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# Evaluation of two Bacillus sp. strains as an alternative to chemical fungicides for controlling *Botrytis cinerea* in roses destined for preservation

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**Abstract**

This study evaluated the efficacy of *Bacillus subtilis* and *Bacillus amyloliquefaciens* as biocontrol agents against *Botrytis cinerea* in rose crops destined for preservation in Cayambe, Ecuador. A randomized complete block design was used with nine treatments: six biological (three concentrations of each species) and three chemical treatments. The incidence and severity of the pathogen were evaluated weekly, along with economic indicators such as the cost-benefit ratio (CBR) and net benefit (NB). The biological treatment B1D3 (*B. subtilis* $1\times10^{12}$ CFU/ml) demonstrated the highest effectiveness, reducing incidence to 21.7% and severity to 7.61%, significantly outperforming the chemical treatments Q1, Q2, and Q3, whose values ranged between 24.2%-30.8% for incidence and 9.25%-10.80% for severity. Economically, B1D3 also stood out, achieving a CBR 10% higher and an NB approximately 40% greater than the closest chemical treatment (Q2). In contrast, lower-concentration biological treatments, such as B2D1 (*B. amyloliquefaciens* $1\times10^{6}$ CFU/ml), were less effective, with incidences of 40.4% and severities of 21.6%. These results position B1D3 as a sustainable and economically viable alternative to traditional chemical fungicides, reinforcing its utility for integrated management of *B. cinerea*. This study supports the use of *Bacillus* as an effective and cost-efficient strategy that enhances the sustainability of floriculture while reducing the environmental impact associated with synthetic chemical use.

**Keywords:** *amyloliquefaciens,* biological control, *Botrytis cinerea, Bacillus, subtilis,* Roses, sustainability

## Introduction

In 2023, Ecuadorian flower exports grew by 4%, reaching approximately 987 million dollars (Expoflores, 2023). Ecuadorian flowers are globally recognized for their exceptional quality and beauty, with preserved flowers standing out due to their extended durability and advanced preservation processes (Yépez et al., 2019). Maintaining such high standards requires rigorous preservation methods that ensure freshness and a longer post-harvest lifespan.

To strengthen its market position, the floriculture industry has adopted technical regulations that enhance product quality, improving customer satisfaction and competitiveness (Cerón, 2017). However, pests and diseases, particularly *Botrytis cinerea*, pose significant challenges. This fungus infects plants during greenhouse production and causes gray mold post-harvest, leading to losses of up to 50% of production (Muñoz et al., 2019). For preserved roses, these losses can be even higher due to stringent quality standards and high remediation costs. Globally, *B. cinerea* causes economic losses estimated between 10 and 100 billion dollars, highlighting the need for effective control strategies (Roca-Couso et al., 2021a).

Traditionally, *B. cinerea* has been managed using fungicides, classified as protective or systemic. While effective, prolonged chemical use has led to resistant *B. cinerea* strains and poses risks to human health and the (Valcárcel & Quintero, 2021). Protective fungicides eliminate spores and hyphae upon contact, but their limitations necessitate exploring alternative strategies (Bika et al., 2021).

Biocontrol microorganisms, such as *Bacillus* strains, are promising alternatives. These bacteria suppress pathogens through mechanisms such as antibiotic production, nutrient competition, hyphal lysis, and stimulation of systemic plant resistance (Cardoso, 2014;


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Valcárcel & Quintero, 2021). *Bacillus amyloliquefaciens* effectively controls plant diseases by synthesizing antimicrobial compounds, competing for resources, and enhancing natural defenses (Chun et al., 2019). Similarly, *Bacillus subtilis* inhibits pathogens, including *B. cinerea*, through antibiotic production and improves seed vigor and seedling development (Jaramillo, 2018; Reyes-Bonilla et al., 2024).

This study evaluates *B. subtilis* and *B. amyloliquefaciens* as biocontrol agents for *B. cinerea* in preserved rose crops in Cayambe, Ecuador. It analyzes the cost-benefit of replacing chemical fungicides with biological agents, aiming to reduce environmental impacts and enhance Ecuadorian floriculture's global competitiveness.

**Materials and Methods**

*Collection and Isolation of Botrytis cinerea as an Experimental Pathogen*

To isolate the pathogen, infected tissue samples were collected from rose plants (*Rosa × hybrida*) of the Melodie variety, cultivated in greenhouses located in Cayambe, Ecuador, during the months of April to June 2024. This period is characterized by high relative humidity, favorable for the development of *Botrytis cinerea*. The selected samples consisted of flowers at the cutting stage showing visible signs of *Botrytis cinerea* infection, identified by the presence of pustules on at least three outer petals. Samples showing signs of other diseases were excluded to ensure the representativeness of the results.

The samples were transported to the laboratory at 4°C under controlled refrigeration conditions. In the laboratory, the samples were immersed in sterilized distilled water and shaken for 5 minutes at 120 rpm using an orbital shaker to release the spores. The suspension was filtered through a fine mesh to remove plant tissue debris and inoculated onto potato dextrose agar (PDA) plates using the drop-plating technique, chosen for its efficacy in recovering single-spore cultures (Indunil Chinthani et al., 2020).

The plates were incubated at 23 ± 1°C for 7 days. Colonies displaying the distinctive morphological characteristics of *Botrytis cinerea* were selected and subcultured onto fresh PDA plates to obtain pure cultures. The features of the mycelium and conidia were evaluated under a stereoscopic microscope as a preliminary confirmation of the pathogen.

*Procedure for Isolation and Cultivation of Bacillus Strains for Biological Control*

The strains of interest were obtained using a BK-BAS-IV biological air sampler, chosen for its efficiency in capturing viable microorganisms in controlled environments. This device allowed the collection of microbial samples from the cultivation environment. The collected samples were inoculated onto Nutrient Agar (NA) to promote the growth and selective isolation of bacterial species of interest.

The microbial load of the selected colonies, preliminarily identified as *Bacillus subtilis* and *Bacillus amyloliquefaciens*, was verified through Gram staining, catalase tests, and morphological characteristics. These colonies were treated in the laboratory using the serial dilution technique, followed by plating on NA to isolate single-spore cultures (Indunil Chinthani et al., 2020). The plates were incubated at 28°C for 48 hours.

After incubation, colonies with the characteristic morphology of *Bacillus* were selected and purified through successive subcultures. Subsequently, spore suspensions were prepared at three different concentrations ($1\times10^6$ CFU/ml, $1\times10^9$ CFU/ml, and $1\times10^{12}$ CFU/ml) for antagonism assays. These concentrations were chosen to evaluate the biocontrol effectiveness across a range of representative doses, including moderate and high levels. The results were compared to the effectiveness of conventional chemical fungicides available on the market.

*Selection and Preparation of Botrytis cinerea-Infected Samples*

A study population comprising 35,200 rose plants distributed across 11 greenhouses was selected. Representative sampling areas of 450 m² were delineated, and a sample size of 30 harvest buds per plot was calculated using the finite population formula (Equation 1) with a 95% confidence level and a 15% margin of error. The 15% margin of error was chosen to balance precision and operational feasibility given the initial population size.

$$n = \frac{Z^2 \cdot p \cdot (1-p)}{e^2}$$ Equation 1

Where:

Z = 1.96 (Z value for a 95% confidence level)

p = 0.5 (maximum variability)

e = 0.15 (margin of error)

For inoculation, a spore suspension of *Botrytis cinerea* at a concentration of $1\times10^6$ CFU/ml was prepared, determined after preliminary tests evaluating different dilutions and inoculation methods. The selected technique was uniform spraying, which ensured complete coverage of the external surface of the petals in all flowers of the experimental group.

After application, the flowers were placed in controlled humidity chambers (80-90%) at a constant temperature of 20-22°C for 3 hours to promote spore

germination and initial pathogen establishment. The infection was monitored through regular observations of visible gray mold symptoms and microscopic confirmation of the characteristic fungal structures after 72 hours.

*Design of Biological and Chemical Treatment Applications*

The experiment included three treatment groups and a control. The treatment groups consisted of applying suspensions of *Bacillus subtilis* and *Bacillus amyloliquefaciens* at three concentrations ($1\times10^6$, $1\times10^9$, and $1\times10^{12}$ CFU/ml). The suspensions were applied using a Truper® sprayer with a 0.3 mm nozzle, calibrated to 20 PSI to ensure uniform distribution at a rate of 1 cc/s over flowers previously inoculated with *Botrytis cinerea*.

As a chemical control treatment, three fungicides commonly used in the farm's integrated management programs were applied: Switch (Cyprodinil + Fludioxinil) at a dose of 0.3 g/l, Cantus (Boscalid) at 0.5 g/l, and Stroby (Kresoxim-methyl) at 0.15 g/l. These fungicides were selected for their proven effectiveness in controlling *B. cinerea* and served as a positive control, enabling a comparison of the effectiveness of *Bacillus* strains against chemical agents.

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*Evaluation of Botrytis cinerea Incidence*

The incidence of *Botrytis cinerea* was evaluated 7 days after the application of treatments. To determine the incidence, the number of infected flowers in each experimental plot was recorded following the protocol described by (Elmhirst et al., 2011). Equation 2 was used to calculate the incidence percentage:

$$\%\ Incidence = \frac{Number\ of\ affected\ flowers}{Total\ number\ of\ evaluated\ flowers}$$

In each plot, 30 flowers were randomly selected for evaluation, and the analysis was performed with 8 repetitions per treatment. Visible infection symptoms considered included the presence of gray spots, necrosis on petals and stems, and characteristic mycelium of *Botrytis cinerea*. The data obtained were recorded on observation sheets that included the total number of flowers evaluated and the number of flowers showing symptoms.

*Evaluation of Botrytis cinerea Severity*

The severity of the infection caused by *Botrytis cinerea* was visually assessed using a severity scale based on the percentage of the affected area in each infected flower. The scale by (Martínez & Moreno, 2008) was used due to its high sensitivity in evaluating infections in floral tissues. **Figure 1** illustrates the progression of the disease in the evaluated variety:

**0:** No visible symptoms
**1:** 1-20% of the area infected
**2:** 21-40% of the area infected
**3:** 41-60% of the area infected
**4:** 61-80% of the area infected
**5:** 81-100% of the area infected

Each flower showing visible symptoms was assigned a severity value, and these values were subsequently summed for all flowers evaluated in each treatment to obtain the total severity. The average severity was calculated by dividing this sum by the total number of flowers evaluated per treatment. The percentage severity was calculated using Equation 3:

$$\%\ Severity = \frac{Total\ \ severity\ of\ the\ treatment}{Global\ \ severity}$$

Where:

**Total severity of the treatment:** Sum of severity values for all flowers evaluated in the treatment.

**Global total severity:** Sum of severity values for all flowers evaluated in the experiment.

These values were used to quantify the effectiveness of bacterial treatments at different concentrations compared to the chemical treatment and the control, assessing their ability to reduce the severity of the infection.

*Cost-Benefit Analysis*

The cost-benefit analysis of this study was conducted by considering the total production costs, the quantity of production obtained, and the revenue generated for each treatment. To calculate the total cost, all inputs used and other expenses associated with the application of biological and chemical treatments

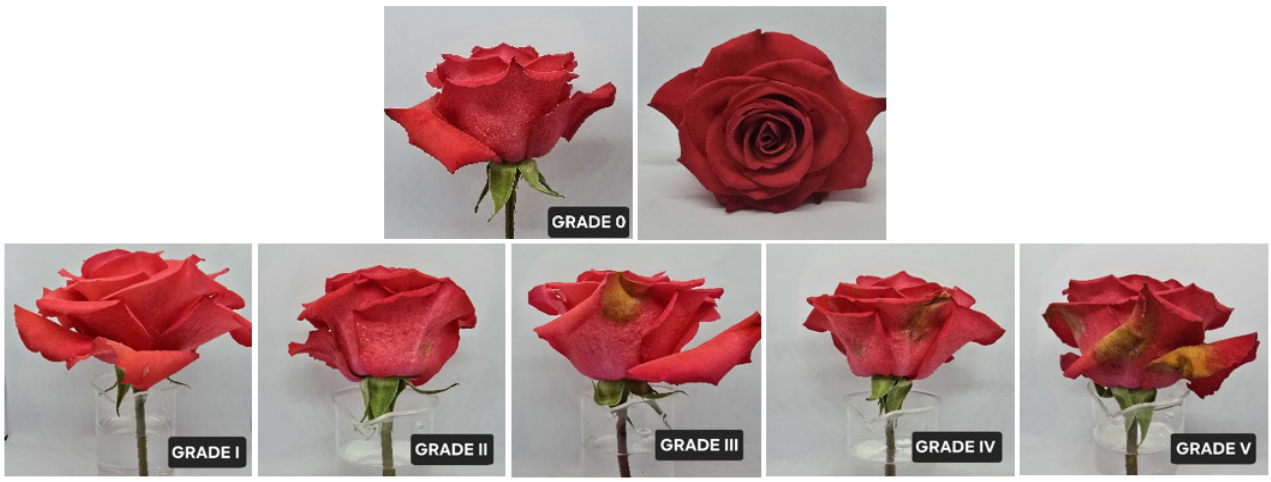


**Figure 1.** Progression of gray mold caused by *B. cinerea*

were included. Production was recorded as the total number of units generated per treatment, while revenue was calculated by multiplying the production by the unit selling price, set at $1.25. Subsequently, the cost-benefit ratio (CBR) was determined by dividing the total revenue by the total cost for each treatment, allowing for the identification of the relative economic efficiency of each strategy.

*Data Recording, Documentation, and Analysis*

A detailed system for recording and documenting data was implemented, including observation sheets with information on treatments, the number of flowers evaluated, incidence, severity values, and additional observations. Additionally, representative photographs of each plot were taken to visually document the disease progression and the effects of the treatments. All data were initially organized in Excel spreadsheets and then imported into R for statistical analysis.

The data on the incidence and severity of *Botrytis cinerea* were analyzed using an analysis of variance (ANOVA) for a completely randomized design, followed by Tukey's post-hoc test to identify significant differences between treatments at a 95% confidence level ($\alpha=0.05$\ alpha = 0.05). Assumptions of normality and homogeneity of variances were verified beforehand using the Shapiro-Wilk and Levene tests, respectively. Additionally, the Pearson correlation coefficient was calculated to evaluate the relationship between incidence and severity, establishing whether a reduction in incidence directly impacts disease severity.

All statistical analyses were conducted in R (version 4.3.0) using specialized packages such as dplyr, car, agricolae, and stats. The results were visualized through bar charts and box plots generated with ggplot2.

**Results and Discussion**

*Comparative Efficacy of Biological and Chemical Treatments in Reducing Incidence and Severity of Botrytis cinerea*

Statistical analysis was conducted to evaluate the efficacy of various biological and chemical treatments in managing *Botrytis cinerea*. Treatments based on different concentrations of *Bacillus subtilis* and *amyloliquefaciens* (B1D1, B1D2, B1D3, B2D1, B2D2, B2D3) were compared with chemical fungicides (Q1, Q2, Q3). The goal was to determine which treatment most effectively reduces the incidence and severity of the disease.

The Kruskal-Wallis test ($\chi2=20.119$, $p=0.0099$) revealed significant differences in the median incidence among treatments, indicating that certain treatments have superior effects in reducing the disease. Correlation analysis showed a positive relationship between incidence and severity, with Pearson ($r=0.4755$) and Spearman ($\rho=0.6124$) coefficients suggesting that higher incidence correlates with increased severity. Furthermore, a linear regression model indicated that incidence significantly influences severity (slope=74.292, $p<0.001$), explaining 22.61% of the total variability.

**Table 1** presents the mean and standard deviations for incidence and severity. Among the evaluated treatments, B1D3 ($1\times10^{12}$ CFU/ml of *Bacillus subtilis*) exhibited the lowest incidence and severity values, with averages of 21.70% and 7.61%, respectively. In comparison, chemical treatments Q1, Q2, and Q3 showed higher severity values (ranging from 9.25% to 10.80%). These findings highlight the superiority of biological treatments in disease management.

These results reinforce the effectiveness of biological strategies, particularly B1D3, in reducing both the incidence and severity of *Botrytis cinerea*. Additionally, the positive relationship between incidence and severity

**Table 1:** Descriptive Summary of Treatments for Incidence and Severity Variables

| Treatment | Incidence% | SD Incidence | Severity% | SD Severity | n |
|---|---|---|---|---|---|
| B1D1 | 36.70 | 10.40 | 20,10 | 8.05 | 8 |
| B1D2 | 28.30 | 8.36 | 12,00 | 5.27 | 8 |
| B1D3 | 21.70 | 8.16 | 7,61 | 2.39 | 8 |
| B2D1 | 40.40 | 13.40 | 21,60 | 11.30 | 8 |
| B2D2 | 38.30 | 7.35 | 17,40 | 6.23 | 8 |
| B2D3 | 28.20 | 8.72 | 10,60 | 3.59 | 8 |
| Q1 | 30.80 | 10.20 | 9,64 | 2.90 | 8 |
| Q2 | 24.20 | 10.40 | 9,25 | 2.68 | 8 |
| Q3 | 30.80 | 17.70 | 10,80 | 2.71 | 8 |

**Note:** Where **B1D1** = *B. Subtilis* $1\times10^{6}$ UFC/ml; **B1D2** = *B. Subtilis* $1\times10^{9}$ UFC/ml; **B1D3** = *B. Subtilis* $1\times10^{12}$ UFC/ml; **B2D1** = *B. amyloliquefaciens* $1\times10^{6}$ UFC/ml; **B2D2** = *B. amyloliquefaciens* $1\times10^{9}$ UFC/ml; **B2D3** = *B. amyloliquefaciens* $1\times10^{3}$ UFC/ml; **Q1** = Switch (Cyprodynil + Fludioxinil) a 0.3 g/l; **Q2** = Cantus (Boscalid) a 0.5 g/l; y **Q3** = Stroby (Kresoxym - Methil) a 0.15cc/l.

underscores the importance of implementing preventive measures to minimize the initial incidence of the disease.

The descriptive analysis of the treatments evaluated for managing *Botrytis cinerea* shows results supporting the use of biological agents as an effective strategy to reduce the incidence and severity of this disease in agricultural crops. In this study, treatments based on *Bacillus subtilis* and *Bacillus amyloliquefaciens* demonstrated notable superiority compared to traditional chemical fungicides, particularly in the case of B1D3, which recorded the lowest incidence (21.70%) and severity (7.61%) values. These findings are consistent with previous research highlighting the potential of these bacteria to inhibit pathogens through the production of antimicrobial compounds such as surfactants and fengycins, which not only directly affect the fungus but also stimulate systemic resistance in plants (Chen et al., 2007; Luna-Bulbarela et al., 2018).

The observed effectiveness of biological treatments may be explained by the ability of these bacteria to efficiently colonize the rhizosphere and compete with pathogens for space and nutrients, a mechanism extensively documented in recent studies (Altieri et al., 2023). Additionally, their multifaceted action, including the induction of defensive responses in plants, reinforces their potential as a sustainable alternative to chemical fungicides, whose effectiveness has decreased due to resistance development in *B. cinerea* strains (Fillinger & Walker, 2016). On the other hand, chemical treatments, while effective in certain contexts, showed higher incidence and severity values, which could be attributed to lower environmental persistence or excessive reliance on specific compounds like kresoxim-methyl, known for its limited effectiveness in cases of resistance (DeLong et al., 2020).

The statistical analysis in this study reinforces the relevance of biological treatments in managing *B. cinerea*. Results from the Kruskal-Wallis test indicate significant differences among treatments, while the positive correlation between incidence and severity suggests that effectively reducing incidence may directly impact mitigating the damages associated with the disease. Although the linear regression model showed that incidence accounts for only 22.61% of the variability in severity, this finding underscores the need to implement integrated management strategies that combine biological control with cultural practices, such as moisture and ventilation management in crops, to maximize results (Droby & Lichter, 2007).

From a practical perspective, the superiority of B1D3 as the most effective biological treatment highlights its potential to partially or completely replace chemical fungicides in Integrated Pest Management (IPM) programs. This is particularly relevant in an agricultural context demanding more sustainable solutions with reduced dependence on chemical inputs. Moreover, incorporating these biological agents into agricultural systems could contribute to preserving the microbial biodiversity of the soil, an aspect often affected by intensive fungicide use (Wang et al., 2024).

It is important to note that while biological treatments showed promising results, their effectiveness may vary depending on environmental conditions and crop type, suggesting the need for additional studies to evaluate their performance in different agroecological contexts. Furthermore, future research should focus on optimizing the formulations and application methods of these bacteria, as well as exploring potential synergies between biological and chemical treatments to maximize efficacy and reduce the risk of resistance in *B. cinerea* (Elad et al., 2016; Fedele et al., 2020).

The results of this descriptive analysis not only support the effectiveness of biological treatments in managing *Botrytis cinerea* but also underscore the importance of adopting integrated and sustainable approaches that consider both pathogen dynamics and the needs of modern agricultural systems. Current scientific evidence reinforces the role of agents such as *Bacillus subtilis* and *B. amyloliquefaciens* as key tools to address phytopathological challenges sustainably and efficiently.

*Evaluation of Incidence by Treatments*

To analyze the differences in disease incidence among the applied treatments, an analysis of variance (ANOVA) was performed. The results revealed that the treatment factor had a significant effect on incidence. The analysis showed an F-value of 2.944 and a p-value

of 0.0074, indicating statistically significant differences between the means of the evaluated treatments. The treatment factor had 8 degrees of freedom, with a sum of squares of 0.2564 and a mean square of 0.03205, while the residuals recorded 63 degrees of freedom, a sum of squares of 0.6858, and a mean square of 0.0109. These results highlight the variability among treatments and underscore their influence in reducing disease incidence, providing key information for identifying more effective management strategies.

Treatment B1D3, composed of *Bacillus subtilis* at a concentration of $1\times10^{12}$ CFU/ml, stood out as the most effective, showing the lowest incidence and dispersion compared to the other treatments (**Figure 2**). This performance can be attributed to the high concentration of viable bacteria in the treatment, which promotes rapid and effective colonization. The high density of *B. subtilis* increases the production of antimicrobial compounds such as iturins, fengycins, and surfactins, which inhibit the growth of *Botrytis cinerea* (Bueno et al., 2022). Additionally, these compounds indirectly stimulate the plant's systemic defenses, enhancing its resistance to infections (Ayaz et al., 2023). The use of *B. subtilis* in pathogen biocontrol has been extensively documented in the literature, with recent studies demonstrating its efficacy against a variety of phytopathogens (Li et al., 2024).

In contrast, treatment B2D1, composed of *Bacillus amyloliquefaciens* at a concentration of $1\times10^{6}$ CFU/ml, showed the highest disease incidence. This result may be attributed to the low microorganism concentration in this treatment, which limits its ability to compete with the pathogen and produce sufficient quantities of bioactive metabolites. Furthermore, previous studies have demonstrated that while *B. amyloliquefaciens* is effective under certain conditions, it performs less effectively than *B. subtilis* in terms of adaptability and colonization speed in highly competitive systems (Esquivel□cervantes et al., 2022; Lv et al., 2020a). This difference in efficacy between the two microorganisms has also been observed in other crops, where *B. subtilis* has demonstrated greater capacity to suppress the growth of soil and rhizosphere pathogens (Tu et al., 2023).

The chemical treatments (Q1, Q2, and Q3) presented intermediate levels of disease incidence. Although chemical fungicides are effective in controlling *Botrytis cinerea*, their action is typically specific and limited to direct inhibition of the pathogen, without providing additional benefits such as stimulation of the plant's defense system. Among these, treatment Q2, based on boscalid, stood out as the most effective due to its mode of action, which interferes with the pathogen's energy metabolism by blocking chitin synthesis in fungal cells (Roca-Couso et al., 2021). However, chemical fungicides have disadvantages, such as the potential for pathogen resistance and adverse effects on non-target organisms, which limit their long-term applicability (Kim et al., 2016).

The Tukey analysis confirmed significant differences between B1D3 and B2D1, with an adjusted p-value of 0.017, reinforcing the superiority of the former (**Figure 3**). This positions B1D3 as the best strategy, not only for its capacity to reduce disease incidence but also for the consistency of the results obtained. The findings align with recent studies demonstrating that *B. subtilis* at high concentrations offers an effective and sustainable alternative against pathogens like *Botrytis cinerea*, improving plant health and reducing dependency on synthetic fungicides (Orozco-Mosqueda et al., 2023).

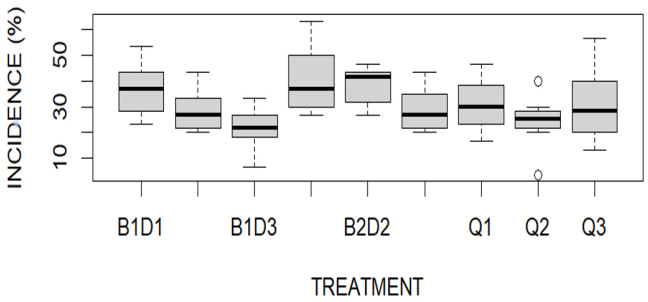


**Figure 2.** Distribution of disease incidence (%) across the evaluated treatments

*Note.* The boxplot represents the distribution of incidence across different treatments. Treatment codes: B1D1 = *B. subtilis* $1\times10^{6}$ CFU/ml; B1D2 = *B. subtilis* $1\times10^{9}$ CFU/ml; B1D3 = *B. subtilis* $1\times10^{12}$ CFU/ml; B2D1 = *B. amyloliquefaciens* $1\times10^{6}$ CFU/ml; B2D2 = *B. amyloliquefaciens* $1\times10^{9}$ CFU/ml; B2D3 = *B. amyloliquefaciens* $1\times10^{3}$ CFU/ml; Q1 = Switch (Cyprodinil + Fludioxonil) at 0.3 g/l; Q2 = Cantus (Boscalid) at 0.5 g/l; and Q3 = Stroby (Kresoxim-methyl) at 0.15 cc/l.

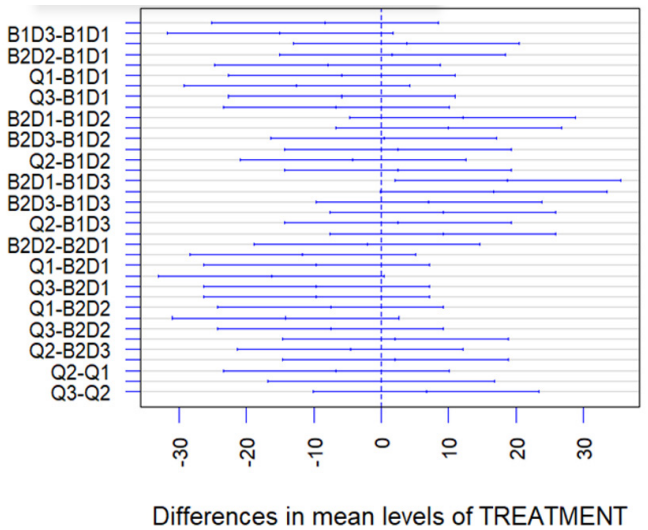


**Figure 3.** 95% Confidence Intervals for Mean Incidence Differences Between Treatments (Tukey HSD Test)

Note. The horizontal bar chart with error lines represents the results of Tukey's statistical significance tests between treatments. Treatment codes: B1D1 = B. subtilis 1×106 CFU/ml; B1D2 = B. subtilis 1×109 CFU/ml; B1D3 = B. subtilis 1×1012 CFU/ml; B2D1 = B. amyloliquefaciens 1×106 CFU/ml; B2D2 = B. amyloliquefaciens 1×109 CFU/ml; B2D3 = B. amyloliquefaciens 1×103 CFU/ml; Q1 = Switch (Cyprodinil + Fludioxonil) at 0.3 g/l; Q2 = Cantus (Boscalid) at 0.5 g/l; and Q3 = Stroby (Kresoxim-methyl) at 0.15 cc/l.

The effectiveness of *Bacillus subtilis* at high concentrations, as observed in treatment B1D3, aligns with previous research. (Archila, 2018) reported that high densities of *Bacillus* enhance pathogen suppression by increasing competition for nutrients and space, as well as boosting the production of antimicrobial compounds. In this study, the combination of high bacterial concentration and antagonistic capacity positions *Bacillus subtilis* as a key tool in the management of *Botrytis cinerea* in high-value crops such as roses. These findings are highly relevant for sustainable production systems, reducing dependency on synthetic fungicides and promoting integrated disease management strategies (Bellone et al., 2023).

*Translation*

*Evaluation of Severity by Treatment*

An analysis of variance (ANOVA) was conducted to assess the effect of treatments on disease severity, aiming to determine whether there were significant differences between the mean severity levels of the evaluated treatments. The ANOVA results showed that the treatment factor had a significant effect on severity, with an F-value of 6.26 and a p-value of 0.00061, indicating that the mean severity levels among treatments were not equal. Specifically, the treatment factor presented 8 degrees of freedom, a sum of squares of 1675, and a mean square of 209.40, while the residuals had 63 degrees of freedom, a sum of squares of 2107, and a mean square of 33.45. These results highlight the importance of selecting appropriate treatments to control disease severity.

In terms of efficacy, treatment B1D3 (*Bacillus subtilis* $1\times10^{12}$ CFU/ml) stood out as the most effective, showing the lowest severity and dispersion, which suggests consistent control over the pathogen. Conversely, B2D1 (*Bacillus amyloliquefaciens* $1\times10^{6}$ CFU/ml) was the least effective, with higher severity and variability. This difference may be explained by the lower concentration of *Bacillus amyloliquefaciens*, which limits its ability to efficiently suppress the pathogen. Literature supports these findings, as previous studies have shown that the concentration of biocontrol microorganisms is critical to their efficacy. For instance, (Jan et al., 2023) reported that *Bacillus* strains, applied under in vitro conditions, significantly inhibited the mycelial growth of various pathogens, emphasizing that higher concentrations have a greater inhibitory effect. Similarly, (Márquez et al., 2020) demonstrated that *Bacillus subtilis*, applied weekly at appropriate concentrations, effectively controls the severity of *Botrytis cinerea* and other pathogens due to its ability to induce systemic acquired resistance (SAR), strengthening the plant's defenses.

Treatment B1D3 likely achieved the highest effectiveness because of the high concentration of *Bacillus subtilis*, which creates a larger microbial population capable of effectively competing against the pathogen and producing antimicrobial compounds that limit disease development. (Lv et al., 2020) highlighted that *Bacillus amyloliquefaciens*, at elevated concentrations, produces antimicrobial compounds such as surfactin and fengycin, which reinforce plant resistance. This mechanism may explain the superior performance of B1D3 compared to B2D1.

On the other hand, chemical treatments like Q1 (Switch), Q2 (Cantus), and Q3 (Stroby) performed better than B2D1 but were less effective than B1D3. However, these chemical treatments depend on precise dosing and application to achieve consistent results, which may limit their long-term effectiveness compared to biocontrol agents that also induce an immune response in plants.

The Tukey analysis confirmed the superiority of treatment B1D3, which was significantly better than B1D1 ($p = 0.0129$) and B2D1 ($p = 0.0032$). Furthermore, Q1 and Q2, while more effective than B2D1 ($p = 0.0199$), did not surpass B1D3 in reducing severity. These results establish B1D3 as the most effective treatment in this study.

**Figure 5** shows that B1D3 exhibits the lowest severity, with lower and more consistent values, whereas B1D1 and B2D1 show higher values and greater dispersion. Similarly, **Figure 4**, which displays the 95% confidence intervals for the mean severity differences between treatments, confirms the superiority of B1D3, standing out significantly compared to other treatments such as B2D1.

Treatment B1D3, based on *Bacillus subtilis* at a high concentration, shows the best results due to several key factors: its high concentration of microorganisms, the ability to induce systemic acquired resistance (SAR) in plants, and the production of antimicrobial compounds that limit pathogen development. These mechanisms align with previous studies that have demonstrated that *Bacillus subtilis*, at elevated concentrations, not only directly combats the pathogen but also strengthens the natural defenses of plants, providing more durable and consistent control (Hashem et al., 2019). This biological approach not only enhances treatment effectiveness but also promotes more sustainable agriculture by reducing dependence on chemical products.

*Cost-Benefit Analysis*

The cost-benefit analysis of the project was conducted considering fixed costs, variable costs associated with inputs, and the profitability derived from

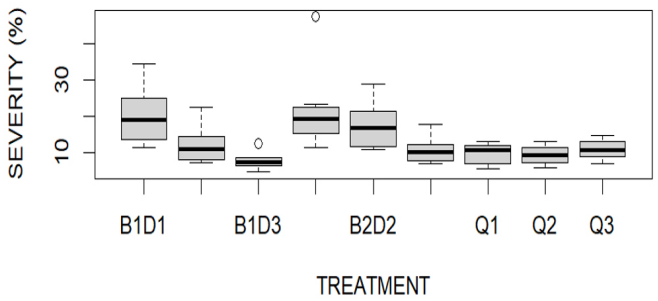


**Figure 4.** Distribution of disease severity (%) across the evaluated treatments

*Note.* The boxplot represents the distribution of severity across different treatments. Treatment codes: B1D1 = *B. subtilis* 1×10⁶ CFU/ml; B1D2 = *B. subtilis* 1×10⁹ CFU/ml; B1D3 = *B. subtilis* 1×10¹² CFU/ml; B2D1 = *B. amyloliquefaciens* 1×10⁶ CFU/ml; B2D2 = *B. amyloliquefaciens* 1×10⁹ CFU/ml; B2D3 = *B. amyloliquefaciens* 1×10³ CFU/ml; Q1 = Switch (Cyprodinil + Fludioxonil) at 0.3 g/l; Q2 = Cantus (Boscalid) at 0.5 g/l; and Q3 = Stroby (Kresoxim-methyl) at 0.15 cc/l.

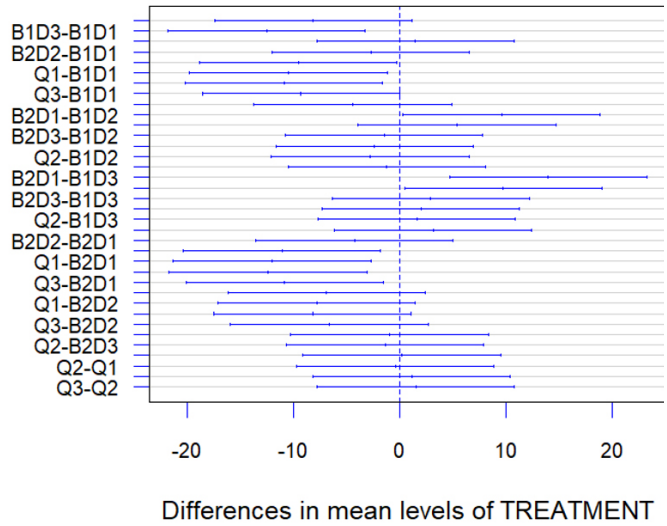


**Figure 5.** 95% Confidence Intervals for Mean Severity Differences Between Treatments (Tukey HSD Test)

*Note.* The horizontal bar chart with error lines represents the results of Tukey's statistical significance tests between treatments. Treatment codes: B1D1 = *B. subtilis* 1×10⁶ CFU/ml; B1D2 = *B. subtilis* 1×10⁹ CFU/ml; B1D3 = *B. subtilis* 1×10¹² CFU/ml; B2D1 = *B. amyloliquefaciens* 1×10⁶ CFU/ml; B2D2 = *B. amyloliquefaciens* 1×10⁹ CFU/ml; B2D3 = *B. amyloliquefaciens* 1×10³ CFU/ml; Q1 = Switch (Cyprodinil + Fludioxonil) at 0.3 g/l; Q2 =

the reduction in the incidence of *Botrytis cinerea* for each evaluated treatment. Two main indicators were used for this analysis: the Cost-Benefit Ratio (CBR) and Net Benefit (NB), calculated individually for each treatment. These indicators assess both the economic efficiency and the viability of the proposed biological and chemical strategies.

The cost-benefit analysis shows that treatment B1D3 (*Bacillus subtilis* 1×10¹² CFU/ml) is the most efficient, achieving a significant 41% increase in revenue compared to the most economical treatment (B1D1) due to its high production and competitive cost per unit. Although B1D1 (*Bacillus subtilis* 1×10⁶ CFU/ml) had the lowest total cost, its revenue performance was lower compared to B1D3 and Q2 (Cantus at 0.5 g/l), the latter standing out as a balanced treatment with moderate costs and good results. Overall, the B1 group treatments showed a better cost-benefit ratio compared to the others, with B1D3 being the most favorable option due to its optimal balance between investment and results, significantly outperforming treatments such as B2D2 and Q1, which, despite generating high revenue, had elevated costs per unit. This analysis underscores the importance of prioritizing treatments that optimize production and reduce unit costs to maximize benefits.

**Figure 6** presents the CBR of the evaluated treatments. The results show that the biological treatment B1D3 (*Bacillus subtilis* 1×10¹² CFU/ml) achieved the highest CBR among the biological options, demonstrating its superiority in terms of economic profitability within this category. Among chemical treatments, Q2 (Cantus at 0.5 g/l) achieved the highest CBR, positioning itself as the most efficient chemical alternative. Conversely, treatments such as B2D1 (*B. amyloliquefaciens* 1×10⁶ CFU/ml) and Q1 (Switch at 0.3 g/l) presented the lowest CBRs, reflecting their limited profitability in comparison.

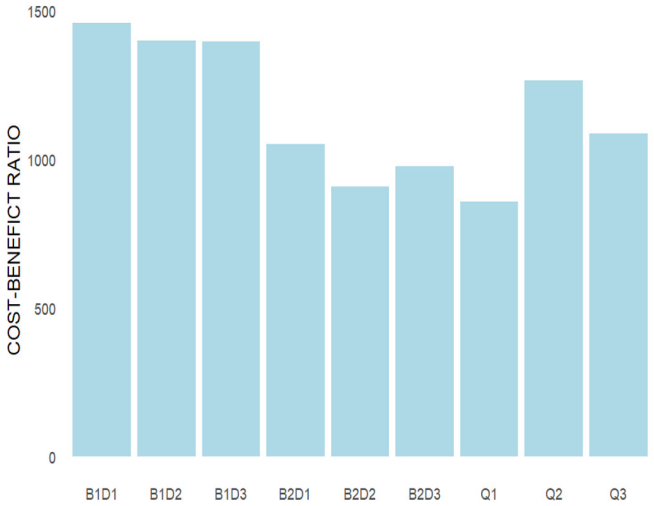


**Figure 6.** Cost-Benefit Ratio (CBR) of the Evaluated Treatments

*Note.* **Bar** chart showing the Cost-Benefit Ratio (CBR) for each treatment. On the X-axis: B1D1 = *B. subtilis* 1×10⁶ CFU/ml; B1D2 = *B. subtilis* 1×10⁹ CFU/ml; B1D3 = *B. subtilis* 1×10¹² CFU/ml; B2D1 = *B. amyloliquefaciens* 1×106 CFU/ml; B2D2 = *B. amyloliquefaciens* 1×10⁹ CFU/ml; B2D3 = *B. amyloliquefaciens* 1×10³ CFU/ml; Q1 = Switch (Cyprodinil + Fludioxonil) at 0.3 g/l; Q2 = Cantus (Boscalid) at 0.5 g/l; and Q3 = Stroby (Kresoxim-methyl) at 0.15 cc/l. The Y-axis represents the cost-benefit ratio, a dimensionless value resulting from the ratio of benefits obtained to the costs associated with each treatment.

Additionally, the net benefit analysis, presented in **Figure 7**, shows that treatment B1D3 also achieved the highest Net Benefit (NB) among all treatments, significantly outperforming both biological and chemical alternatives. This result reinforces its position as the most efficient and profitable treatment. Chemical treatments Q2 and Q3 exhibited high and similar net benefits, although they were still lower than those achieved by B1D3. In contrast, treatments B2D1 and Q1 again stood out for their low net benefits, which limits their adoption as economically viable strategies.

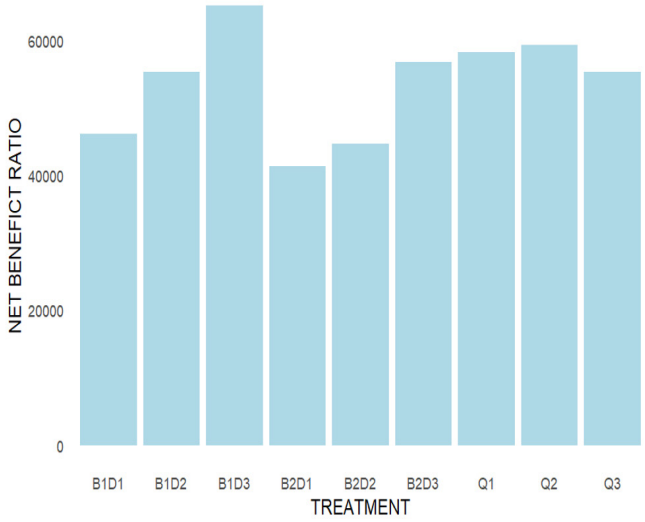


**Figure 7.** Net Benefit Ratio of the Evaluated Treatments

*Note.* Bar chart showing the Net Benefit for each treatment. On the X-axis: B1D1 = *B. subtilis* $1\times10^{6}$ CFU/ml; B1D2 = *B. subtilis* $1\times10^{9}$ CFU/ml; B1D3 = *B. subtilis* $1\times10^{12}$ CFU/ml; B2D1 = *B. amyloliquefaciens* $1\times10^{6}$ CFU/ml; B2D2 = *B. amyloliquefaciens* $1\times10^{9}$ CFU/ml; B2D3 = *B. amyloliquefaciens* $1\times10^{3}$ CFU/ml; Q1 = Switch (Cyprodinil + Fludioxonil) at 0.3 g/l; Q2 = Cantus (Boscalid) at 0.5 g/l; and Q3 = Stroby (Kresoxim-methyl) at 0.15 cc/l. The Y-axis represents the net benefit in monetary units. This net benefit is a dimensional measure calculated as the difference between generated revenue and the total costs associated with each treatment.

The results obtained in this study align with previous findings reported by (Santos et al., 2021), who observed that the implementation of *Bacillus subtilis* and *Bacillus amyloliquefaciens* in agricultural systems significantly improves the cost-benefit ratio, particularly when technical limitations, such as incompatibility with chemical pesticides, are overcome. Additionally, (Ngalimat et al., 2021) and (Han et al., 2023) highlighted that these bacteria not only suppress pathogens through the production of antimicrobial compounds but also induce systemic resistance in plants, contributing to higher yields and long-term sustainability.

The analysis conducted demonstrates that treatment B1D3 is not only effective in reducing the incidence and severity of *Botrytis cinerea* but also maximizes economic profitability. This sustainable approach has the potential to transform disease management in ornamental crops, providing benefits for both producers and the environment.

## Conclusions

This study confirmed that the biological treatment B1D3, based on *Bacillus subtilis* at a concentration of $1\times10^{12}$ CFU/ml, is an effective and sustainable solution for managing *Botrytis cinerea* in roses intended for preservation. B1D3 significantly reduced disease incidence (21.7%) and severity (7.61%), outperforming both chemical and biological treatments evaluated, and exhibited the highest cost-benefit ratio—10% higher than the nearest chemical treatment and approximately 40% greater net benefit. Its high efficacy is attributed to its ability to efficiently colonize plant tissue, compete with the pathogen, and produce antimicrobial compounds, in addition to inducing systemic resistance in plants.

In contrast, while chemical fungicides were effective, their lower profitability and dependence on repeated applications limit their long-term viability. These results highlight the potential of biological strategies not only to improve the productivity and quality of ornamental crops but also to reduce dependency on chemical inputs, enhance the competitiveness of the floriculture sector in international markets, and meet the demands for environmental sustainability and chemical-free production.

It is recommended to expand research to optimize formulations and application methods for these biological agents and to evaluate their performance in different agroecological contexts, establishing them as an effective, profitable, and sustainable solution for floriculture.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.