controls ($p < 0.01$).

QUANTITATIVE EFFICACY AGAINST *FUSARIUM*

In vitro assays: The power of inhibition

The study by Naik et al. (2010) remains a key reference, validated by subsequent investigations. Upon evaluating *Trichoderma harzianum* (strain Th-3) against six virulent isolates of Fov using dual culture technique on potato dextrose agar (PDA), consistently high mycelial inhibition rates were observed (Table 1). Mean inhibition reached 83.63% (range 74.11%-90.50%), with isolates Fov-3 and Fov-6 showing very high inhibition (> 90%). These data are corroborated by Sandheep and Jisha (2014), who reported 80%-90% inhibitions using native strains of *T. harzianum* (strains TvTh-02, TvTh-05) and *T. virens* (strain TvTv-03) isolated from vanilla rhizosphere in Kerala, India. Consistency of these results across different strains, geographies (India, Mexico, Colombia), and experimental conditions (temperature 25-28 °C, pH 5.5-6.5) suggests intrinsic robustness in control mechanism.

Table 1. In vitro antagonistic efficacy of *Trichoderma harzianum* strain Th-3 against six isolates of *Fusarium oxysporum* f. sp. *vanillae* (Fov). [1]Radial growth of pathogen measured 7 d after inoculation in dual culture assays on potato dextrose agar at 28 °C. Values are means ± standard error (n = 5 replicates per isolate). [2]Inhibition percentage calculated as: [(Control growth - Dual culture growth)/Control growth] × 100. Classification: Moderate-high (70%-80%), High (80%-90%), Very high (> 90%). Source: Elaborated from data of Naik et al. (2010).

Pathogen isolate	Radial growth[1]	Inhibition[2]	Classification
	mm	%	
Fov-1	10.70 ± 0.8	82.83	High
Fov-2	12.10 ± 1.1	80.58	High
Fov-3	5.92 ± 0.5	90.50	Very high
Fov-4	10.13 ± 0.9	83.74	High
Fov-5	12.86 ± 1.2	74.11	Moderate-high
Fov-6	6.21 ± 0.6	90.03	Very high
Mean	9.82	83.63	-

Microbial synergies: The consortium effect

Research has evolved toward use of microbial consortia. Sandheep et al. (2013) demonstrated that co-inoculation of *T. harzianum* (10^8 spores mL^{-1}) with bacterium *Pseudomonas fluorescens* (10^9 CFU mL^{-1}) generated synergistic effect superior to individual use. In greenhouse assays with *V. planifolia* 'Indian', this consortium achieved 90%-95% disease suppression (DSI reduced from 4.2 in pathogen-only control to 0.3 in consortium treatment), in addition to maximizing growth parameters: Plant height increased 2.8-fold (32.5 cm *vs*. 11.6 cm in non-treated control), leaf number increased 3.1-fold (18.2 *vs*. 5.9), and chlorophyll content (SPAD units) increased 1.7-fold (42.3 *vs*. 24.8), all differences significant at $p < 0.001$.

Similarly, Manrique-Barros et al. (2023) explored potential of natural orchid mycorrhizal fungi (*Tulasnella calospora*, *Ceratobasidium* spp.) as biocontrol agents. Although their primary function is nutritional, root niche occupation by these symbionts also confers protection: In vitro confrontation assays showed 52%-68% inhibition of Fov by mycorrhizal fungi, suggesting that ideal biotization protocol for *V. odorata* could include triad of *Trichoderma* (rapid defense), plant growth-promoting rhizobacteria (PGPR) (biostimulation), and mycorrhizae (long-term nutrition).

TRICHODERMA AS BIOSTIMULANT: PHYSIOLOGICAL PLANT ENGINEERING

Biotization not only seeks to protect plants but also to optimize its development. In micropropagation, where the objective is to obtain vigorous plants in the shortest possible time, plant growth promotion (PGP) effect of *Trichoderma* is invaluable. *Trichoderma*-plant interaction triggers profound physiological responses.

Auxin production and rhizogenesis

Many *Trichoderma* strains produce indole-3-acetic acid (IAA) via tryptophan-dependent pathways or induce endogenous auxin synthesis in plants. Quantification via HPLC-MS shows that *T. harzianum* culture supernatants contain 15-45 µg mL^{-1} IAA (Gutiérrez-Miceli et al., 2008). This stimulates cell division in root meristem, resulting in more extensive, branched root system with greater quantity of root hairs (surface area increase 2.5-4.0-fold). For epiphytic/hemiepiphytic orchid like *V. odorata*, rapid establishment of functional roots is critical for survival.

Nutrient solubilization

Trichoderma modifies rhizosphere pH (from 6.5 to 5.2-5.8) through excretion of organic acids (citric, gluconic, oxalic) and secretes phosphatases (acid phosphatase EC 3.1.3.2, activity 50-120 µmol *p*-nitrophenol min^{-1} mg^{-1} protein) and phytases (EC 3.1.3.8). This solubilizes inorganic P (conversion of $Ca_3(PO_4)_2$ to $H_2PO_4^-$) and chelates micronutrients (Fe, Mn, Cu), making them bioavailable (Zehra et al., 2017). In often inert or poor acclimatization substrates (pine bark, perlite), this capacity increases available P by 2.5-3.8-fold.

Ethylene regulation

Under stress conditions (such as *ex vitro* transplant), plants produce ethylene (10-50 nL L^{-1}), which inhibits root growth and accelerates senescence. Some *Trichoderma* strains possess enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (EC 4.1.99.4), which degrades ethylene precursor ACC, reducing stress hormone levels by 40%-70% and allowing plants to maintain growth (Soumare et al., 2021).

Synergistic AgNPs-Trichoderma interactions in *V. odorata* micropropagation: Secondary metabolism, antifungal defense, and phytotoxicity constraints

The integration of Ag nanoparticles (AgNPs) with *Trichoderma*-based biotization represents an emerging strategy to enhance sanitation and bio-stimulation in micropropagation; however, in *V. odorata*, it remains a hypothesis supported by indirect evidence—not experimentally validated. Gunasena et al. (2024) report AgNPs can reduce microbial contamination, induce callus, and elevate secondary metabolites, yet warn of potential phytotoxicity and environmental risk. Metabolically, AgNPs may act as elicitors, triggering ROS, signaling cascades, phenolics, or lignification—potentially enhancing *Fusarium* defense—but without proven induction of vanillin precursors in *V. odorata*. Mayo-Mosqueda et al. (2025) observed hormetic effects in *Laelia rubescens*: 25-100 mg L^{-1} AgNPs promoted root growth, while 200 mg L^{-1} caused necrosis and dose-dependent ROS/lignin accumulation. Li et al. (2025) confirmed strain-specific compatibility: *Trichoderma koningiopsis* showed only 5.76% mycelial inhibition and synergistically reduced Fusarium wilt in melon. Thus, AgNPs-*Trichoderma* synergy in *V. odorata* must be framed as promising but conditional on validating dosage, fungal viability, explant response, oxidative stress, and pathogen control. A critical synthesis of available evidence supporting this integrative approach is presented in Table 2, which evaluates key dimensions including contamination control, metabolic modulation, phytotoxicity, fungal compatibility, and pathogen suppression.

Table 2. Critical synthesis of evidence for Ag nanoparticles (AgNPs)-*Trichoderma* integration in *Vanilla odorata* micropropagation. ROS: Reactive oxygen species.

Analysis axis	Available evidence	Interpretation for *V. odorata*	Risk/Limitation	Validation criterion
In vitro contamination control	AgNPs reduce microbial load (Gunasena et al., 2024); potential alternative to chemical sterilants	May complement sanitation during establishment/multiplication	Efficacy depends on concentration, exposure time, explant type	Measure contamination, explant survival, necrosis, regeneration
Secondary metabolism and defense	AgNPs elicit ROS, gene expression, phenolics, lignification (Gunasena et al., 2024)	Suggests possible link to phenolic defense against *Fusarium*	No evidence of vanillin precursor induction in *V. odorata*	Quantify total phenolics, antioxidant activity, lignin, phenylpropanoid genes
Phytotoxicity in orchids	Hormetic response in *Laelia rubescens*: 100 mg L^{-1} optimal; 200 mg L^{-1} causes necrosis (Mayo-Mosqueda et al., 2025)	Useful proxy for orchid tissue culture; species-specific responses expected	Not directly transferable to *V. odorata*	Establish species-specific dose-response curve
AgNPs-*Trichoderma* compatibility	*Trichoderma koningiopsis* shows high AgNP tolerance (Li et al., 2025); minimal mycelial inhibition	Some strains may coexist with AgNPs under optimized conditions	Compatibility is strain-dependent; not universal	Assess mycelial growth, sporulation, viability, antagonism
Fusarium control	AgNPs + *T. koningiopsis* reduced Fusarium incidence in melon (Li et al., 2025)	Supports integrated control hypothesis for vanilla	Evidence from non-orchid host; not confirmed in *Vanilla*	Test against vanilla-associated *Fusarium* isolates; measure disease severity, colonization, survival

Species-specific mechanisms of *Trichoderma* spp. in *V. odorata*: Biocontrol *vs*. Biostimulation

The functional value of *Trichoderma* as biocontrol and biostimulant cannot be generalized across the genus; efficacy is strain-, host-, and system-dependent (Guzmán-Guzmán et al., 2023; Andrade-Hoyos et al., 2023). While species like *T. harzianum*, *T. virens*, and *T. asperellum* are widely used, their mechanisms—and outcomes—vary significantly. For example, *T. asperellum* inhibits *Fusarium oxysporum* via chitinase, phosphate solubilization, and root colonization in tomato (Sehim et al., 2023), while *T. harzianum* QT20045 enhances peanut growth through IAA production and ethylene suppression against *Sclerotium rolfsii* (Wang et al., 2024)—not *Fusarium*. *Trichoderma virens* modulates root architecture and defense signaling via volatiles and auxin-like compounds, but evidence in orchids remains indirect.

Critically, in *V. odorata*, rhizospheric *Trichoderma* isolates show 50%-80% in vitro inhibition of Fov (Franco-Galindo and Mosquera-Espinosa, 2023), yet field or plant-level validation is lacking. No strain has been experimentally confirmed to control vanilla-specific pathogens under micropropagation or acclimatization conditions. Thus, current evidence supports these species as *candidates*, not proven solutions. Table 3 summarizes key strains, reported mechanisms, and applicability to *V. odorata*.

Table 3. Comparative mechanisms and *Vanilla odorata*-applicability of key *Trichoderma* species/strains. IAA: Indole-3-acetic acid; ACS: 1-aminocyclopropane-1-carboxylate synthase; ACO: 1-aminocyclopropane-1-carboxylate oxidase; ISR: induced systemic resistance.

Strain/Species	Reported mechanisms	Key outcome (non-*Vanilla*)	Evidence in *V. odorata*	Interpretive caution
T. asperellum	Chitinase, antifungal metabolites, IAA, P-solubilization, root colonization	Inhibits *Fusarium oxysporum*; improves tomato biomass	Indirect: based on tomato model	Requires validation with vanilla-associated *Fusarium*
T. harzianum QT20045	Mycoparasitism, IAA ↑, ethylene ↓, ACS/ACO regulation	78.5% biocontrol *vs*. *Sclerotium rolfsii*; enhanced root growth	None (peanut host, non-*Fusarium* pathogen)	Hormonal modulation promising, but pathogen/host mismatch
T. virens	Volatile compounds, auxin-like signals, lateral root promotion, ISR induction	Enhances root architecture and defense in model systems	None	Include as comparative candidate; lacks vanilla validation
Rhizospheric *Trichoderma*	Competition, mycoparasitism, lytic activity	50%-80% *in vitro* inhibition of *Fusarium oxysporum* f. sp. *vanillae*	Only in vitro; no in vivo or acclimatization data	Most relevant, but insufficient for practical application

Impact on orchid acclimatization

Acclimatization phase is "valley of death" in micropropagation. Plants come from environment with 100% humidity, low light (40-60 µmol m^{-2} s^{-1} photosynthetic photon flux density, PPFD), and exogenous C (30 g L^{-1} sucrose). Upon passing to greenhouse, they must activate photosynthesis and control transpiration under 200-400 µmol m^{-2} s^{-1} PPFD and 60%-80% relative humidity.

Classic study by Gutiérrez-Miceli et al. (2008) on orchid *Guarianthe skinnerii* (Bateman) Dressler (ecologically relevant model for *Vanilla*) illustrates power of biotization. Inoculation with *T. harzianum* (10^6 spores g^{-1} substrate) radically transformed plantlet performance during 120-d acclimatization period (Table 4). Survival rate increased 1.6-fold (85% *vs*. 53% in non-inoculated control, $\chi^2 = 12.45$, $p < 0.001$), plant height 3.6-fold (18.2 cm *vs*. 5.1 cm, $t = 8.92$, $p < 0.001$), leaf number 2.5-fold (12.8 *vs*. 5.1, $t = 7.34$, $p < 0.001$), and shoot number 1.4-fold (3.2 *vs*. 2.3, $t = 2.87$, $p = 0.008$).

Recent results by Serrano-Fuentes et al. (2024) confirm this trend specifically in *V. planifolia*. By inoculating micropropagated plantlets with mycorrhizal fungus *Rhizophagus intraradices* (20 spores g^{-1} substrate) during acclimatization, they achieved survival rates between 95%-100% (*vs*. 45%-60% in non-inoculated controls), validating principle that reintroduction of fungal symbionts is essential for successful "hardening" of vanilla plantlets.

Table 4. Impact of *Trichoderma harzianum* inoculation on growth parameters and survival of micropropagated orchid *Guarianthe skinnerii* during acclimatization (120 d period). [1]Non-inoculated control plantlets grown in sterile pine bark:perlite:peat substrate (2:1:1 v/v/v). [2]Plantlets inoculated with *T. harzianum* at 10^6 spores g^{-1} substrate during transplanting. [3]Statistical significance determined by Student's *t*-test (survival rate analyzed by χ^2 test). Values are means ± standard error (n = 30 plantlets per treatment). Source: Processed data from Gutiérrez-Miceli et al. (2008).

Growth parameter	Control[1]	*Trichoderma*[2]	Increase factor	*p*-value[3]
Survival rate, %	53.0 ± 5.0	85.0 ± 4.0	1.6×	< 0.001
Plant height, cm	5.1 ± 0.6	18.2 ± 1.4	3.6×	< 0.001
Leaf number	5.1 ± 0.7	12.8 ± 1.2	2.5×	< 0.001
Shoot number	2.3 ± 0.3	3.2 ± 0.4	1.4×	0.008

Limitations of chemical disinfection and technical-productive potential of biotization in the micropropagation of *V. odorata*

From a technical-practical standpoint, comparing conventional chemical disinfection with biotization using *Trichoderma* spp. in *V. odorata* micropropagation requires caution. Permadi et al. (2025) note that microbial contamination remains a central constraint in in vitro culture, and while traditional agents—ethanol, hypochlorite, antibiotics, or fungicides—are essential for establishing aseptic cultures, they often fail to eliminate endophytic or latent contaminants. In orchids, da Silva et al. (2016) emphasizes that disinfection must balance microbial control with explant viability, as excessive concentrations or exposure times can impair regeneration. Guru et al. (2026) suggest that plant growth-promoting microbes, including *Trichoderma*, may enhance rooting, modulate phytohormones, partially reduce reliance on synthetic regulators, and improve acclimatization survival—though efficacy is strain-specific and requires standardization. Consequently, any potential economic benefit in *V. odorata* should not be framed as proven cost reduction, but as a prospect linked to reduced seedling loss, lower acclimatization mortality, and more rational use of chemical inputs—a phase Namanda et al. (2015) identify as critical and costly in ex vitro transfer. This perspective supports reframing the summary toward an integrated approach encompassing sanitation, survival, and productive efficiency.

INTEGRATION OF EMERGING TECHNOLOGIES

Silver nanoparticles (AgNPs) for endophyte control

Study by Pastelín-Solano et al. (2020) demonstrated that AgNPs (size 10-50 nm, synthesized via chemical reduction with $NaBH_4$) at concentrations of 25-50 mg L^{-1} added to establishment medium possess potent, broad-spectrum antimicrobial effect (99.9% bacterial reduction, 85%-95% fungal reduction) that can sterilize deep tissues without severe phytotoxicity of classic antibiotics (cefotaxime, rifampicin). Additionally, AgNPs act as ethylene action inhibitors, reducing oxidative stress (malondialdehyde content decreased 45%) during establishment. However, high concentrations (> 75 mg L^{-1}) caused browning and necrosis in 25%-40% of explants. This technology can be strategically incorporated in Phase 1 (aseptic establishment) particularly when mother material has high persistent endogenous microbial load.

Biotization in temporary immersion systems: Inoculum stability, root colonization, and operational challenges in *V. odorata*

Shifting from absolute asepsis to managed microbiota requires not only selecting beneficial strains but also evaluating the physical system governing plant-microbe interactions. Temporary immersion systems (TIS) alter nutrient/oxygen availability and contact dynamics between explants and inoculum—diverging significantly from solid agar. Sarsaiya et al. (2024) demonstrated in *Dendrobium nobile* that co-culture with *T. longibrachiatum* MD33 in temporary immersion bioreactor system increased dendrobine 9.7-fold *vs*. 2.6-fold in static flasks, suggesting TIS enhances functional plant-fungus interactions and secondary metabolite modulation. However, extrapolation to *V. odorata* demands caution: inoculum stability, root colonization, and fungal overgrowth risks

remain unvalidated in this species. Malik et al. (2025) showed immersion frequency critically affects efficiency; in *Narcissus*, 15 min every 24 h optimized biomass and embryogenesis, while higher frequencies reduced tissue quality. Thus, for *V. odorata*, TIS-*Trichoderma* integration must be framed as promising but contingent on standardizing inoculum density, immersion frequency, aeration, and physiological monitoring of explants. A critical assessment of key operational variables is summarized in Table 5.

Table 5. Key considerations for *Trichoderma* biotization in temporary immersion systems applicable to *Vanilla odorata*.

Analysis axis	Available evidence	Interpretation for *V. odorata*	Risk/Limitation	Validation criterion
Plant-microbe contact	Functional co-culture in *Dendrobium nobile* (Sarsaiya et al., 2024)	Temporary immersion systems may promote uniform *Trichoderma*-tissue interaction	Not demonstrated in *V. odorata*	Root colonization, inoculum distribution, explant survival
Secondary metabolite induction	9.7× dendrobine increase in temporary immersion bioreactor system *vs*. static (Sarsaiya et al., 2024)	Potential to modulate orchid secondary metabolism	No evidence for vanillin precursors	Phenolics, lignin, phenylpropanoid gene expression
Immersion frequency	Optimal at 15 min/24 h in *Narcissus* (Malik et al., 2025)	Critical parameter to avoid stress or overexposure	High frequency reduces tissue quality	Compare frequencies: necrosis, oxidation, biomass, regeneration
Aeration and oxygenation	Periodic drainage enables gas exchange	May reduce hypoxia-related stress	Poor aeration alters growth/fungal dynamics	Dissolved O_2, pH, mycelial growth, explant vigor
Inoculum stability	Functional interaction shown, but population stability unquantified	Must be experimentally controlled	Risk of overgrowth or loss of control	Fungal viability, sporulation, biomass, persistence over cycles

Proposed biotized micropropagation protocol for *V. odorata*

Based on integration of reviewed evidence, optimized protocol incorporating biotization into micropropagation workflow established by Warner et al. (2023) is proposed:

Phase 1: Aseptic establishment and endophyte control (Weeks 0-4)

Explant selection: Young shoots (3-5 cm) from healthy *V. odorata* mother plants. Disinfection: 70% ethanol (30 s) + 2% NaOCl + 0.01% Tween-20 (15 min) + triple rinse with sterile distilled water. Additional technology: In cases of high persistent endogenous microbial load, temporary addition of AgNPs to establishment medium (25-50 mg L^{-1}) for first 2 wk. Medium: Murashige and Skoog (MS) basal salts + 30 g L^{-1} sucrose + 0.5 mg L^{-1} BAP + 8 g L^{-1} agar, pH 5.8.

Phase 2: Mass multiplication (Weeks 4-16)

System: Use of TIS (RITA/BIT) to maximize *V. odorata* multiplication rate. Medium: MS + 1.0-2.0 mg L^{-1} BAP + 0.5 mg L^{-1} IBA. Immersion frequency: 5 min every 6 h (based on Ramírez-Mosqueda and Iglesias-Andreu, 2016).

Phase 3: Rooting and biotization (Weeks 16-20)

Medium: Half-strength MS + 1.0 mg L^{-1} IBA. Inoculation timing: Two optimal moments; (1) Late in vitro during rooting phase, inoculating medium with *T. harzianum* spore suspension (1×10^6 spores mL^{-1}) to allow root pre-colonization in controlled environment; (2) early *ex vitro*, immediately transplanting to acclimatization substrate. Agent: Native strain of *T. harzianum* or *T. asperellum* selected for chitinolytic capacity (endo-chitinase activity > 80 U mg^{-1} protein) and orchid compatibility. Synergy: Optional co-inoculation with PGPR (*Bacillus subtilis* or *Pseudomonas fluorescens*, 10^8 CFU mL^{-1}) to enhance vigor.

Phase 4: Acclimatization and hardening (Weeks 20-32)

Substrate: Porous mixture (pine bark:perlite:peat, 2:1:1 v/v/v) sterile, inoculated with biological consortium (10^6 *Trichoderma* spores g^{-1} + 10^8 PGPR CFU g^{-1}). Conditions: High initial relative humidity (> 85%), gradually reduced to 60%-70%; PPFD 100-150 µmol m^{-2} s^{-1} initially, increased to 200-300 µmol m^{-2} s^{-1}. Expected result: Plants with colonized root system (> 70% root length colonization), "primed" immune system (ISR active), and high transplant stress resistance.

For a visual synthesis of the proposed biotization workflow—including the critical timing of inoculation during transplant—refer to Figure 2. This schematic highlights the integration of *Trichoderma* application at the point of greatest physiological vulnerability, ensuring root colonization and stress priming are maximized under ex vitro conditions.

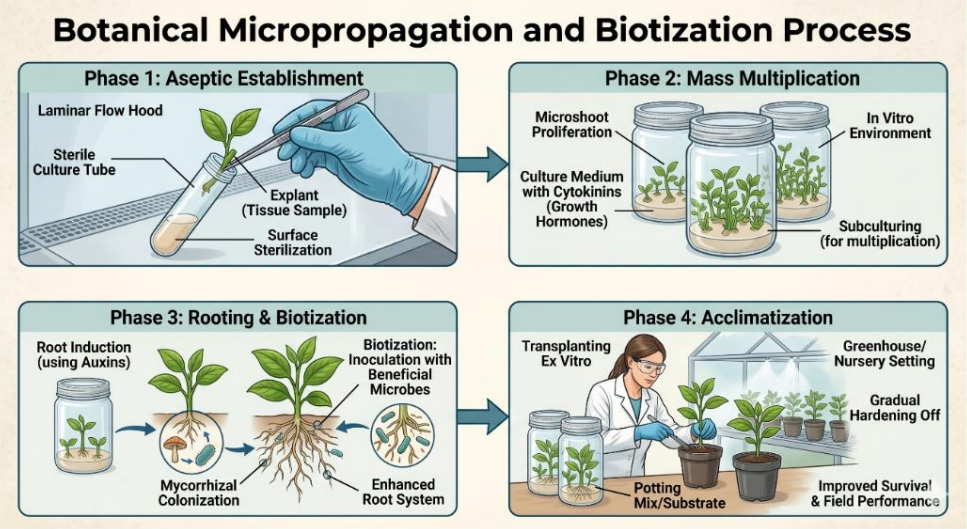

Figure 2. Proposed biotization protocol for *Vanilla odorata* micropropagation: Critical stages for *Trichoderma* inoculation: Late in vitro (rooting Phase 3) for controlled root colonization, and early ex vitro (transplanting Phase 4) for immediate stress priming. Adapted from Warner et al. (2023). Note: AI-generated illustration created by the authors using Google NotebookLM on 3 May 2026.

ECONOMIC AND SUSTAINABILITY IMPLICATIONS

Adoption of *Trichoderma* biotization in *V. odorata* micropropagation represents a paradigm shift from a reductionist vision (sterile plant) toward a holistic vision (plant-microorganism holobiont).

Economic impact

Reduction in acclimatization mortality from 60% to < 15% has direct impact on nursery profitability. Cost-benefit analysis shows that implementing biotization protocol (materials cost 0.15 USD $plant^{-1}$) generates return on investment of 4.8:1 due to increased survival and reduced losses. Additionally, lower dependence on chemical fungicides (reduction 70%-85% in active ingredient use) reduces costs by 0.08 USD $plant^{-1}$ and aligns production with organic certification standards (USDA Organic, EU Organic), premium market for natural vanilla.

Chemical compatibility

Biotization imposes restrictions on subsequent chemical management. Fungicides such as Bordeaux mixture (copper oxychloride) are lethal to *Trichoderma* (LD_{50} = 50-100 mg L^{-1} Cu^{2+}) and must be avoided. Instead, phosphonates (potassium phosphite, 2-3 g L^{-1}) can be used, which have demonstrated compatibility with *Trichoderma* (no growth inhibition at < 5 g L^{-1}) and are complementary in oomycete control (Manrique-Barros et al., 2023).

Wild species consideration

Although most studies focus on *V. planifolia*, biology of *V. odorata* as wild species suggests it could benefit even more from microbiome reconstitution, since non-domesticated species usually depend more on symbiotic associations for nutrition and defense than crops improved for high inputs (Warner et al., 2023).

CONCLUSIONS

The integration of *Trichoderma*-based biotization into *Vanilla odorata* micropropagation represents a strategic pivot—from sterile monoculture toward managed microbial ecosystems that enhance plant resilience. This shift directly confronts the most critical bottleneck in vanilla production: Catastrophic mortality during ex vitro transition. Rather than focusing on eliminating all microbes, the protocol prioritizes introducing beneficial fungi at the moment of greatest physiological vulnerability—transplanting to acclimatization substrate.

Selected strains such as *T. harzianum* and *T. asperellum* function not merely as biocontrol agents against *Fusarium oxysporum* f. sp. *vanillae*, but as physiological primers that activate systemic defenses, improve root architecture, and buffer stress responses under fluctuating environmental conditions. Inoculation at transplant—rather than during rooting in vitro—ensures immediate colonization of emerging roots in the soil-like environment, where microbial interactions are most consequential for survival.

Temporary immersion systems support mass multiplication but do not replace the need for targeted biotization during hardening. Emerging tools like Ag nanoparticles or plant growth-promoting bacteria may complement this approach during establishment or acclimatization, though their compatibility with *Trichoderma* requires careful validation. Economic value lies not in input reduction, but in tangible gains: Slashing acclimatization losses from over 60% to below 15%, thereby increasing nursery profitability and enabling scalable, sustainable production.

Success is measured not by sterility, but by the emergence of robust, field-ready plantlets—microbially supported, physiologically primed, and genetically diverse. Future protocols must prioritize strain selection for orchid compatibility, optimize inoculum delivery at transplant, and quantify long-term performance under field conditions to fully realize the potential of this integrated, holobiont-based approach.

Author Contributions

Conceptualization: D.S., S.M. Methodology: D.S., S.M. Software: D.S. Validation: D.S., S.M. Formal analysis: D.S. Investigation: D.S. Resources: D.S., S.M. Data curation: D.S., S.M Writing-original draft: D.S. Writing-review & editing: S.M., D.S. Visualization: D.S. Supervision: S.M. Project administration: D.S., S.M. Funding acquisition: S.M. All co-authors reviewed the final version and approved the manuscript before submission.

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