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Evaluation of soil fungistasis for the control of the phytopathogen *Botrytis* sp. in rose buds

Tesis en torno a una hipótesis o problema de investigación y su contrastación

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DEDICATION

To my parents Hilda and Isaías, because none of this would have been possible without their support.

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ABSTRACT

This study examines soil fungistasis as a sustainable biological control strategy against *Botrytis* sp., a necrotrophic fungus causing up to 30% of losses in rose production. The research evaluates the fungistatic capacity of Ecuadorian non-agricultural and floricultural soils, the impact of biocides, and the potential of directed evolution to optimize soil microbial communities for pathogen suppression. Soils from Pintag, Cayambe, and Lasso showed inhibition rates of 70.93%, 80.94%, and 82.28%, respectively, confirming fungistasis prevalence in Ecuadorian Andean soils. Biocide application reduced fungistatic capacity, notably in Lasso, where EC50 rose to 0.000117g soil/mL from 6.43×10^{-7} g soil/mL in untreated soils, reflecting a 181-fold increase in EC50, indicating a lower inhibition capacity. To extract the fungistatic potential for *in vivo* application, a soil tea was prepared, achieving *in vitro* inhibition rates of 82.01% (Cayambe) and 81.07% (Lasso). However, inhibition using soil tea in *in vivo* rose petals assays decreased to 14.44% and 13.34% with high variability, suggesting microbial activity is more effective under controlled conditions. To optimize fungistasis in *in vivo* applications, Cayambe's microbial communities underwent 14 generations of directed evolution. "Top" communities achieved 17.47% inhibition, while "Bottom" communities reached 4.23%, generating a simplified consorti5um with replicable inhibitory capacity and potential for *in vivo* applications.

These findings confirm that soil fungistasis, driven by microbiota, offers a sustainable strategy for controlling *Botrytis* sp. in rose production.

Keywords: fungistasis, *Botrytis* sp., soil microbiota, biological control, directed evolution, roses, floriculture, microbial consortia, pathogen suppression.

RESUMEN

Este estudio examina la fungistasis del suelo como una estrategia de control biológico sostenible contra *Botrytis* sp., un hongo necrotrófico responsable de una pérdida de productividad hasta del 30% alrededor del mundo. La investigación evalúa la capacidad fungistática de los suelos no agrícolas y florícolas ecuatorianos, el impacto de los fungicidas y el potencial de la evolución dirigida para optimizar las comunidades microbianas del suelo con el fin de suprimir patógenos. Los suelos de Pintag, Cayambe y Lasso mostraron tasas de inhibición del 70,93%, 80,94% y 82,28%, respectivamente, lo que confirma la prevalencia de la fungistasis en los suelos andinos ecuatorianos. La aplicación de fungicidas redujo la capacidad fungistática, especialmente en Lasso, donde el EC50 aumentó a 0,000117 g de suelo/ml desde $6{,}43\times10^{-7}$ g de suelo/ml en suelos no tratados, reflejando un incremento de 181 veces en el EC50, lo que indica una reducción significativa en la capacidad de inhibición. Para extraer el potencial fungistático para su uso *in vivo*, se preparó té de suelo, logrando tasas de inhibición *in vitro* del 82,01% (Cayambe) y 81,07% (Lasso). Sin embargo, la inhibición utilizando té de suelo en ensayos *in vivo* con pétalos de rosa disminuyó al 14,44% y 13,34%, con alta variabilidad, lo que sugiere que la actividad microbiana es más efectiva bajo condiciones controladas. Con el fin de optimizar la fungistasis en aplicaciones *in vivo*, las comunidades microbianas de Cayambe se sometieron a 14 generaciones de evolución dirigida. Las comunidades "Top" alcanzaron una inhibición del 17,47%, mientras que las comunidades "Bottom" lograron un 4,23%, lo que permitió generar un consorcio microbiano simplificado con capacidad inhibitoria replicable y potencial para aplicaciones *in vivo*.

Estos hallazgos confirman que la fungistasis del suelo, impulsada por el microbiota, ofrece una estrategia sostenible para el control de *Botrytis sp.* en la producción de rosas.

Palabras clave: fungistasis, *Botrytis* sp., microbiota del suelo, control biológico, evolución dirigida, rosas, floricultura, consorcios microbianos, supresión de patógenos.

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1. *Botrytis cinerea*

1.1 Biology and Life Cycle of *B. cinerea*

Botrytis cinerea is a necrotrophic pathogen that causes gray mold in various plants, including grapes, strawberries, and roses. It utilizes virulence factors such as cell wall-degrading enzymes, toxins (botrydial and botcinine), and cell death-inducing proteins (CDIPs) like BcCrh1 and BcXyn11A1 [1]. Its life cycle includes three main stages: germination, penetration, and establishment. Germination occurs under conditions of at least 93% relative humidity and temperatures between 10°C and 23°C. Penetration occurs via germ tubes or appressoria, culminating in biomass accumulation in dead tissues [2]. Its adaptability to diverse hosts and conditions establishes it as a high-impact agricultural pathogen.

1.2 Limitations of Current Control Methods

The chemical control (biocides) of *Botrytis cinerea* faces critical limitations due to multiple (MLR) and multidrug (MDR) resistances, resulting from mutations in genes such as *β-tubulin, cytb, sdhB, erg27,* and *Bcpos5*, which affect key biocides: SDHIs (boscalid, fluopyram), SBIs (fenhexamid), and APs (cyprodinil). Furthermore, the overexpression of efflux transporters (*atrB*, *mfsM2*) amplifies cross-resistance to pyraclostrobin, iprodione, and fludioxonil. These resistances, documented at rates exceeding 80% of frequency, drastically reduce the efficacy of biocides and management strategies such as crop rotations [3,4]. Additionally, the overuse of biocides leads to environmental contamination and affects non-target organisms, highlighting the urgent need for sustainable and integrated alternatives.

The management of *Botrytis cinerea* through technological and biological alternatives offers promising solutions to the limitations of conventional methods. For example, poly (lactic-co-glycolic acid) (PLGA) nanoparticles encapsulating biocide fluopyram penetrate

conidia and mycelium within minutes, maximizing antifungal efficacy and protecting the compounds from degradation. Pterostilbene inhibits conidial germination, while fluopyram reduces fungal growth by 72% after 48 hours [5]. On the other hand, in biological options, an example is the use of *Bacillus amyloliquefaciens* QST713 (Ba QST713), which acts through antibiosis, competition for nutrients, production of antimicrobial compounds (surfactins, iturins, and fengicins), and the induction of systemic resistance. It has shown both preventive and curative efficacy, particularly at low temperatures (10°C) [6]. However, biological strategies face limitations regarding adhesion to the petal and resistance to UV rays.

Among the biological strategies for controlling *Botrytis cinerea*, soil fungistasis emerges as a crucial natural process in the suppression of fungal pathogens, providing an ecological foundation for the sustainable management of agricultural diseases.

2. Soil Fungistasis

2.1 Definition and Basic Concepts

Soil fungistasis is a phenomenon in which fungal propagules, such as spores or hyphae, fail to germinate or exhibit inhibited growth in the soil despite favorable temperature and humidity conditions. This inhibition is due to factors such as the production of fungistatic compounds by the microbial communities in the soil or nutrient competition exerted by the microorganisms present. This process plays a crucial role in soil ecology as it regulates the life cycles of fungi and contributes to the general suppression of diseases caused by soil-borne pathogens[7,8]. As soil pathogens, *Fusarium* species such as *Fusarium culmorum* and *Fusarium oxysporum* have been commonly used in the analysis of soil fungistasis. However, the study of soil fungistasis has expanded to include other fungi, such as nematophagous fungi like *Pochonia chlamydosporia* and *Lecanicillium psalliotae*. Mycoparasitic fungi such as *Clonostachys rosea*, *Trichoderma viride*, *Trichoderma harzianum*, *Trichoderma koningii*, and

Trichoderma hamatum have also been analyzed. Furthermore, entomopathogenic fungi used for biological control, such as *Beauveria bassiana* and *Metarhizium robertsii*, as well as saprophytic/antagonistic fungi like *Chaetomium globosum*, have been studied [9–11].

2.2 Mechanisms Responsible for Soil Fungistasis

Bacteria and fungi, play a key role in the soil fungistasis mechanisms. A study on the importance of root commensal bacteria (BRCs) demonstrated how they play a crucial role in regulating fungal and oomycete communities in the roots of *Arabidopsis thaliana*. These interactions are characterized by predominant negative correlations between bacteria such as *Pseudomonas* and *Variovorax* and filamentous eukaryotes like *Davidiella* and *Alternaria*, indicating competition for resources and direct antagonistic mechanisms. Furthermore, microbiota reconstitution experiments showed that in the absence of BRCs, fungi and oomycetes dominate the roots, negatively affecting plant growth and survival [12]. Therefore, this microbial imbalance highlights the essential role of BRCs in maintaining fungistasis, as their presence limits the proliferation of pathogenic organisms and ensures a functional balance in the root microbiota to provide plant fitness.

A previous study further expands this concept on *Fusarium graminearum*, which demonstrated that temperature sterilization reduces soil fungistasis by altering bacterial diversity and composition. Thermal treatments up to 121°C eliminated key bacteria such as *Pseudomonas* and *Acidobacteria*, resulting in the near-total loss of fungistasis. In this regard, complex microbial interactions determine the fungistasis mechanism in the soil [13].

2.2.1 Competition for nutrients and space

It has been reported that bacterial richness and the presence of key genera such as *Flavobacterium*, *Mycoplana*, *Gelidibacter*, and *Pseudeurotium* are essential to restrict the proliferation of *Fusarium graminearum* [14]. Specifically, these bacteria limit the pathogen

through competition for resources, direct antagonism, and the production of inhibitory compounds. In a study on suppressive soils, the role of the microbiota in inhibiting diseases caused by *Fusarium* was analyzed. The importance of bacterial communities such as *Bacillus* and *Pseudomonas* was highlighted, as they release siderophores that limit the pathogen's access to iron, while *Paenibacillus* produces fusaricidin, a compound that activates plant defense pathways [15].

On the other hand, fungal diversity also plays a crucial role, as synthetic community trials have shown that fungal communities with higher species richness significantly increase fungistasis, reducing the germination and growth of *Trichoderma harzianum* and *Mucor* sp. This effect is attributed to competition for resources and the partitioning of ecological niches, which contributes to the suppression of invasive fungi and creates more stable fungistatic conditions [16].

Finally, the competition and interaction between bacteria and fungi driven by the limited availability of carbon and nutrients in the soil is another factor affecting fungistasis. On one hand, bacteria predominate in the uptake of simple compounds such as sugars and amino acids, while fungi dominate the decomposition of complex molecules like lignin and cellulose through specialized exoenzymes [17]. This differentiation determines the structure of chemical and spatial niches in the soil. On the other hand, co-occurrences between these groups reflect key ecological interactions such as competition, mutualism, and parasitism. For example, saprophytic fungi facilitate bacterial activity by releasing enzymes that break down complex polymers, while certain bacteria solubilize phosphorus and fix nitrogen, supporting fungal activity in the decomposition of lignocellulose [18].

2.2.2 Production of Antimicrobial Compounds

The capacity of microorganisms to produce antimicrobial compounds and volatile organic compounds is fundamental to soil fungistasis, with specific metabolites and their mechanisms of action being particularly significant. For instance, *Aspergillus fumigatus*, isolated from soil beneath a lichen thallus, synthesizes N-formyl-4-hydroxyphenyl-acetamide and atraric acid. The latter exhibits antifungal activity, inhibiting up to 87% of *Pleurotus ostreatus* growth [19]. Similarly, *Bacillus velezensis* synthesizes cyclic lipopeptides such as surfactin, which facilitate biofilm formation in symbiotic interactions with the beneficial fungus *Rhizophagus irregularis*. Additionally, it produces antifungal metabolites like bacillisin, which protect *Rhizophagus irregularis* from mycopathogens including *Trichoderma harzianum* [20]. Although *Trichoderma* species are generally regarded as beneficial due to their role in biological control, their mycoparasitic behavior posing a threat to symbiotic fungi [21].

Furthermore, a comparative study with soil microorganisms, including *Bacillus*, *Streptomyces*, *Actinomycetes*, *Penicillium notatum*, and *Hortea werneckii*, highlighted their ability to synthesize antimicrobial compounds and extracellular enzymes such as proteases and cellulases, which are essential in microbial dynamics [22]. Another study identified various antimicrobial compounds produced by bacteria isolated from the rhizosphere of *Arabidopsis*, specifically *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Acinetobacter sp.* These microorganisms demonstrated the ability to inhibit the growth of *Phytophthora* species, including *P. capsici*, *P. citricola*, *P. palmivora*, and *P. cinnamomi*. The main compounds identified comprised diketopiperazines (DKPs), cellulases, proteases, catechol-type siderophores, and a range of volatile compounds whose chemical nature could not be characterized[23]. Finally, a study with *Paenarthrobacter ureafaciens*, isolated from disease-suppressive soils, reported that this microorganism emits microbial volatile compounds (mVCs) that inhibit up to 73.41% of the growth of *Saccharomyces cerevisiae*, inducing

oxidative stress, accumulation of reactive oxygen species (ROS), and mitochondrial dysfunction [24].

2.3 Factors Affecting Soil Fungistasis

Abiotic factors play a crucial role in soil fungistasis, directly affecting the survival and proliferation of pathogens such as *Fusarium graminearum*. The resistance of soil to *F. graminearum* is linked to concentrations of manganese (Mn) and iron (Fe). Manganese showed a significantly inverse correlation with pathogen viability, suggesting that optimal levels of Mn may inhibit fungal growth by interfering with metabolic processes. Iron, while essential for the fungus, can limit its proliferation when the native microbiota produces siderophores that sequester this nutrient [14].

Regarding agricultural practices, tillage strategies and crop management also play a key role in regulating soil fungistasis. For example, it was found that no-tillage and reduced-tillage systems increase soil organic carbon (SOC) content and microbial biomass in surface layers, which is associated with greater fungistatic activity and disease suppression [25]. However, in another similar study, a different pattern was observed: fungistasis was higher in soils with conventional tillage and lower in no-tillage systems, negatively correlating with the DNA content of *Fusarium graminearum*. Additionally, crop residues with low C:N ratios, such as legumes, decompose more rapidly and reduce the persistence of the pathogen, whereas cereal residues promote its survival, especially in no-tillage systems [26]. These differences suggest that the impact of tillage practices on fungistasis may vary depending on the context, possibly due to differences in climate, soil type, or residue management.

In addition to minerals and agricultural practices, soil moisture is another critical abiotic factor for fungistasis. High moisture levels (90% and 50% of maximum water holding capacity) have been shown to promote the germination of microsclerotia of *Verticillium longisporum* in

sterilized soils, while low moisture levels (6% MWHC) significantly reduce germination, regardless of the presence of active microbiota [27]. This indicates that moisture influences fungal activity beyond microbial effects.

Finally, the agrochemicals used to treat the soil can also deeply influence fungistasis. Fumigation with chloroform significantly reduced fungistasis, increasing the growth of *Fusarium graminearum* by up to 80% in treated soils, compared to non-fumigated soils. This effect is attributed to removing competing microorganisms and releasing of labile carbon compounds from dead microbial cells, creating favorable conditions for the pathogen's development [26]. Additionally, in another study, the effects of three chemical disinfectants on soil were analyzed, highlighting that the biocide dazomet significantly reduced microbial diversity, affecting key genera such as *Nitrospira* and *Massilia*, while favoring *Pseudomonas* and *Gemmatimonas*. This disinfectant inhibited essential enzymatic activities such as urease and sucrase, altering carbon and nitrogen cycles. Fenaminosulf, a specific biocide, primarily reduced fungal diversity without significantly affecting bacteria, while kasugamycin showed milder and more transient effects on enzymatic activity [28]. These results highlight that the effects and recovery of soil depend on the spectrum and persistence of the application a myriad of disinfectants.

While natural fungistasis inherently regulates a variety of pathogens, its application in field settings remains limited due to the variability of microbial communities across soils, driven by specific soil characteristics that can lead to unstable effects. Directed evolution offers a solution by enabling the optimization and stabilization of microbial communities, thereby enhancing their potential for agricultural disease control.

3. Directed Evolution

3.1 Introduction to the concept of directed evolution and its relevance in the design of microbial communities

Directed evolution in microbial communities emerges as a key tool for the design and optimization of functional microbiomes, directly addressing the complexity of linking composition and function at the community level. This approach is based on two essential principles: first, heritable phenotypic variation within communities, meaning that differences in collective functions must have a reproducible basis that allows for selection; and second, the ability to regenerate such variation after each selection cycle, achieved through techniques such as bottlenecks, which reduce initial complexity to refocus selection on desirable traits, or through environmental disturbances that induce adaptive changes [29]. These tools allow for the maintenance of the functional diversity necessary to achieve optimal microbial evolution, while mitigating common issues such as chaotic ecological succession or generational instability. This iterative cycle approach of artificial selection explores the "structure-function ecological landscape," a conceptual framework that describes how interactions between community members determine emergent collective capabilities [30]. Complementing this perspective, it is noted that these landscapes can be interpreted as extensions of genotype-phenotype maps, adapted to the non-linear and synergistic interactions between species [31]. These interactions allow the identification of "soft modes" that reduce the dimensionality of the search space, facilitating navigation through complex landscapes and optimizing specific community functions, such as biodegradation or metabolite production. Unlike traditional strategies like "migrant-pool" or "propagule" methods, which attempt to optimize communities through simple reassemblies this fail to achieve a consistent functional maximum meanwhile directed evolution combines enrichment and compositional exploration processes to generate robust and resilient communities [30].

3.2 Use of Bottlenecks as an Experimental Tool to Select Key Functional Traits

Bottlenecks are tools that simplify microbial communities by reducing their initial diversity. This process is related to genetic drift, as the reduction in diversity can cause certain metabolic traits to become fixed in the population, even if they do not provide an adaptive advantage. However, it is also possible that some traits may be lost, as genetic drift is governed by random events without direct selective pressures. It all starts from the initial constraints that promote resource partitioning and density-dependent metabolic interactions, such as competitive exclusion and cooperative growth, which steer the assemblages towards stable and multistable functional states, especially in resource-limited systems [32]. This perspective is reinforced by highlighting those bottlenecks guide communities toward multistable states, where different stable functional configurations can emerge under identical conditions. These transitions are driven by density-dependent cooperative interactions, such as Allee effects. In this context, a strong Allee effect can lead to extinctions below critical thresholds, while a weak Allee effect favors coexistence or the dominance of specific species [33]. This perspective is reinforced by highlighting that bottleneck guide communities toward multistable states, where different stable functional configurations can emerge under identical conditions. These transitions are driven by density-dependent cooperative interactions, such as Allee effects. In this context, a strong Allee effect can lead to extinctions below critical thresholds, while a weak Allee effect favors coexistence or the dominance of specific species [34]. This perspective is reinforced by highlighting those bottlenecks guide communities toward multistable states, where different stable functional configurations can emerge under identical conditions. These transitions are driven by density-dependent cooperative interactions, such as Allee effects. In this context, a strong Allee effect can lead to extinctions below critical thresholds, while a weak Allee effect favors coexistence or the dominance of specific species [35].

3.3 Examples of Functional Convergence through Bottlenecks

This perspective is reinforced by highlighting those bottlenecks guide communities toward multistable states, where different stable functional configurations can emerge under identical conditions. These transitions are driven by density-dependent cooperative interactions, such as Allee effects. In this context, a strong Allee effect can lead to extinctions below critical thresholds, while a weak Allee effect favors coexistence or the dominance of specific species[33].

The first example occurs under controlled conditions, with glucose as the sole carbon source. Through successive passages, microbial communities converge towards similar functional structures at the family level despite high initial variability. These taxonomic families, such as Enterobacteriaceae and Pseudomonadaceae, are functionally redundant. The drivers of this convergence are cross-metabolic interactions and interspecific competition, which play a key role in stabilizing the emerging function [36]. In another study, the explanation of this behavior was expanded by noting that both stochastic and deterministic dynamics govern community assembly. In other words, deterministic processes, such as migration rates and interspecific competition, interact with the random variability inherent in the initial composition or environmental fluctuations, leading to consistent functional patterns regardless of the initial community composition [37].

Similarly, the impact of copper stress on siderophore production, a key metabolite in metal detoxification, was investigated. In this case, copper induced a stabilizing selection that favored intermediate levels of siderophores by penalizing both low microbial producers due to their lower tolerance to stress and high producers due to metabolic costs and diminishing returns. This process occurred through two mechanisms acting simultaneously: ecological selection, which rearranged the relative abundance of species towards taxa with moderate production, such as *Cupriavidus*, and adaptive evolution in *Pseudomonas fluorescens*, which reduced its investment in siderophores to optimize metabolic efficiency [38].

On the other hand, of genetic algorithms was used to predict and optimize key microbial functions through iterative cycles of community assembly and disassembly in a previously optimized environment. In their study, continuous bottlenecks optimized microbial functions of four-species communities based on their degradative capacity for industrial pollutants. After 18 rounds, the optimized communities integrated effective degraders, metabolic facilitators, and "free-riders" (not involved in degradation). Most species in the evolved communities did not significantly differ from their ancestors in their phenotype, suggesting that genetic evolution plays a small role at this temporal scale [39].

In another study, nine cycles of iterative passage were employed to select microbial communities in the phyllosphere, which significantly suppressed bacterial blight disease in tomato, caused by *Pseudomonas syringae* pv. *tomato*. Initially, the severity of the disease progressively increased until passages 4-5, with an average of 38-45%. However, starting from passage 7, it decreased drastically, reaching levels below 10% by passage 9. Notably, the removal of these communities through thermal treatment reversed the suppressive effect, confirming the relevance of microbial activity [40].

Finally, using mangrove soil microcosms enriched with PET and an artificial selection system to isolate plastic-transforming microbial consortia, six transfer cycles were performed. Adjustments such as the addition of peptone (T1-T3) and an increase in temperature to 40°C (T4-T6) directed selection towards key taxa such as *Pseudoxanthomonas winnipegensis*, *Brevibacillus*, and *Paenibacillus*. By the end, the selected consortia were able to partially modify PET, as evidenced by spectroscopy, demonstrating the potential of iterative selection under controlled conditions to optimize microbial communities in polymer degradation [41].

Together, these studies illustrate how different mechanisms can lead to functional convergence in microbial communities through bottlenecks. Understanding these processes is

essential for the design and manipulation of microbiomes with applications in agriculture, medicine, and biotechnology.

PART 2: SCIENTIFIC ARTICLE

Evaluation of soil fungistasis for the control of the phytopathogen *Botrytis* sp. in rose buds

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Introduction

Roses (from the genus *Rosa* of the Rosaceae family) are valued for its diversity of colors, fragrances, and ornamental applications. With approximately 30,000 to 35,000 cultivated varieties, are used in floral arrangements, perfumery, and the pharmaceutical industry[42]. In Ecuador, roses are one of the main non-oil export products for export. By 2023, the total production of roses reached 3.64 billion cut stems, with an annual production generating estimated revenues of 809 million dollars, making Ecuador the second largest exporter of this product worldwide [43-44]. The impact of roses extends beyond the economic level, as they provide approximately 50,000 jobs in Ecuador [45].

However, this sector faces significant challenges, primarily due to diseases that affect plants in the field. These include bacterial diseases, such as *Pseudomonas syringae*, which causes necrosis in stems, and nematodes like *Meloidogyne spp.* However, the greatest concerns in the field stem from fungal diseases, such as *Diplocarpon rosae* (black spot), *Peronospora sparsa* (downy mildew), *Oidium* sp. (powdery mildew), *Phragmidium spp.* (rust), and *Botrytis cinerea*, which represents not only a serious issue in the field but also in post-harvest [46]. *Botrytis cinerea* is a necrotrophic fungus that causes losses of at least 30% in the annual rose production,

affecting quality and marketability [47]. In the field, it infects petals and damaged tissues through stomata, wounds, or the cuticle, thriving in high humidity (>93%) and temperatures of 10-20°C, which are the standard conditions in greenhouses [48]. This leads to latent infections that exacerbate as tissues mature, producing conidia and sclerotia that perpetuate the disease. Its persistence in plant residues and genetic variability complicate control efforts. This issue extends to post-harvest, where lesions and visible mold diminish the shelf life and market value of flowers [47,49].

Chemical control has proven insufficient due to the emergence of multiple resistances caused by mutations in key genes affecting biocides such as SDHIs (boscalid, fluopyram), SBIs (fenhexamid), and APs (cyprodinil)[3,50]. In intensively treated crops, resistance exceeds 80%, and a continuous increase in multiresistant strains is observed, indicating a gradual accumulation of resistances due to selection pressure [50].

On the other hand, biological methods also face challenges due to inconsistencies caused by unpredictable environmental conditions, such as fluctuations in temperature, humidity, and air, which affect their activity. In addition, issues related to plant adhesion and the production of chemical exudates, influenced by factors such as age, humidity, and light, also modify the surface microflora, impacting the effectiveness of biocontrol, which in some cases may be either promoted or interfered with by the natural microbiota[6,51].

In this context, soil fungistasis emerges as a potential approach. Fungistasis is an intrinsic soil phenomenon that inhibits the growth of fungal propagules through competition for nutrients and the production of inhibitory compounds. It affects various fungal species, regardless of their previous interaction with the soil [8]. This phenomenon is directly related to soil microbial activity; when the soil is sterilized, it loses its inhibitory capacity, highlighting the crucial role of the microbiota in fungistasis [8,52]. Although fungistasis has been extensively studied in soil fungi such as *Fusarium* sp., its potential for the control of foliar pathogens, such

as *Botrytis* sp., has yet to be explored. This is partly due to the lack of studies that allow for extracting of the soil's fungistatic potential and its application to other parts of the plant.

Soil has an impressive diversity; a single gram of soil can harbor up to 10^{10} bacterial cells and an estimated species diversity ranging from 4 x 10^3 to 5 x 10^4 species, resulting in extremely complex interactions [53]. This diversity complicates the identification of bacteria responsible for specific phenomena, such as fungistasis. Despite advances in the study of soil microbiota, no effective protocol yet exists to reduce this complexity without compromising the desired phenotype. Directed evolution techniques, such as bottlenecks with serial passes and enrichment through fermentation, allow for the generation of bacterial subcommunities with reduced diversity but capable of maintaining specific phenotypes[30,35].

Ecuadorian floriculture, particularly in Pichincha and Cotopaxi, faces an economic and social challenge due to the impact of *Botrytis cinerea* on roses during the raining season. These regions account for 68% and 15% of national production, respectively due to the optimal conditions for rose cultivation, such as altitudes of 2,600-3,000 masl, temperatures ranging from 5-25°C, and fertile soils; however, these environmental conditions also favor the proliferation of this pathogen. [54,55]. In this context, it is crucial to investigate how the agricultural practices of local flower growers, particularly the use of biocides, affect the fungistatic capacity of the soil and to determine whether soils exposed to both *Botrytis* and biocides retain a useful fungistatic effect.

The present study aims to evaluate the fungistatic capacity of soils from floricultural farms in the inhibition of *Botrytis* sp. and to explore how the use of directed evolution techniques can stabilize and optimize an inoculum capable of effectively controlling the pathogen in rose petals. In addition, we will evaluate whether the microorganisms present are sufficient to generate this effect, analyze the impact of biocides on the inhibitory capacity of the soil and determine whether the inhibitory effect can be extracted both *in vitro* and *in vivo*.

In doing so, we aim to explore new possibilities for biological control agent and promote more sustainable farming practices.

Results

Soil microorganisms are necessary and sufficient to establish soil fungistasis

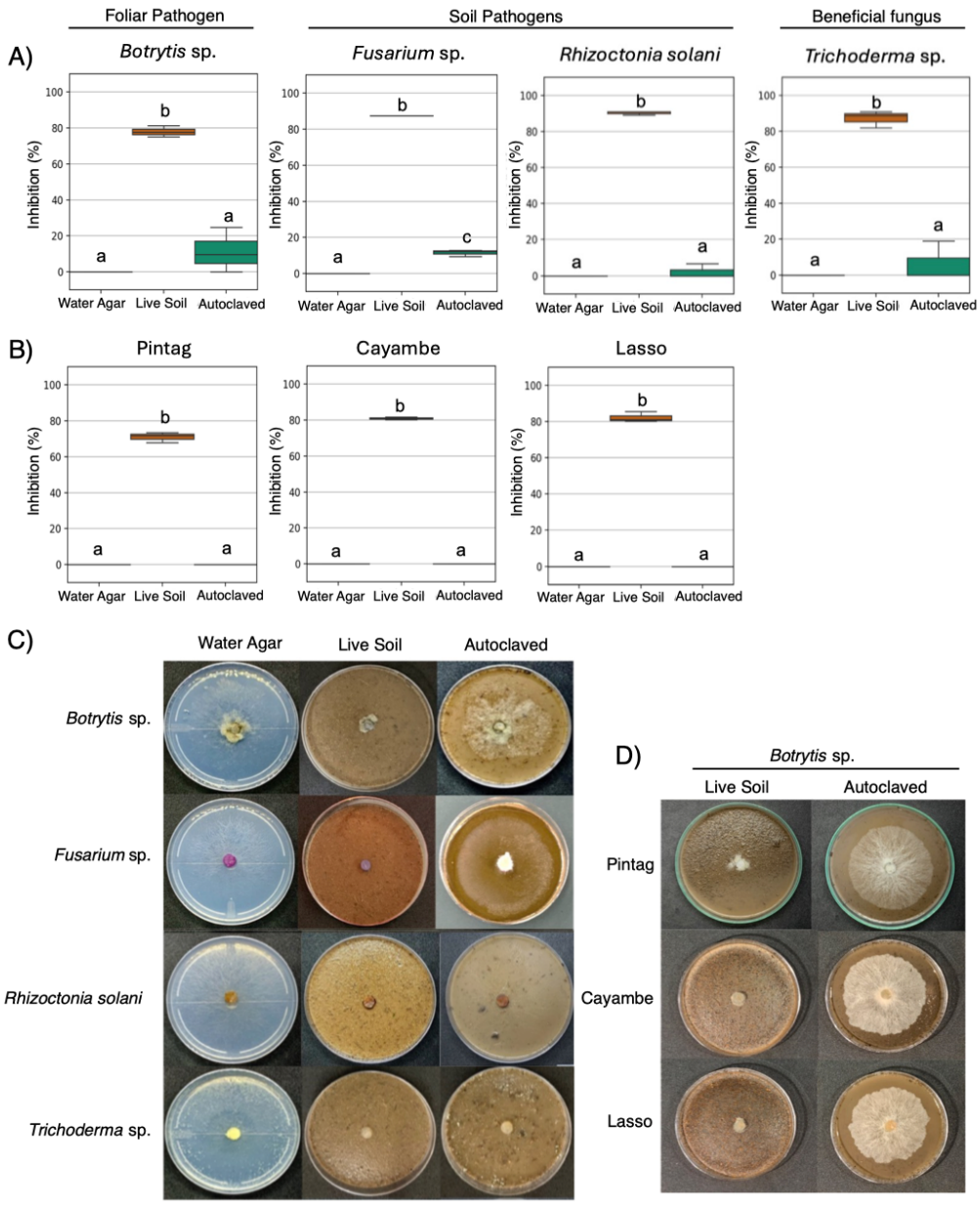


Figure 1. Fungistatic effect of live and autoclaved soil on pathogens and beneficial fungi in different locations.
A and C) Inhibition of growth of foliar and soil pathogens (*Botrytis* sp., *Fusarium* sp., *Rhizoctonia solani*) and the beneficial fungus *Trichoderma* sp. in three treatments: water agar, live soil, and autoclaved soil, using soil samples from Pintag. **B and D)** Comparison of the fungistatic effect of live and autoclaved soil on the foliar pathogen *Botrytis* sp. across the locations of Pintag, Cayambe, and Lasso. These results confirm that live soil exerts a significant

fungistatic effect in contrast to autoclaved soil, indicating that the microorganisms present in the soil are both necessary and sufficient for pathogen inhibition. Different letters indicate statistically significant differences between treatments according to ANOVA followed by Tukey's HSD post-hoc test ($p < 0.05$).

The fungistasis analysis assays that are described in Figure 6.1 y 6.2 showed that the microorganisms present in the soil are essential for the inhibitory effect against fungal pathogens and beneficial fungi, as live soil exhibited a significantly greater inhibition capacity compared to autoclaved soil (Figures 1A and 1C). In the Pintag soil, sterilization drastically reduced the inhibition capacity against *Botrytis* sp., *Fusarium* sp., *Rhizoctonia solani*, and *Trichoderma* sp. For the foliar fungus *Botrytis* sp., the inhibition percentage in live soil was 77.94%, dropping to only 11% in autoclaved soil ($p = 0.0001$). For soil pathogens, *Fusarium* sp. showed an inhibition of 87.36% in live soil, reducing to 11.53% in autoclaved soil ($p < 0.001$); *Rhizoctonia solani* presented an inhibition of 90.26% in live soil, decreasing to 2.25% in autoclaved soil ($p =0.0495$). Finally, the beneficial fungus *Trichoderma* sp. experienced an inhibition of 87.15% in live soil, which fell to 6.36% after sterilization ($p = 0.0495$). It is important to note that there were no significant differences between the water agar control and autoclaved soil ($p > 0.05$), except for *Fusarium* sp. ($p < 0.001$), where autoclaved soil retained some residual resistance (Figure 1A).

These results suggest that the loss of microorganisms in the soil eliminates its inhibitory capacity, indicating that the fungistatic effect of live soil is broad, affecting fungi with different origins and physiologies. To verify that this effect is not exclusive to Pintag soil, an additional assay was performed comparing soils from three locations: Pintag, Cayambe, and Lasso. Under live soil conditions, inhibition percentages of 70.93%, 80.94%, and 82.28% were observed, respectively, while in autoclaved soils, inhibition was 0% across all locations ($p < 0.001$)

(Figures 1B and 1D). This reinforces the conclusion that soil microorganisms are both necessary and sufficient to exert a robust fungistatic effect against various fungi.

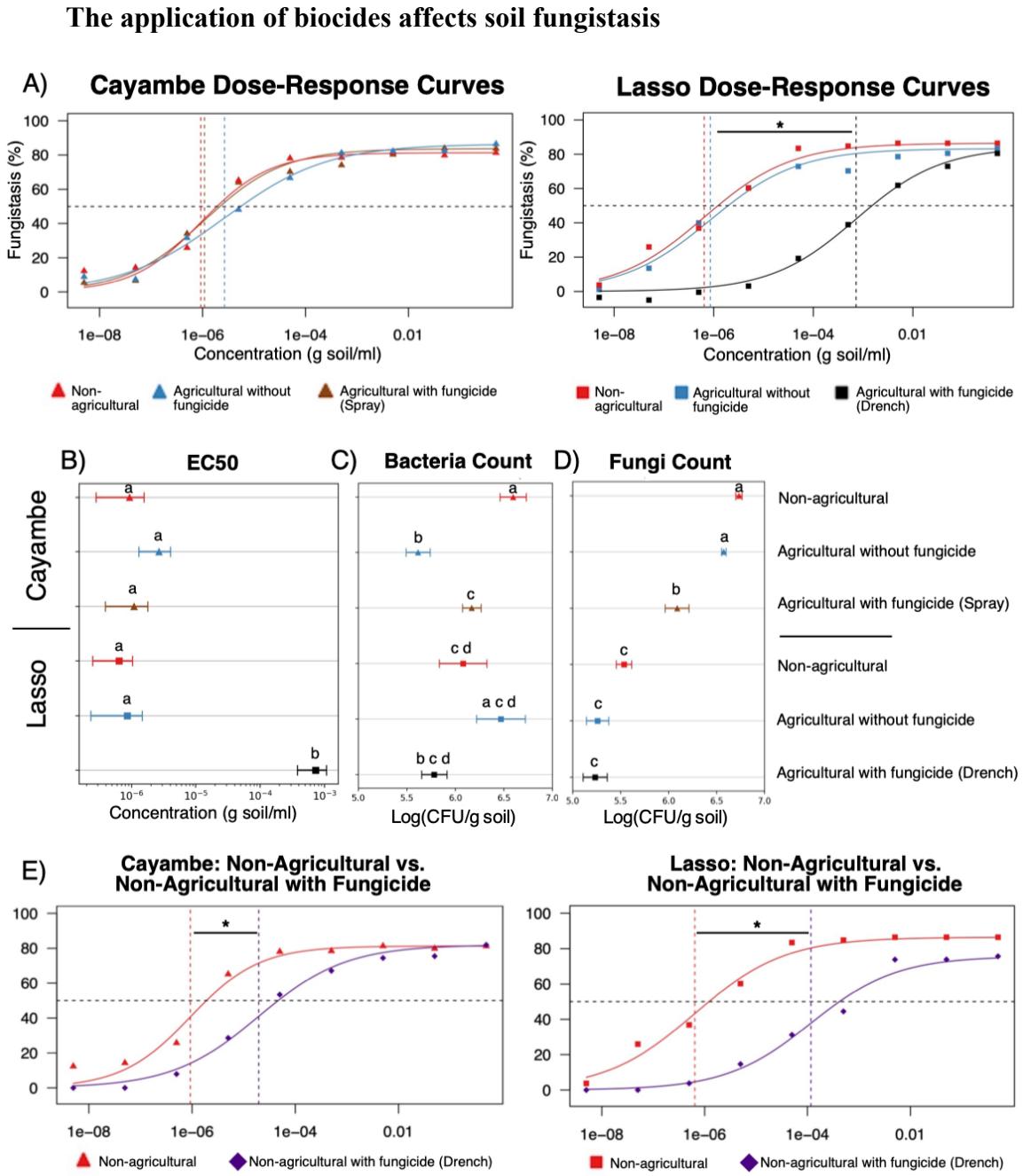


Figure 2. Impact of Biocides on Fungistatic Effect and Microbial Count in Soil.
A) Dose-response curves for agricultural and non-agricultural soils from Cayambe and Lasso against *Botrytis* sp., evaluating the fungistatic effect based on soil concentration (g soil/mL). **B)** EC50 values in soils from Cayambe and Lasso, comparing different treatment conditions. **C)** Bacterial count (CFU/g soil) in non-agricultural soils, agricultural soils without biocide, and agricultural soils with spray and drench treatments. **D)** Fungal count (CFU/g soil) in non-agricultural soils, agricultural soils without biocide, and agricultural soils with spray and drench treatments. **E)** Effect of biocide treatment applied using the drench technique on non-

agricultural soils from Lasso and Cayambe. Biocide application by drench decreases the fungistatic effect of the soil, with no direct influence from the quantity of fungi or bacteria. Different letters indicate statistically significant differences between treatments according to the ANOVA test followed by Tukey's HSD post-hoc analysis ($p < 0.05$). (*) indicate statistically significant differences between treatments according to the Wald test ($p < 0.05$).

The serial dilution process to assess the fungistatic effect of live soil , described in Figure 7 revealed high resistance to fungistatic activity. The gradual loss of this activity began at the $1:1 \times 10^4$ dilution and disappeared completely at the $1:1 \times 10^8$ dilution. In contrast, no significant differences were observed between dilutions in autoclaved soil, which was used as a control (see Figure S1). This methodology facilitated the acquisition of data points, which were fitted to a curve to calculate the EC50 value and analyze each curve independently for the Cayambe and Lasso soils, along with their respective fit errors (see Figure S2).

In the dose-response curves for Cayambe (Figures 2A and 2B), no significant differences in EC50 values were found between non-agricultural soils ($EC50 = 9.25\times10^{-7}$, CI: $2.81\times10^{-7} – 1.57\times10^{-6}$), agricultural soils without biocide ($EC50 = 2.67\times10^{-6}$, CI: $1.30\times10^{-6} – 4.03\times10^{-6}$) ($p= 0.05$), and soils treated with spray biocide ($EC50 = 1.09\times10^{-6}$, CI: $3.9\times10^{-7} – 1.79\times10^{-6}$) ($p = 1$). These results suggest that biocide application does not significantly affect the fungistatic capacity of Cayambe soil, regardless of the treatment.

On the other hand, in Lasso (Figures 2A and 2B), biocide application by drench showed a notable impact on soil fungistatic capacity. In non-agricultural soils ($EC50 = 6.43\times10^{-7}$, CI: $2.50\times10^{-7} – 1.04\times10^{-6}$) and agricultural soils without biocide ($EC50 = 8.54\times10^{-7}$, CI: $2.34\times10^{-7} – 1.47\times10^{-6}$), EC50 values remained low and don’t have significant differences ($p = 1$), indicating high fungistatic activity. However, in agricultural soils treated with biocide by drench ($EC50 = 7.2\times10^{-4}$, CI: $3.78\times10^{-4} – 1.07\times10^{-3}$), the EC50 increased significantly, showing an approximate increase of 1119 times compared to agricultural soils without biocide ($p < 0.001$) and 843 times relative to non-agricultural soils ($p < 0.001$), respectively, reflecting

a drastic reduction in fungistatic capacity. This suggests that biocide application in agricultural soils significantly affects fungistatic capacity, especially when using the drench technique, while spray application does not have a significant effect.

Regarding microbial biomass (CFU) (Figures 2C and 2D), in Cayambe, non-agricultural soils showed the highest bacterial (6.59 ± 0.14) and fungal (6.73 ± 0.03) counts, followed by soils treated with spray biocide (bacteria: 6.17 ± 0.10, fungi: 6.09 ± 0.12). Agricultural soils without biocide had the lowest bacterial counts (5.61 ± 0.12), although fungal counts were like those in non-agricultural soils (6.57 ± 0.02). In Lasso, agricultural soils without biocide had the highest bacterial counts (6.47 ± 0.25), followed by non-agricultural soils (6.08 ± 0.25), while soils treated with biocide by drench showed the lowest values (5.78 ± 0.13). For fungi, non-agricultural soils had the highest counts (5.53 ± 0.08), but no significant differences were observed compared to agricultural soils without biocide (5.26 ± 0.12) or those treated with biocide by drench (5.23 ± 0.12).

Overall, Cayambe soils showed higher CFU values than Lasso soils; however, the EC50 differences do not correlate, which may indicate a lack of influence of microorganisms. This is confirmed in Lasso, where variations in bacterial and fungal counts were not significant between non-agricultural soils and those treated with biocide by drench ($p > 0.05$), suggesting that biocide application did not significantly affect microbial biomass.

To confirm the effect of biocide applied by drench in reducing fungistatic capacity, non-agricultural soils treated with biocide were used, following the procedure described in Figure 8. As shown in Figure 2E, in untreated soils from Cayambe, the EC50 value was 9.25×10^{-7} (CI: 2.81×10^{-7} – 1.57×10^{-6}). After biocide application, EC50 increased to 1.96×10^{-5} (CI: 1.02×10^{-5} – 2.89×10^{-5}), indicating a 21-fold increase in EC50 which means worst inhibition capacity ($p < 0.001$). In Lasso soils, the EC50 value for untreated soils was 6.43×10^{-7} (CI: 2.50×10^{-7} – 1.04×10^{-6}), and after biocide application, EC50 increased to 0.000117 (CI: 0.0000388 –

0.000195), reflecting a 181-fold increase in EC50 which means worst inhibition capacity ($p < 0.001$).

Based on this assay and evaluating both EC50 values and bacterial and fungal counts, we conclude that non-agricultural soils have the highest fungistatic potential for *in vivo* use. Although no significant differences were observed due to the higher average in EC50, we will prioritize the non-agricultural soil from Cayambe for further analysis.

Fungistatic effect on *Botrytis* sp. in *in vivo* and *in vitro* assays: Extraction and application

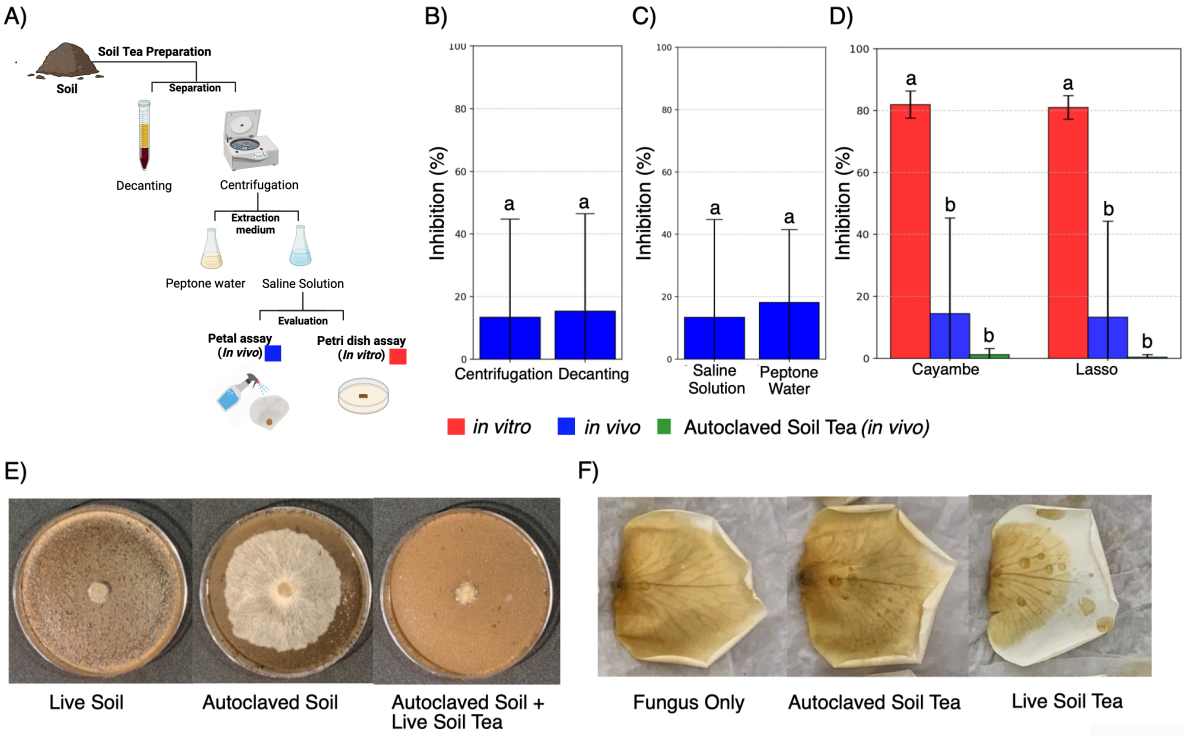


Figure 3. Comparison of the fungistatic effect *in vitro* and *in vivo* on rose petals treated with soil tea.
A) Diagram of the *in vitro* and *in vivo* assays, both using soil samples and employing soil tea as a treatment. **B)** Comparison of soil tea extraction methods (centrifugation vs. decantation) in the inhibition of *Botrytis* sp. **C)** Comparison between saline solution and peptone water as extraction media for soil tea using centrifugation. **D)** Comparison of *in vitro* vs. *in vivo* inhibition. **E)** *In vitro* plate images where the fungistatic effect is recovered in autoclaved soils treated with soil tea. **F)** Images of petals treated under *in vivo* conditions, where the fungistatic effect is not maintained with the same intensity as observed *in vitro*. These results show that soil tea exhibits a more pronounced fungistatic effect under *in vitro* conditions compared to direct applications on plant tissue. Different letters indicate statistically significant differences between treatments according to the t-test ($p < 0.05$).

The use of soil tea of the non-agricultural soil from Cayambe using the process described in Figure 9A demonstrated a robust fungistatic effect under *in vitro* conditions, highlighting its potential for controlling *Botrytis sp.* When its effectiveness was evaluated under *in vivo* conditions using the petal assay described in Figure 9A and 9B, no significant differences were observed between extraction methods using centrifugation (13.41 ± 31.41%) and decantation (15.37 ± 31.18%) ($p = 0.80$) (Figure 3B). However, the high variability in the data suggests that these methods are equally limited for extracting soil tea and applying it directly to plant tissues. This result emphasizes the complexity of maintaining the fungistatic activity of the tea under less controlled conditions. Regarding the extraction media (Figure 3C), no statistically significant differences were found between saline solution (13.41 ± 31.41%) and peptone water (18.19 ± 23.36%) ($p = 0.91$). Although peptone water showed a slightly higher performance, the high data dispersion limits its reliability as a preferred method. Both media allow for the recovery of active compounds, but the effectiveness of the tea appears to be influenced by factors beyond the extraction medium.

Finally, when comparing the localities and experimental conditions, it was evident that the activity of the soil tea was significantly higher under *in vitro* conditions than *in vivo* (Figure 3D). In the *in vitro* assays, average inhibition rates of 82.01% in Cayambe and 81.07% in Lasso were achieved, while in the *in vivo* assays, these figures drastically dropped to 14.44% and 13.34%, respectively. Additionally, autoclaved tea exhibited minimal inhibition (<1.21%), confirming that the active compounds responsible for the fungistatic activity are heat sensitive. These differences highlight the influence of environmental and biological factors on the effectiveness of soil tea when applied directly to plant tissue.

Thus, despite the absence of significant differences in the protective performance between the non-agricultural soils of Lasso and Cayambe, the non-agricultural soil from

Cayambe will be used for further directed evolution analysis, as it still shows, on average, a slightly better performance compared to Lasso.

Directed evolution as a method for stabilizing the fungistatic effect

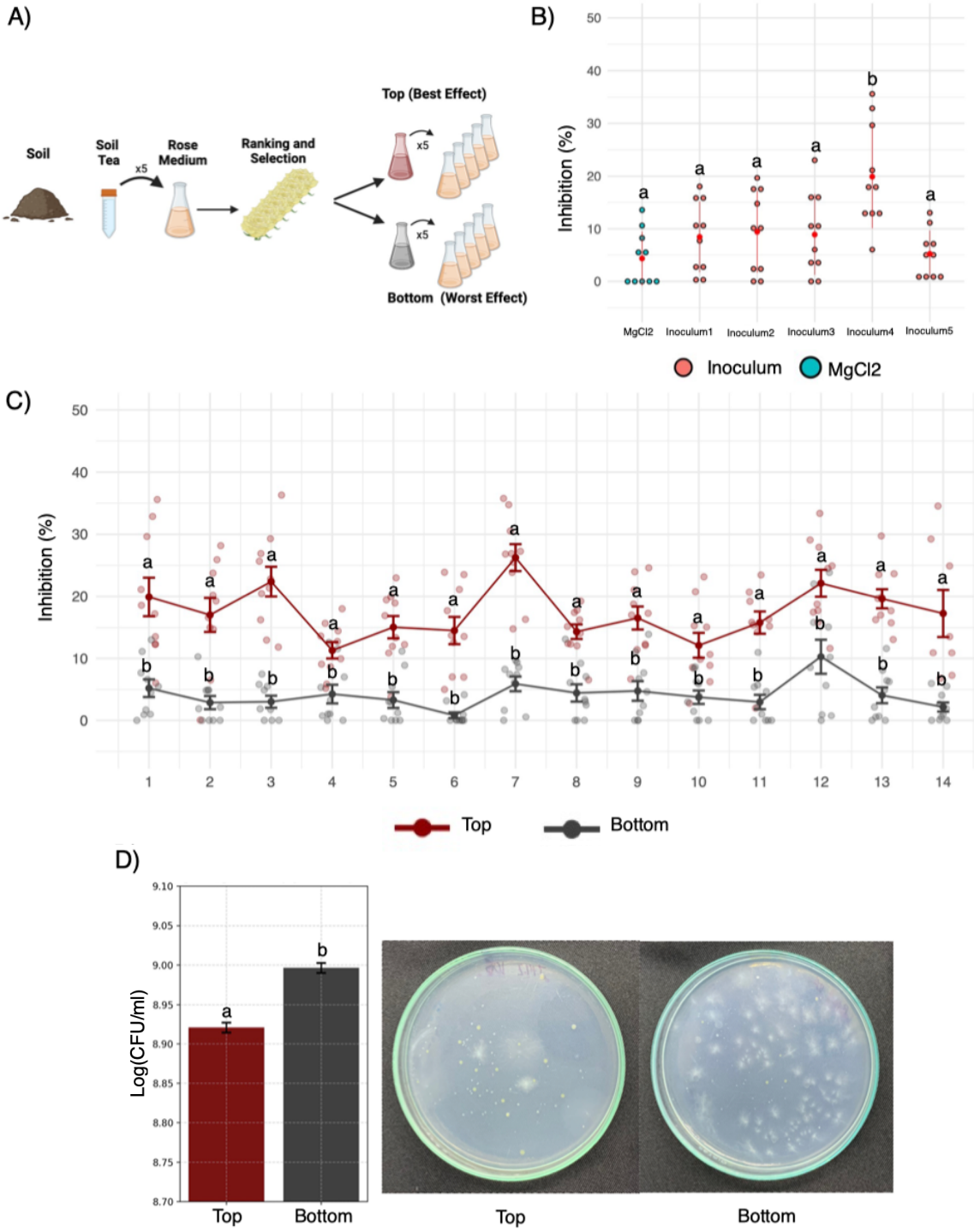


Figure 4. Stabilization of fungistatic effect through directed evolution.
A) Experimental diagram of the directed evolution process to select microbial communities with high (Top) and low (Bottom) fungistatic effect against *Botrytis* sp. **B)** Initial inhibition

percentage of the five inocula evaluated. Letters indicate statistically significant differences between groups according to ANOVA and Tukey's HSD post-hoc test ($p < 0.05$). **C)** Evolution of the inhibition percentage over 14 generations. Letters indicate statistically significant differences according to the t-test ($p < 0.05$). **D)** Comparison of bacterial growth Log (CFU/ml) between Top and Bottom communities, accompanied by representative images of the culture plates. Letters indicate statistically significant differences according to the t-test ($p < 0.05$). The Top communities show a high and stable fungistatic effect, while the Bottom communities maintain a consistently low fungistatic effect.

Directed evolution was initiated using the non-agricultural soil from Cayambe and the fresh rose medium, according to the scheme presented in Figure 11. The initial step of directed evolution allowed for the selection of microbial communities with the best and worst fungistatic capacities, identified as Top and Bottom, respectively. The five initial inoculum, cultivated for 72 hours, exhibited significant differences in *Botrytis* sp. growth inhibition, as shown by ANOVA (Figure 4B). The inoculum with the highest inhibition (Top) achieved 19.92% with significant differences with the other inoculum ($p < 0.001$), while the one with the lowest effect (Bottom) reached 5.21%. The remaining inoculum, with intermediate values, were discarded to focus selection on the extremes. This initial selection is critical for establishing the conditions necessary for directed evolution, ensuring maximum divergence between Top and Bottom.

Throughout 14 generations, the Top communities significantly maintained their fungistatic effect, while the Bottom communities remained at low inhibition levels. In all generations, the differences between Top and Bottom were significant according to a statistical analysis (t-test). The Top group exhibited an average inhibition rate of 17.47%, with peaks reaching 26.25% (Figure 4C). On the other hand, the Bottom group maintained an average inhibition of 4.23%, never exceeding 10.29%. This divergence indicates that the Top communities effectively responded to the selection, while the Bottom communities failed to adapt, resulting in low fungistatic capacity.

The comparison between the initial and final generations confirms that directed evolution is effective in stabilizing the fungistatic effect in the Top communities. At the beginning of the experiment, the Top and Bottom communities exhibited an inhibition difference of 14.71 percentage points. In contrast, at the end of the generations, this difference increased to 21.03 percentage points, with an average difference along the generations of 13.24%. This increase suggests that selective pressure not only maintains desirable traits but also has the potential to optimize fungistatic ability over time, ensuring its efficacy for *in vivo* applications.

The differences observed between the Top and Bottom communities were also evaluated by plating the last generation and performing colony-forming unit (CFU) counts. Statistical analysis via a t-test showed a significant difference between both groups (Figure 4D). The Bottom group exhibited an average of 8.996 Log (CFU/ml), while the Top group reached 8.921 Log (CFU/ml) with a significant difference.

These differences reflect distinct behaviors between the Top and Bottom communities. The Bottom group showed a higher colony density, while the Top group exhibited slightly reduced growth under the same conditions. This pattern is consistent with the divergent properties selected during the experiment. However, further studies are needed to determine the specific causes of this difference.

Discussion and limitations

In conclusion, this study has confirmed that soil fungistasis is a widespread phenomenon that depends on the microorganisms present within the soil. The thermal sterilization experiments demonstrated that the soil's inhibitory power is completely lost against a broad spectrum of four fungi with different ecologies, regardless of the geographic origin of the soils.

These findings expand on previous reports where exposure of soil to 121°C facilitates the colonization of *Fusarium graminearum* in Petri dishes[13].

Regarding the generalized inhibition of *Botrytis* sp., *Fusarium* sp., *Rhizoctonia solani*, and *Trichoderma* sp., it is suggested that this inhibitory effect could extend to other fungi of agricultural importance, such as *Sclerotinia sclerotiorum*. This fungus belongs to the same class, *Leotiomycetes*, and the family *Sclerotiniaceae*, like *Botrytis* sp., with both being necrotrophic fungi [56]. Similarly, *Verticillium* spp. could also be included, as although it belongs to a different family, it shares the class *Sordariomycetes* with *Fusarium*. Both are characterized by causing vascular wilt and generating co-infections in crops such as cotton or potato[57,58]. In the same way, *Ceratobasidium*, related to *Rhizoctonia solani*, and types of *Trichoderma*, such as *Trichoderma harzianum* or *Trichoderma virens*, could also be affected by fungistasis [59,60].

Therefore, due to the ecological and pathogenic relationships of the fungi studied with other pathogenic and beneficial fungi, and considering their different ecologies, as some are foliar and others are soil-borne, we can affirm that fungistasis is indeed a general phenomenon in soil.

On the other hand, assays conducted on soils from three locations (Pintag, Cayambe, and Lasso) suggest that the fungistatic capacity is consistently high across high Andean soils. However, compositional and textural differences are evident. Soils from Cayambe exhibit an organic matter content of 5-11% and a loamy-sandy texture, while Lasso soils contain only 1-3% organic matter and are predominantly sandy[61,6263,64]. These differences in organic matter and texture may influence the specific dynamics of fungistatic activity at each site. Nevertheless, this evidence suggests that fungistasis is not solely dependent on soil composition, as similar fungistatic behavior can be observed across diverse soil types in the high Andean region.

However, the elimination of thermolabile compounds during sterilization by the high temperatures of the autoclave could be an important factor in this loss of activity. For this reason, gamma ray sterilization is recommended to eliminate microorganisms without significantly altering the chemical composition of the soil. These authors suggest that a dose of between 20-70 kGy can eliminate microorganisms, modifying only the carbon and nitrogen reserves, which should be considered in experiments [65,66]. Finally, it would be valuable to expand the assays to soils from coastal and Amazonian regions to observe the behavior of fungistasis in these ecosystems. This would allow for a more conclusive determination of whether this fungal inhibition phenomenon is applicable to any type of soil.

This work also revealed that fungistasis is a persistent effect and does not depend on the number of fungi or bacteria in the soil. Through serial dilutions, it was determined that the effect can be maintained up to four dilutions. The application of the EC50 as a quantitative metric represents a conceptual advance by treating soil, a complex biological system, as if it were a pharmaceutical drug. This approach enables a more precise evaluation of its inhibitory capacity.

The EC50 analysis, along with fungal and bacterial counts, indicates that Lasso soil treated with biocides based on copper hydroxide and cymoxanil, applied via drench, exhibited a greater loss in fungistatic capacity. This result suggests that these biocides specifically alter the composition and dynamics of microbial communities beyond merely affecting their abundance or count. In fact, no significant differences were observed in the quantity of fungi or bacteria between non-agricultural soils and agricultural soils treated with biocides by drench, despite a wide and significant difference in their EC50 values.

Bacterial dynamics, however, showed greater variability compared to fungal dynamics, particularly in Lasso, where no significant differences in fungi count were observed between treatments. This finding suggests that cymoxanil has a lesser effect on bacterial dynamics, with

copper hydroxide appearing to play a more influential role. This conclusion is supported by the limited available information on the impact of cymoxanil on microbial communities. However, it is well-documented that intensive copper use in soils reduces the diversity of active bacterial communities, favoring the prevalence of copper-tolerant taxa such as *Nitrospira* and *Acidobacteria*, while groups like *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* tend to decrease. Such shifts in microbial structure can influence the mineralization of organic compounds and the carbon cycle [67]. In this context, the reduction in fungistasis may be linked to the decline of these specific microbial groups, especially considering that floricultural applications in Cayambe do not involve active ingredients based on copper.

The impact of biocides on fungistatic activity was further confirmed through the application of the copper hydroxide-cymoxanil mixture to non-agricultural soils, where a significant reduction in fungistasis was observed compared to untreated non-agricultural soils. Cu-based agrochemicals exert microbiological effects by disrupting cellular structures, binding to biomolecules, and disturbing cellular homeostasis. Their interaction with soil microbiota raises concerns about potential shifts in microbial balance, the development of resistance, and broader ecological impacts[68].

To better understand this effect, it is recommended to conduct assays using cymoxanil independently to completely rule out its role in the reduction of soil fungistasis. Additionally, it would be useful to conduct tests that evaluate the fungistatic response following the removal of specific microbial groups. This could be achieved by applying targeted antibiotics, as demonstrated in previous studies using fluoroquinolones and β-lactams to target Gram-negative bacteria [69].

An important point to highlight is that, according to our assay, tillage or agricultural management does not significantly affect fungistasis, which agrees with the variable results obtained in previous studies [25,26] since fungistasis can be influenced by climate, soil type,

residue management, and not solely by whether the soil has undergone tillage or not. With our assay, this is particularly clear, as the soils from floriculture farms are intensively tilled, and the lack of significant differences when compared to virgin soils rules out the influence of tillage.

The extraction of the inhibitory effect of fungistasis through soil tea for *in vivo* application yielded highly variable results, regardless of the mechanical extraction methods or medium used. This indicates that, even when a nutrient-rich medium is employed, the *in vivo* inhibition is not significantly affected. This is likely due to the abiotic and biological limitations of the petals as a substrate, as they must compete with the native microbiota of the petals and adapt to a smooth, homogeneous environment, which is more exposed compared to the porous, heterogeneous environment of the soil. This has already been observed in previous studies, where adhesion and radiation tolerance are among the main concerns when using biological control [2]. This environmental factor becomes more relevant, as when we use soil tea to recover the effect in an *in vitro* assay with sterilized soil, we achieve a complete recovery of the fungistatic effect. Another potential explanation is the concentration of microorganisms, as approximately 2 mL of soil tea is applied per petal, whereas 25 mL is used per 100 mL of soil medium in *in vitro* assays. This difference may necessitate concentrating the soil tea for petal applications.

Finally, the directed evolution experiments demonstrated the phenotypic variability inherent in microbial communities, even when originating from the same sample. Additionally, the selection and classification of phenotypes using the petal assay proved to be quite fast and efficient. Thus, from the initial classification step, we were able to obtain varied phenotypes: "Top" as the best protection against the pathogen, and "Bottom" as the worst. The consecutive passages, generating subpopulations of the "Top" and "Bottom" from the initial passage over 14 generations, showed how a sudden and severe reduction in the size of a population with each bottleneck and subsequent enrichment allowed the structuring of simplified communities that

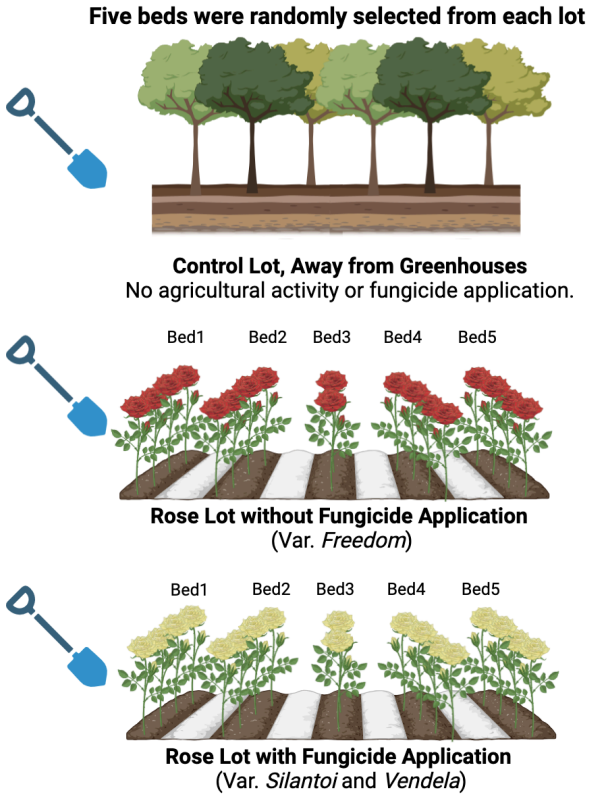
maintain the protection phenotype in the case of the "Top" and the absence of protection in the case of the "Bottom" [36,70]. These simplified communities would facilitate practical applications, as they maintain a stable effect compared to the application of raw soil tea, which was highly variable. Furthermore, they facilitate the study and analysis of key members or interactions responsible for rose petal protection from soil communities, as directed evolution reduces the search space in the communities, optimizing specific community functions [30] [31]. Thus, we have also met the principles of directed evolution, including heritable phenotypic variation, which in this case is the protection against *Botrytis* sp., and the ability to regenerate this variation after each selection cycle with our special medium of fresh rose, $MgCl_2$, and spore solution [29].

However, an important observation arises as this is an indirect evolution experiment, and even though the spore solution is used to expose the community to the fungus, better methods could be explored. Similar studies conclude that optimization in selection is a critical step due to the high dynamics of microbial communities. Proper selection can prevent the dominance of cheaters and grazers, thereby enhancing community function and more effective trait selection[71]. For example, we can use more concentrated spore solutions (higher than 1×10^6 spores/mL) could increase selective pressure on the bacteria and facilitate the selection of communities with more robust protective effects. Additionally, the use of oxalic acid, a well-characterized virulence factor of *Botrytis cinerea*, could provide a more controlled selection model that is less dependent on the intrinsic variability of the spores. Furthermore, studies are showing the possibility of degrading oxalic acid using biological agents [72].

This work makes significant progress in understanding soil fungistasis as a microorganism-dependent phenomenon and proposes innovative methodologies for its study and application. The simplified microbial communities selected through directed evolution and the development of more standardized techniques such as enhanced selective media provide a promising framework for managing fungal diseases in agricultural systems.

Materials and methods

Sampling site and sample collection




Two shovels of soil were taken at a depth of 20 cm for each bed

Figure 5. Soil sampling design in different rose cultivation lots.
Five beds were randomly selected in each lot: 'Rose Lot without Biocide Application' (Var. *Freedom*), 'Rose Lot with Biocide Application' (Var. *Silantoi* and *Vendela*), and a 'Control Lot' with no agricultural activity or biocide application. Two shovels were taken at a depth of 20 cm from each bed.

Soil samples were collected from three key locations: Pintag, Cayambe, and Lasso. Pintag (0°20'28.5"S 78°22'55.8"W) was used as the control location, not associated with floriculture or intensive biocide application, representing reference natural conditions. In Cayambe, samples were taken from rose lots without biocide application of the *Var. Freedom* (0°00'48"N 78°09'52"W), another lot with biocide spray application (Active compounds: Polyoxin, Cyprodinil, Iprodione, Procloraz, Carboxin) of the *Var. Vendela* (0°00'45"N 78°09'57"W), and a control soil located away from the greenhouses, with no agricultural contact

or biocides (0°00'40"N 78°09'48"W). For Lasso, the same approach was followed with a *Var. Freedom* lot (0°45'03.2"S 78°37'56.6"W), in the case of the rose lot with biocide application, it was treated with the Drench technique (Active compounds: Cymoxanil and copper hydroxide) and was of the *Var. Silantoi* (0°44'59"S 78°37'57"W) and finally a control lot (0°44'59"S 78°37'57"W). A composite sampling method was employed to ensure representativeness. Five beds were randomly selected per lot, collecting two shovelfuls of soil at a depth of 20 cm (Figure 5). The 20 cm sampling depth was selected as it aligns with international standards, representing the biologically active soil layer critical for assessing microbial activity and fungistasis.[73–75]. This ensures compatibility with the analysis standards used. The collected samples were stored in breathable sacks and transported to the greenhouse at the Universidad San Francisco de Quito, where they were air-dried for one month, following low-temperature drying recommendations to preserve the microbiological properties of the soil [73]. Subsequently, the samples were sieved through a 5 mm mesh to remove coarse particles and stored in glass jars for further analysis (Table 1).

Code	Location	Agricultural Activity	Biocides	Altitude (masl)	Position
P-NA-NF	Pintag-Pichincha	NO	NO	2714 masl	0°20'28.5"S 78°22'55.8"W
L-NA-NF	Lasso-Cotopaxi	NO	NO	3050 masl	0°44'58.4" S 78°38'01" W
L-SA-NF	Lasso-Cotopaxi	YES	NO	3050 masl	0°45'03.2"S 78°37'56.6"W
L-SA-SF	Lasso-Cotopaxi	YES	YES (Drench)	3050 masl	0°44'59" S 78°37'57" W
C-NA-NF	Cayambe-Pichincha	NO	NO	2740 masl	0°00'40"N 78°09'48"W
C-SA-NF	Cayambe-Pichincha	YES	NO	2740 masl	0°00'48" N 78°09'52" W
C-SA-SF	Cayambe-Pichincha	YES	YES (Spraying)	2740 masl	0°00'45"N 78°09'57"W

Table 1. Details of location and conditions of soil samples collected in three locations.
The table shows the code for each sample, its location, the presence of agricultural activity, the type of biocide treatment, the altitude (masl), and the exact position coordinates for each sampling site.

Fungistasis analysis

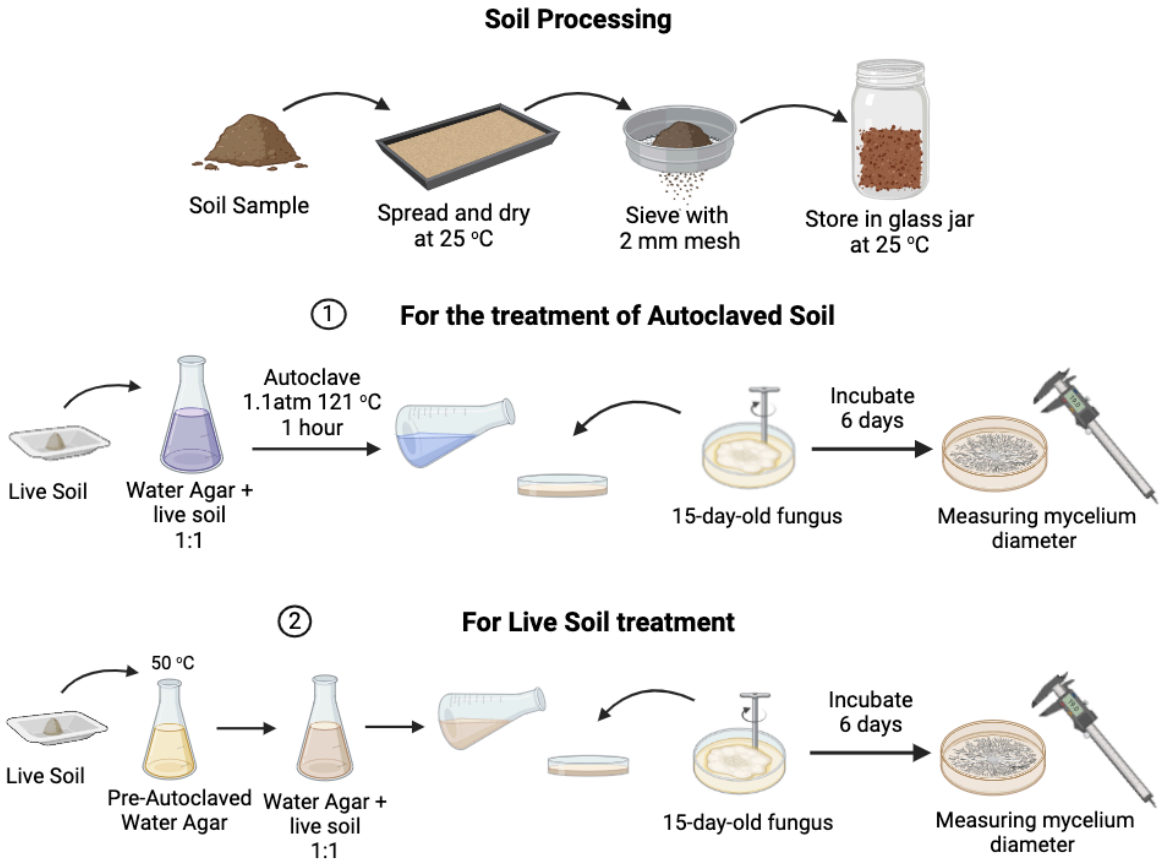


Figure 6. Processing and fungistasis assays of soil samples.
The soil samples were processed and subjected to three treatments: 1) Autoclaved Soil, 2) Live Soil, and 3) Recovery of Effect using Soil Tea. Each treatment was evaluated by measuring the fungal growth diameter after 6 days.

This assay was conducted based on previous studies [76,77]. To prepare this, a 1:1 (weight/volume) mixture of sieved soil in a 2mm mesh and 1.5% water-agar was made, evaluating two treatments: autoclaved soil, live soil. In the autoclaved soil treatment, the soil and water-agar mixture were sterilized in an autoclave at 1 atm of pressure and 120°C for 60 minutes to eliminate all microbial activity, before pouring it into Petri dishes (Figure 6.1).

In the live soil treatment, the soil was not subjected to sterilization, preserving its original microbial composition. Only the water agar was sterilized, and when it reached a temperature of 48-50°C, live soil was added, carefully mixed to achieve a homogeneous distribution before pouring the mixture into Petri dishes (Figure 6.2).

In all treatments, once the medium solidified, a 9 mm diameter mycelium disk from *Botrytis* sp., *Fusarium* sp., *Trichoderma* sp., and *Rhizoctonia solani* (previously cultured for 15 days) was inoculated at the center of each plate. The fungi were obtained from the collection of the agrobiotechnology laboratory of the Universidad San Francisco de Quito. These species were selected for their biological relevance: *Botrytis* sp., as the primary foliar pathogen of interest in this study; *Fusarium* sp. and *Rhizoctonia solani*, as representative soil pathogens; and *Trichoderma* sp., as a beneficial fungus, to observe the differential impact of the fungistatic effect.

Each treatment was performed in quadruplicate, ensuring the statistical robustness of the experimental design. A laser thermometer was used to monitor the temperature of the water agar during preparation, and all implements were autoclaved beforehand to ensure sterility. The plates were incubated at 37°C for 6 days. At the end of the incubation period, the mycelium growth diameter was measured across the plate using a precision caliper, and the average was calculated as the final fungal growth value.

With the mycelial growth diameters, the percentage of inhibition was calculated using the equation (1):

$$(1)\ \%inhibition = \frac{Mycelium\ diameter\ in\ water\ agar - Mycelium\ diameter\ in\ water\ agar\ with\ soil}{Mycelium\ diameter\ in\ water\ agar}$$

For the statistical analysis, an analysis of variance (ANOVA) was performed following a Shapiro-Wilk normality test. A Tukey post-hoc test was then applied to identify significant differences between treatments.

Dilution of the fungistatic effect for comparison between soils and biocide treatments

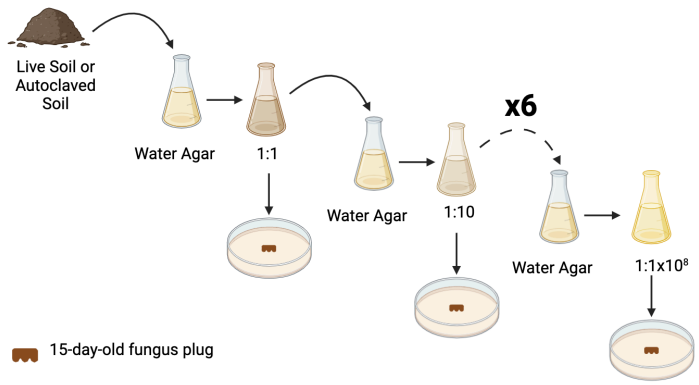


Figure 7. Serial dilutions to evaluate the reduction of the soil fungistatic effect.
Serial dilutions were performed in water-agar, from 1:1 to 1:1×10⁸, starting from live or autoclaved soil. In each dilution, a 15-day-old fungal plug was inoculated to observe the fungistatic effect at each level. Each dilution was evaluated by measuring the fungal growth diameter after 6 days.

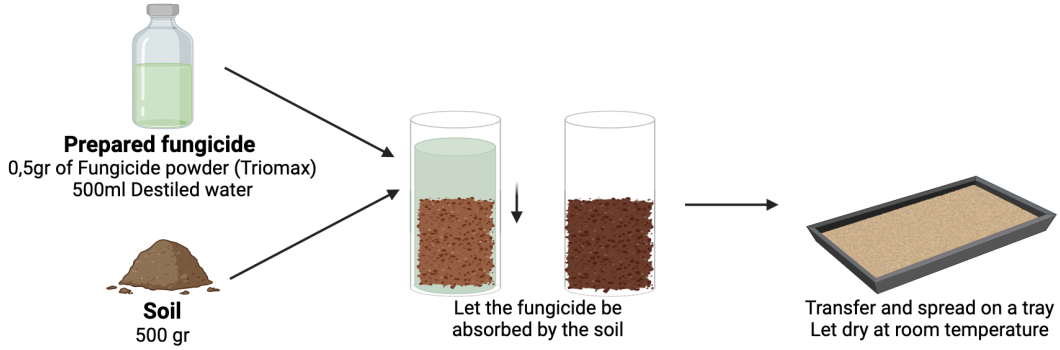


Figure 8. Preparation of biocide-treated soil via drench-like technique.
Biocide solution is poured over soil, allowed to absorb, and dried at room temperature to simulate field application.

Using the same procedure described previously in Figure 6 for preparing the soil and water agar mixture in a 1:1 ratio, serial dilutions were made from this initial solution to evaluate the reduction of the soil fungistatic effect based on its concentration (Figure 7). The dilutions were performed in water agar, starting from a 1:1 concentration and continuing until reaching a final dilution of 1×10^{-8} through successive passages. Each dilution step was carried out while maintaining the water agar at a temperature between 48 and 50°C to ensure the homogeneity of the mixture. Each dilution was then dispensed into Petri dishes in quadruplicate and inoculated with a 9 mm diameter mycelial disk of *Botrytis* sp., grown for 15 days. The plates were

incubated at 37°C for a period of 6 days. At the end of the incubation period, the mycelial diameter was measured in each dilution using a precision caliper.

To assess the impact of biocides on fungistatic activity, 500gr of non-agricultural soils from Cayambe and Lasso were treated with 0.5 g of Triomax (Active compounds: Copper oxychloride, Mancozeb, and Cymoxanil) dissolved in 500 mL of distilled water. The soil-biocide mixture was left to absorb the liquid completely, then air-dried at room temperature. The treated soils were subsequently employed in petri dish assays and dilution-based fungistatic effect tests, as detailed in Figures 6 and 8 in triplicate.

For comparison between soils and treatments, the EC50 was calculated by fitting the percentage of inhibition, now referred to as % fungistasis, since the mycelial diameter in autoclaved soil (instead of the water agar control) was compared with the mycelial diameter in live soil. The % fungistasis was calculated using the equation (2):

$$(2)\ \%Fungistasis = \frac{Mycelium\ diameter\ of\ autoclaved\ soil - Mycelium\ diameter\ of\ live\ soil}{Mycelium\ diameter\ of\ autoclaved\ soil}$$

The obtained % fungistasis values for each of the serial dilutions were fitted to a four-parameter logistic curve, using the model with the equation (3) [78,79]:

$$(3)\ f(x) = c + \frac{d-c}{1+exp^{b(\log(x)-\log(e))}}$$

c represents the lowest point of the curve

b determines the slope of the curve

d represents the highest point of the curve

e is the EC50 value

This approach allowed for the determination of EC50, defined as the concentration of soil required to achieve 50% inhibition of fungal growth, providing a quantitative parameter to compare the fungistatic capacity between different soils and treatments.

For the microorganism count in the soil, 1/10 R2A medium was used for bacteria in triplicated, and the counting was performed after 3 days. For fungi, DRBC medium was used in duplicated, and the counting was performed after 5 days.

Confidence intervals (CI) for EC50 values were calculated using the profile likelihood method, which provides two-tailed 95% confidence intervals based on the fitted four-parameter logistic curve model. The 2.5% and 97.5% percentiles represent the lower and upper bounds of the intervals, respectively. Additionally, the Wald test was used to evaluate significant differences in EC50 values between non-agricultural and agricultural soils treated with biocides. For the statistical analysis of fungal and bacterial counts, an analysis of variance (ANOVA) was performed after assessing data normality using the Shapiro-Wilk test. A Tukey post-hoc test was then applied to identify significant differences between treatments. Results were expressed as mean ± standard deviation (SD).

In vitro and *in vivo* evaluation of tea soil

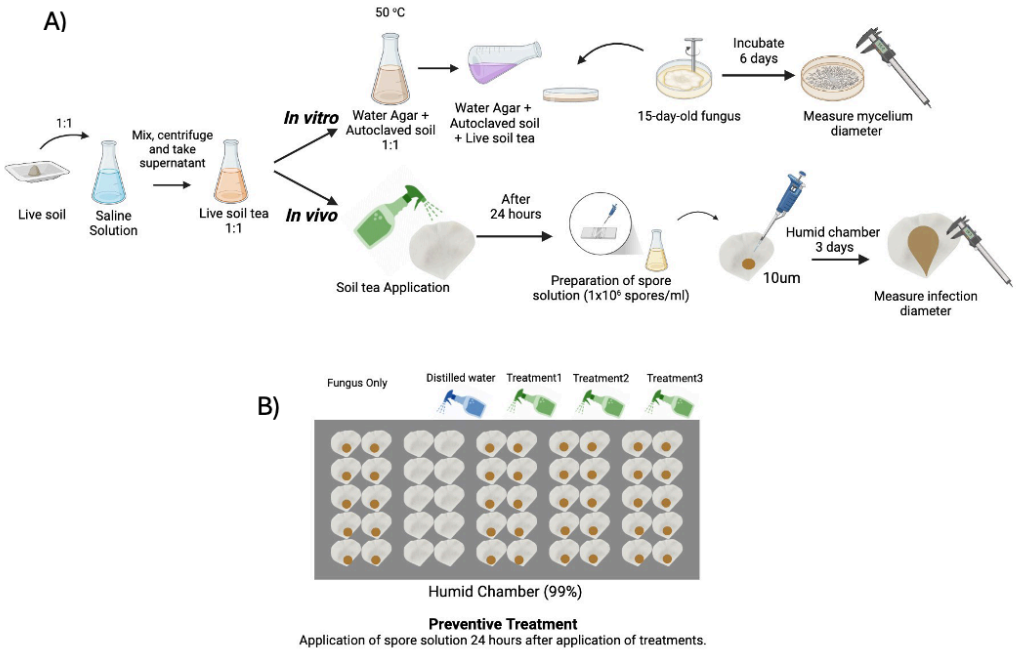


Figure 9. Protocol for evaluating soil tea effectiveness in *in vitro* and *in vivo* assays.
(A) *In vitro*: Soil tea mixed with autoclaved soil and water agar is inoculated with a fungus plug; mycelial growth is measured after 6 days. *In vivo*: Soil tea-treated rose petals receive

spore solution after 24 hours and are incubated for 3 days to measure infection diameter. **(B)** *In vivo* petal assay: Layout of 10 repetitions per treatment, including fungus-only control and distilled water control.

In vitro evaluation

To evaluate the *in vitro* efficacy of soil tea, a 1:1 mixture of live soil and saline solution was prepared. The suspension was agitated at 195 rpm for 10 minutes and subsequently centrifuged at 1,400 g for 15 minutes. The resulting supernatant was filtered through a 10 μm filter, yielding the soil tea. This soil tea was incorporated into a sterilized soil and water-agar mixture, ensuring the tea constituted 20% of the total volume. The homogeneous mixture was poured into Petri dishes in quadruplicate, and after solidification, a 9-mm mycelial disc of *Botrytis* sp., previously cultured for 15 days, was inoculated at the center of each plate (Figure 9).

In vivo evaluation

Humidity Chamber

For the preventive assay on rose petals (Figure 9B), a humidity chamber was prepared using plastic containers thoroughly washed with soap, bleach, and alcohol. Then, autoclaved paper soaked in sterilized distilled water was placed inside, maintaining a constant humidity of 99% to create an optimal environment for fungal development.

Preventive Assay for Inhibition of Botrytis sp.

For the assay, white rose petals from the *Var. Vendela* were selected, removing the first four petals from each flower and using petals numbered 14 and 15, which were checked to ensure the absence of visible pathogens or mechanical damage. Ten petals were placed for each treatment. As this was a preventive assay, the treatments were applied to the petals 24 hours before spore inoculation.

The *Botrytis* sp. spore solution was prepared at a concentration of 1×10^6 spores/mL, using a Neubauer chamber to accurately quantify the spore concentration, and PDB as the medium. Each petal was inoculated with 10 µL of this solution applied to the center. The spores were obtained from previously infected petals or from *Botrytis* sp. cultures grown for 15 days, which were exposed to Dark light for 3 days to stimulate spore production. The treated and inoculated petals were placed in the humidity chamber and incubated for 72 hours. After this period, the results were evaluated by measuring the fungal growth diameter on each petal using a caliper in cross-section, and the average was calculated for each treatment. This protocol allowed the determination of the efficacy of the preventive treatments in inhibiting fungal infection development (Figure 9). For statistical analysis, a t-test was conducted to identify significant differences between treatments. Results were expressed as mean ± standard deviation (SD).

Directed evolution

Preparation of Culture Media

Category	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6
Tea Medium and Spore Solution	MgCl2	MgCl2	MgCl2	Peptone Water	Peptone Water	1/10 TSB
Rose	No	Fresh (10%)	Powder (1%)	No	Fresh (10%)	Fresh (10%)
pH Buffer	0.05 g MES					

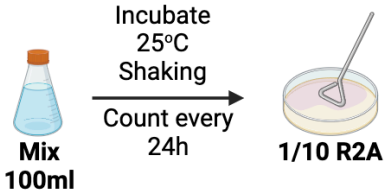


Figure 10. Selection of Culture Media for Directed Evolution Assays.
Six mixes with different combinations of $MgCl_2$, peptone water, TSB broth, and rose (fresh or powder) as carbon sources, along with MES as a pH buffer, were tested. The samples were incubated at 25°C with agitation, with counts taken every 24 hours in 1/10 diluted R2A medium to assess microbial growth under each condition.

For the directed evolution assay, different culture media were prepared to provide a suitable environment for the growth and selection of bacteria (Figure 10). The basic

components of the medium included soil tea, a *Botrytis* sp. spore solution at a concentration of 1×10^6 spores/mL, rose petals (fresh crushed or dried powdered), and a pH buffer, in this case MES (0.05 g). Different bases were used for the preparation of the media: peptone water, 1/10 TSB, and $MgCl_2$. The proportion of the components was 10% soil tea, 10% spore solution, 10% fresh rose or 1% dried rose powder, and the medium was completed with the remaining volume using the same base solution used in each treatment.

Six specific mixes were established: (1) $MgCl_2$ with fresh rose, (2) $MgCl_2$ with dried rose, (3) $MgCl_2$ with MES and fresh rose, (4) peptone water with fresh rose, (5) peptone water with dried rose, and (6) 1/10 TSB with fresh rose. Each preparation was incubated at 25°C with agitation. Bacterial growth was monitored every 24 hours through colony counting using 1/10 R2A medium and the plate spread technique. This procedure allowed the evaluation of the influence of different combinations of components and carbon sources on bacterial growth and adaptation. The chosen medium consists of a mixture of $MgCl_2$ and fresh rose, as this combination allows for the highest bacterial count at three days compared to other mixtures. This characteristic is ideal for maintaining robust bacterial growth, especially under bottleneck conditions. Since $MgCl_2$ does not provide nutrients, the bacteria rely exclusively on the roses as a carbon source, as seen in the growth curves in Supplementary 3A. Additionally, the rose exerts pressure on the bacterial community, as it reduces the bacterial count compared to media without rose, as shown in the growth curves in Supplementary 3B. This approach is optimal because it favors the growth of bacterial communities that must adapt to the compounds present in the petals, which will facilitate *in vivo* application.

General scheme of Directed Evolution

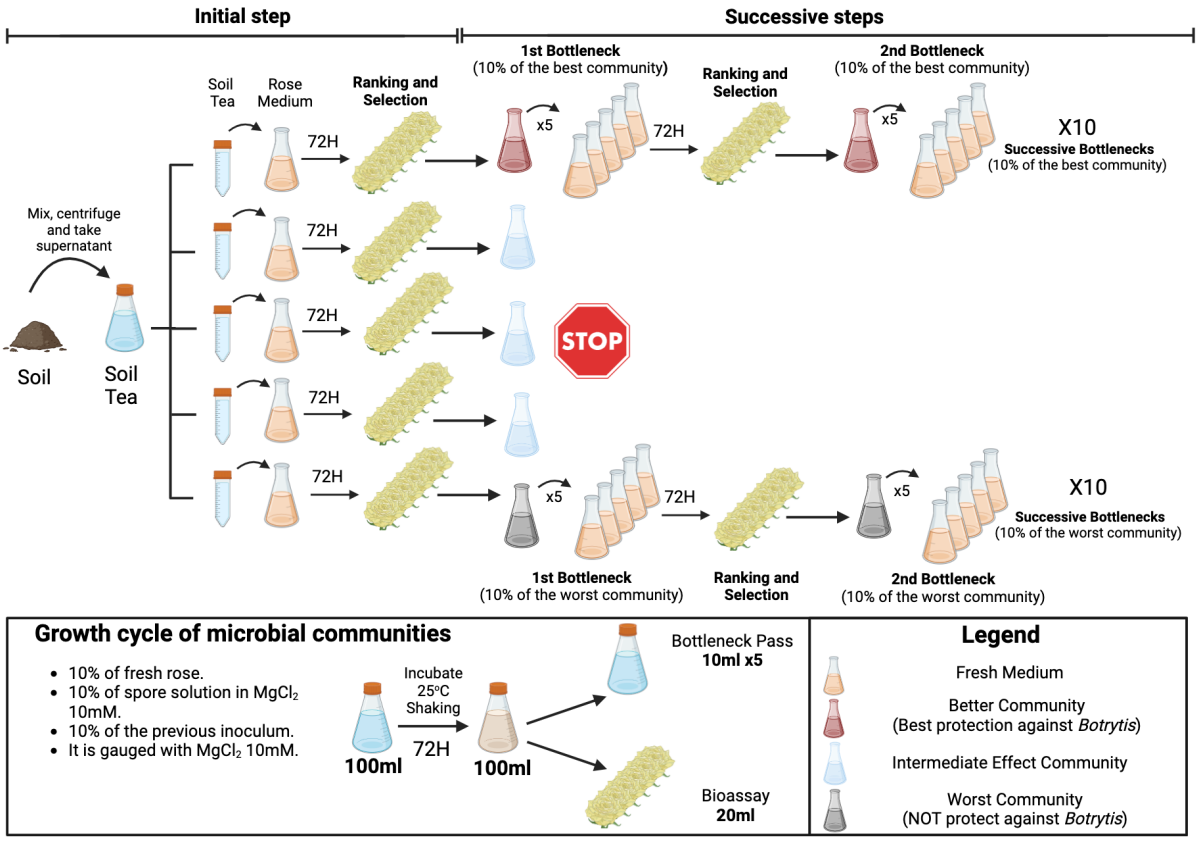


Figure 11. General Scheme of the Directed Evolution Process for Selecting Microbial Communities with Fungistatic Effect.
Starting from soil tea and media supplemented with rose, microbial communities undergo bottleneck cycles to select those with greater or lesser protection against *Botrytis* sp. In each cycle, 10% of the best and worst communities are transferred to new media for successive selection steps. Each growth cycle includes incubation at 25°C with agitation, and the process is repeated until the evolution goal is achieved.

For the directed evolution of bacterial communities with fungistatic effect, a medium based on $MgCl_2$ and fresh rose petals was used, as this combination encourages bacteria to utilize the petal as the sole carbon source (Figure 11). The process begins with the extraction of soil tea using a 10 mM $MgCl_2$ solution as a diluent. Ten 50 mL Falcon tubes are prepared, each containing a 1:1 mixture of soil and $MgCl_2$ solution. The mixture is agitated at 195 rpm and centrifuged at 1,400 g to obtain the supernatant, which is subsequently filtered through a 10 µm filter. The filtered liquid from all 10 tubes is combined in a sterile flask, producing a mother soil tea. This mother soil tea is then evenly distributed into five tubes, designated for both media preparation and subsequent analyses. Then, 10ml of each tube is transferred to fresh

media with rose petals and incubated for 72 hours at 25°C with constant agitation, allowing the initial growth of the microbial communities. The application of the communities in the bioassay, after 72 hours of growth, is based on monitoring the bacterial count of the rose media with soil tea for 7 days in 1/10 R2A medium. At 72 hours, bacterial growth reaches the end of the exponential phase and begins to stabilize, remaining constant from day 4 to day 7 as can be seen in the Supplementary4. This pattern establishes an optimal time window for conducting the bioassay, allowing for adequate growth to occur before proceeding with the evaluation. After incubation, a bioassay on rose petals, as previously explained, is conducted to evaluate the effectiveness of each community in inhibiting *Botrytis* sp. Based on the results of the bioassay, the best and worst communities in terms of fungistatic effect are classified and selected. Subsequently, 10% of the best and worst communities are transferred to five jars each, continuing the selection process through successive bottleneck cycles. This process, in which a bottleneck pass is used in each cycle, allows for the selection of communities with greater protective capacity against *Botrytis* sp., repeating until the directed evolution goal is achieved. At the end of the experiment, the Top and Bottom communities from generation 14 are plated on 1/10 R2A using the plate spread technique, and colony counts are performed after 72 hours. For statistical analysis, an analysis of variance (ANOVA) was performed to determine significant differences between all inoculum, with data normality assessed using the Shapiro-Wilk test. A Tukey post-hoc test was applied to identify significant differences between treatments. In addition, a t-test was used for comparisons between the top and bottom of each generation and for the graph the standard error was used instead of the standard deviation for the error bars.

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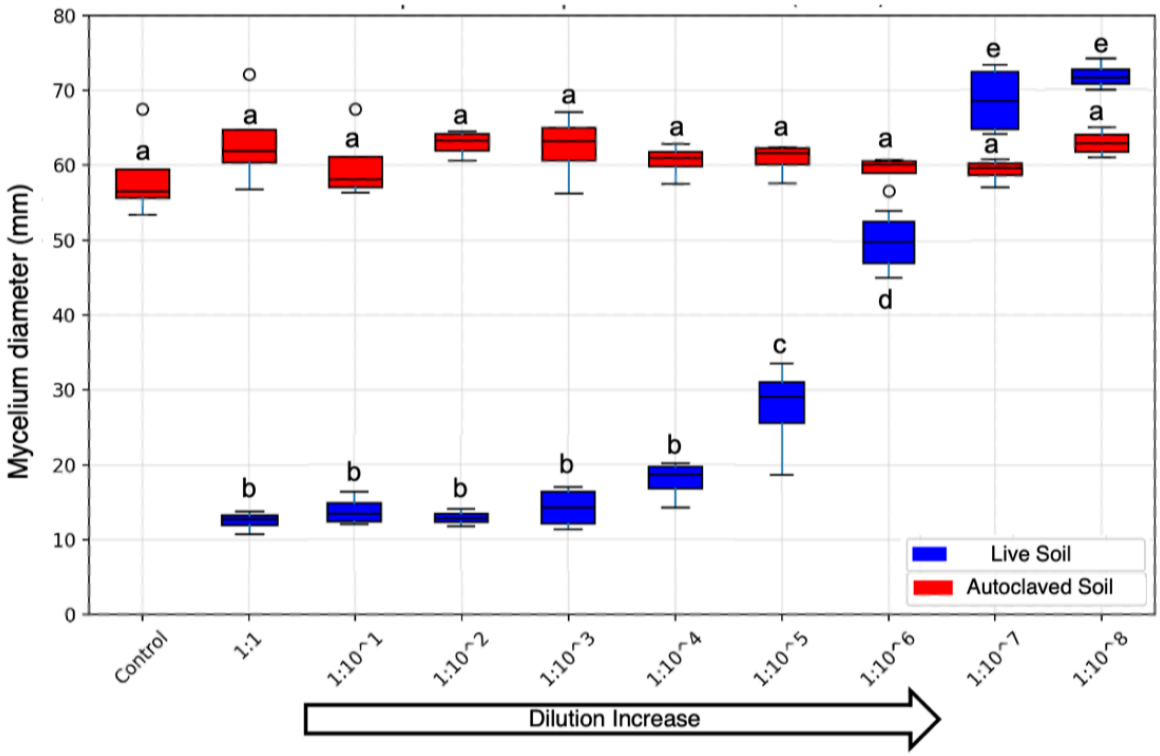
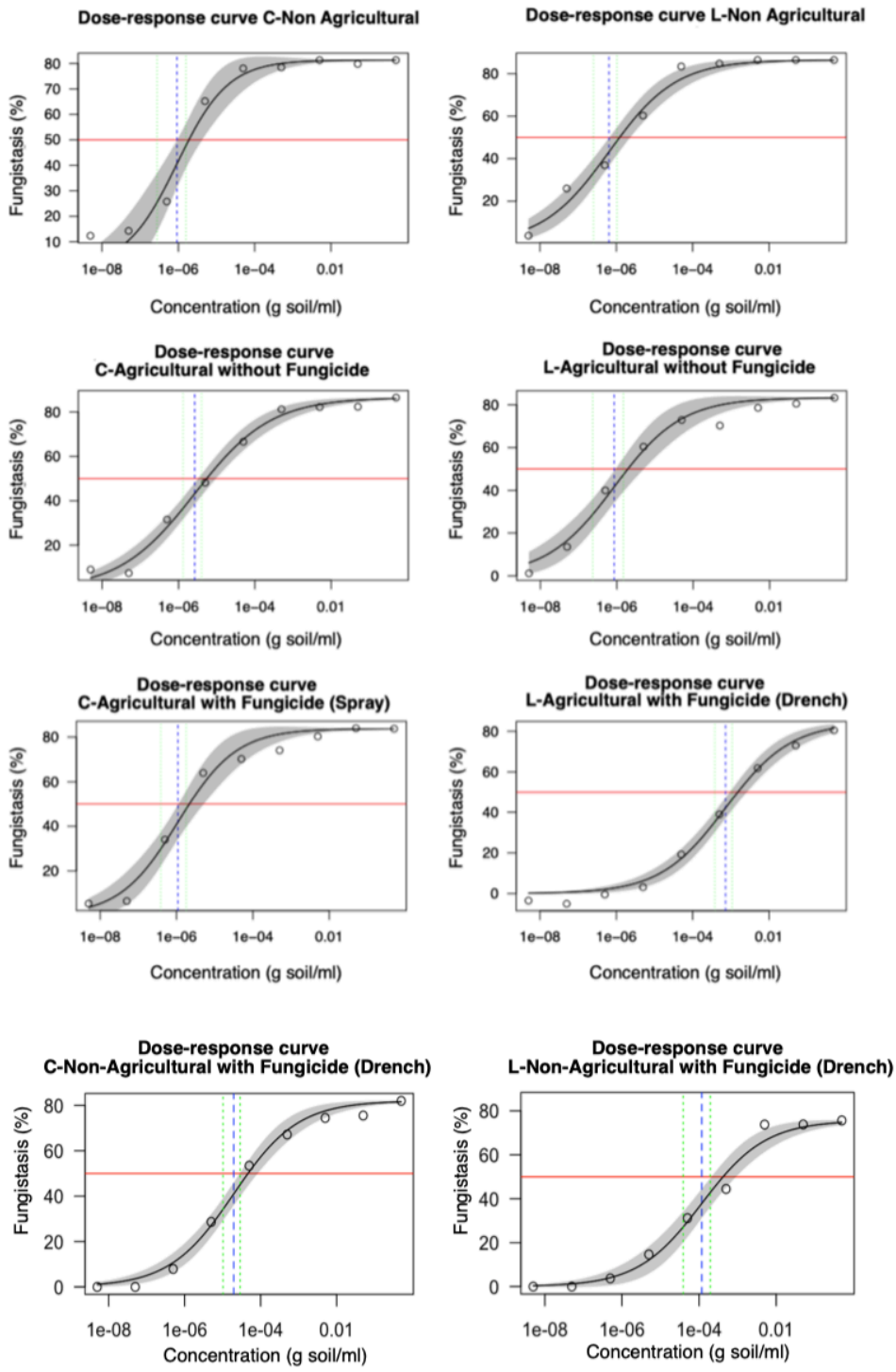
SUPLEMENTARY MATERIAL



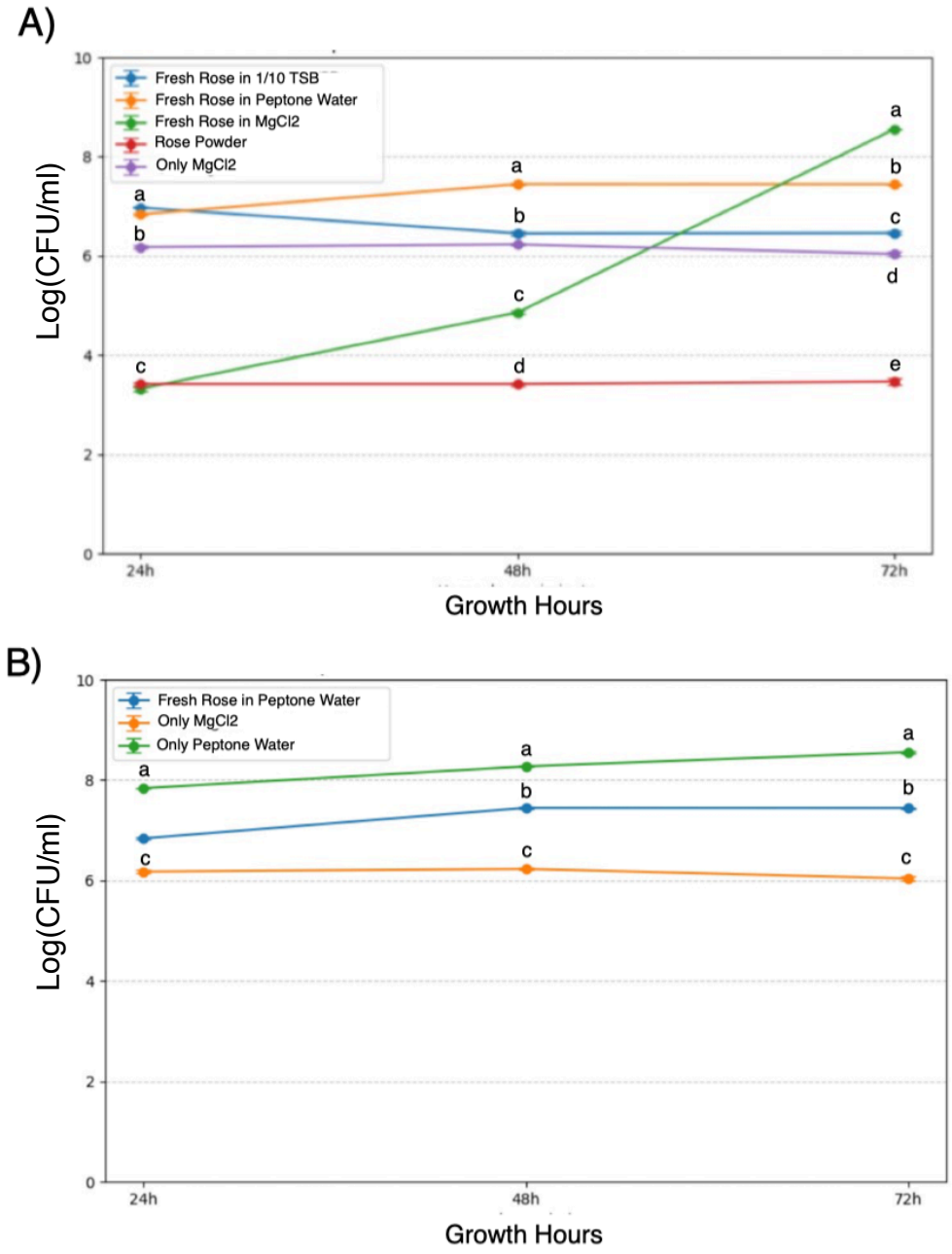
S 1. Effect of serial dilutions on mycelial growth reduction in live and autoclaved soil.

Successive dilutions reduce mycelial growth in live soil, while in autoclaved soil growth remains constant. Letters indicate significant differences between treatments ($p < 0.05$).



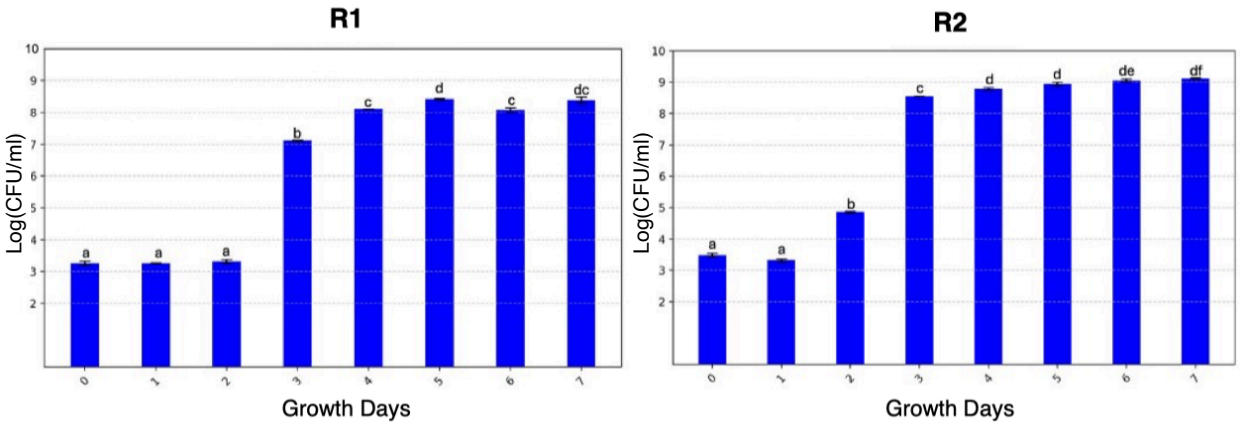
S 2. Individual dose-response curves modeled with a four-parameter logistic equation.

For Cayambe (C) and Lasso (L) soils under non-agricultural and agricultural conditions, with and without biocide. EC50 (blue vertical line), 50% Fungistasis (red horizontal line), error of fit (shaded area) and 95% confidence interval of EC50 (green vertical lines) are shown.



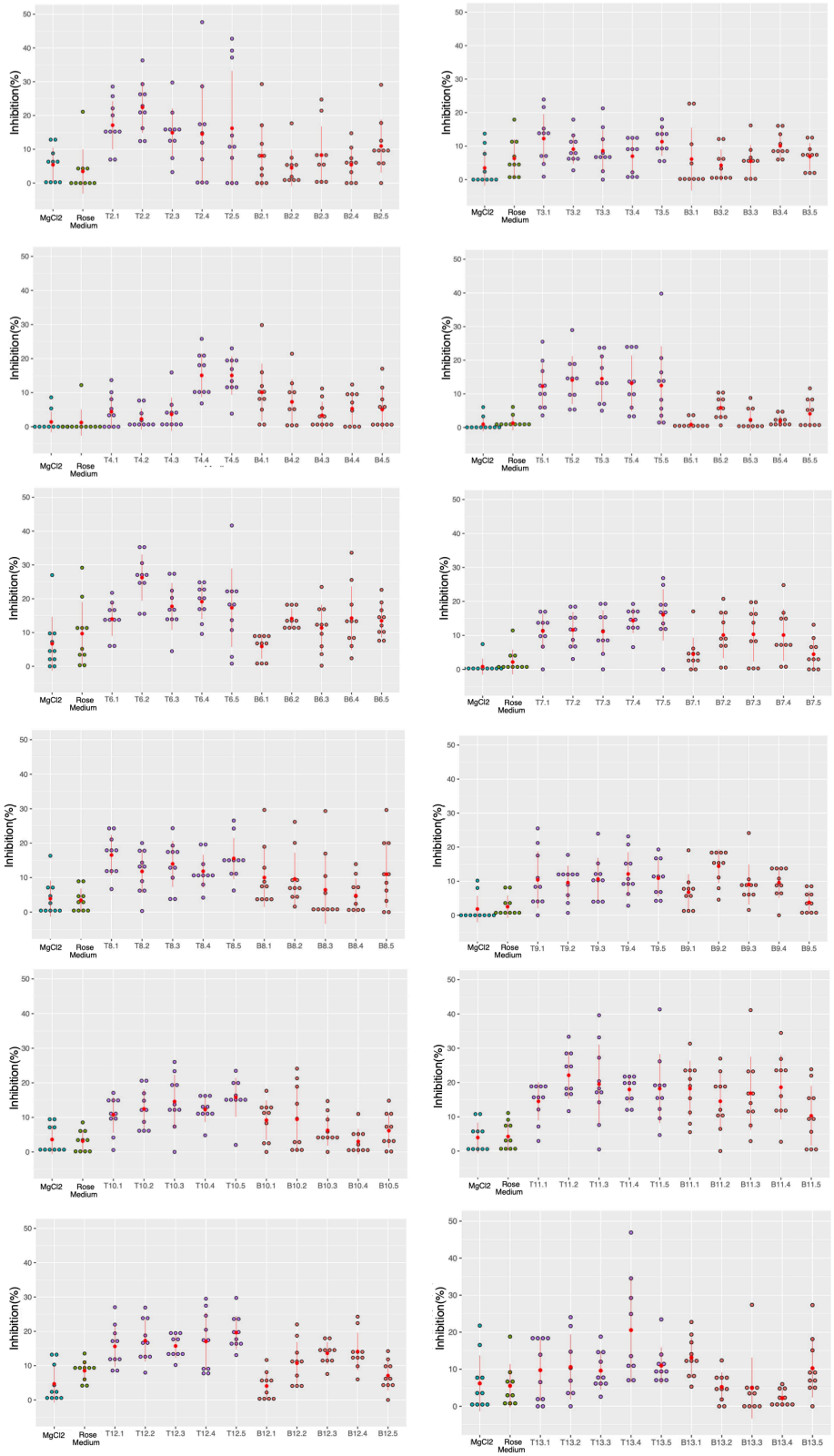
S 3. Selection of Media to Optimize the Directed Evolution Experiment

A) Comparison of growth on fresh rose in different media (1/10 TSB, peptone water, $MgCl_2$) and rose powder. B) Comparison between peptone water with and without rose petal, and $MgCl_2$ as a control with no effect on growth. Different letters indicate significant differences ($p < 0.05$).



S 4. Microbial growth in fresh rose medium with $MgCl_2$.

The count stabilizes from day 3 in both replicas (R1 and R2), remaining constant until day 7. Letters indicate significant differences between days ($p < 0.05$).



S 5. Complete results of fungal inhibition in each generation of selection during the process of directed evolution.

Percentage of fungal inhibition in each generation for all communities: Top (high fungistatic effect), Bottom (low effect), and those with intermediate effect, which were discarded.