centrosur
e-ISSN:2706-6800
INSTITUTO TECNOLÓGICO CORPORATIVO
EDWARDS DEMING

Evaluation of the Antagonistic Activity of *Bacillus subtilis* Against Pathogenic and Beneficial Agricultural Fungi

Evaluación de la Capacidad Antagonista de *Bacillus subtilis* frente a Hongos Agrícolas Fitopatógenos y Benéficos

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Published
Instituto Tecnológico Superior Corporativo
Edwards Deming. Quito - Ecuador

Frequency
April–June
Vol. 1, No. 29, 2026
pp. 63–76
http://centrosuragraria.com/index.php/revista

Dates of receipt
Received: January 22, 2026
Approved: March 25, 2026

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Abstract: Conventional agriculture has caused degradation of ecosystems and contributes to the greenhouse effect, the development of genetic resistance, and adverse effects on human health. The use of microorganisms in biological control promotes sustainable agriculture. The objective of this research was to evaluate the antagonistic capacity of Bacillus subtilis F. Cohn in vitro against strains of phytopathogenic and beneficial fungi. The dual challenge and volatile compound methods were used at two growth stages. Mycelial growth was measured, and the percentage of inhibition and growth rate were determined. Spores were counted to determine the percentage of inhibition in fungal sporulation. The dual method showed that the highest percentage of inhibition occurred in Moniliophthora roreri, with 61.85% vertical and 27.43% horizontal inhibition, while in Aspergillus spp. and Trichoderma harzianum, the percentage was less than 25%. In the volatile compound method, Arthrobotrys conoides and Beauveria bassiana exhibited an inhibition percentage greater than 50%, whereas Penicillium spp. and Metarhizium anisopliae exhibited a percentage less than 25%. B. subtilis exhibited antagonism against most phytopathogenic fungi, while it had a minimal effect on the growth of Aspergillus spp. and T. harzianum and a medium-to-high effect on the growth of nematophagous fungi. Furthermore, B. subtilis colonies caused changes in the macroscopic and microscopic characteristics of the studied fungi, due to the wide range of compounds they can produce.

Keywords: Bacillus subtilis, Trichoderma harzianum, secondary metabolites, nematophagous fungi, entomopathogenic fungi, phytopathogenic fungi.

Resumen: La agricultura convencional ha generado un desgaste en los ecosistemas y contribuye al aumento del efecto invernadero, al desarrollo de resistencia genética y afectaciones a la salud humana. El uso de microorganismos en el control biológico favorece una agricultura sostenible. El objetivo de esta investigación fue evaluar la capacidad antagonista de Bacillus subtillis F. Cohn in vitro, frente a cepas de hongos fitopatógenos y benéficos. Se usaron los métodos de enfrentamiento dual y de compuestos volátiles en dos etapas de crecimiento. Se realizó la medición del crecimiento micelial y se determinó el porcentaje de inhibición y la tasa de crecimiento. Se contabilizó las esporas para determinar el porcentaje de inhibición en la esporulación de los hongos. El método dual mostró que el mayor porcentaje de inhibición ocurrió en Moniliophthora roreri con un 61,85 % vertical y 27,43 % horizontal, mientras que, en Aspergillus spp., y Trichoderma. harzianum el porcentaje fue menor al 25 %. En el método de compuestos volátiles Arthrobotrys conoides y Beauveria bassiana presentaron un porcentaje de inhibición mayor al 50%, por su parte, Penicillium spp. y Metarhizium anisopliae presentaron un porcentaje menor al 25 %. B. subtilis provocó antagonismo frente a la mayoría de los hongos fitopatógenos, mientras que, causó un efecto mínimo en el desarrollo de Aspergillus spp., y T. harzianum y, un efecto medio-alto en el desarrollo de hongos nematófagos. Además, las colonias de B. subtilis provocaron cambios en las características macroscópicas y microscópicas de los hongos estudiados, debido al amplio número de compuestos que pueden producir.

Palabras clave: Bacillus subtilis, Trichoderma harzianum, metabolitos secundários, hongos nematófagos, hongos entomopatógenos, hongos fitopatógenos.

Introduction

Conventional agricultural practices are becoming increasingly unsustainable, negatively impacting environmental, economic, and social aspects in the areas where they are carried out. The excessive use of pesticides and chemical fertilizers, along with inappropriate farming practices and the expansion of agriculture, has damaged ecosystems and contributed to the greenhouse effect (Baquero et al., 2007) .

It is important to recognize that agriculture is a crucial activity for the world’s economic, social, and environmental development, as it provides approximately 80% of the food consumed. However, pests and diseases—including bacteria, nematodes, viruses, insects, and especially fungi—affect approximately 20% to 30% of annual agricultural production (FAO, IFAD, PAHO, WFP, 2020) .

Using microorganisms for the biological control of crop-affecting pathogens is an ecological and effective option that promotes sustainable agriculture, as it reduces the problems associated with the use of pesticides and chemicals (Ruiz-Sánchez et al., 2016) . Research has focused on identifying native microorganisms that function under the specific environmental conditions of each region and can be used to restore soil microbiota interactions. In this way, they could be used as biofertilizers and/or biocontrol agents, mitigating the impact of agrochemicals and reducing production costs (Orberá et al., 2014) .

Certain microorganisms produce nutrients that serve as food for other microorganisms (Albuquerque, Elizabeth Albuquerque, 2009) . Bacteria in the body's normal flora control the growth of harmful microorganisms. Among the microorganisms used for this purpose are bacteria of the genus Bacillus, which have high potential due to their ability to exert antagonistic activity through competition, the production of antibiotics, and the production of lytic enzymes such as chitinases (Tejera et al., 2012) .

The development of bioproducts for disease control focuses on the ecological preservation of the interaction between the plant and the microorganism, strategies for applying inoculants, the isolation of new strains, and the identification of innovative mechanisms of action (Cobo, 2017) . Bacillus species have great potential as antagonists due to the large number of biocidal substances they produce, which are capable of controlling plant pathogens (Villarreal et al., 2017) .

This research is of great importance in the field of sustainable agriculture, as it proposes the use of microorganisms as a biocontrol strategy against pathogens that affect crops. The identification and application of native microorganisms allow for the restoration of soil microbiota interactions, improved nutrient availability, and reduced production costs, thereby contributing to the mitigation of the adverse effects of agrochemicals.

This research offers the potential to identify effective combinations of microorganisms in sustainable agriculture, opening up a promising field. These organisms, primarily beneficial bacteria and fungi, perform key functions in agricultural ecosystems and can improve agricultural productivity while reducing the need for chemical pesticides and fertilizers, thereby promoting sustainability in agricultural and industrial production.

Methodology

The research was conducted at the Biological Sciences Laboratory, located in the Faculty of Natural Resources at ESPOCH, in the Lizarzaburu Parish, Riobamba Canton, Chimborazo Province. The environmental conditions inside the laboratory average a temperature of 23 °C and relative humidity of 67%.

Microorganisms Used

The Bacillus subtilis F. Cohn isolate and the seven strains of beneficial fungi were obtained from the fungal culture collection of the Biological Sciences Laboratory, and the seven strains of phytopathogenic fungi were obtained from the fungal culture collection of the Plant Pathology Laboratory at the Faculty of Natural Resources of ESPOCH.

Calculation of the percentage of mycelial growth inhibition

To determine the effect of Bacillus subtilis F. Cohn on fungal growth, the " method was used, which involves measuring the horizontal and vertical diameters in the presence of the bacterium, while Petri dishes containing only the fungi were used as controls. Data were collected every 24 hours. Using the measurements obtained, the percentage of mycelial growth inhibition was calculated using Equation 1.

$$Porcentaje\ de\ inhibición = \left(1 - \frac{diámetro\ tratamiento}{diámetro\ control}\right) * 100$$
(1)

To determine the degree of inhibition, the scale described in Table 1 was used.

Table1 . Scale of percentage of inhibition

Low	0%–25%
Medium	25%–50%
High	> 50%

Calculation of the growth rate

Mycelium growth was recorded in the Petri dish (Corrales Ramírez et al., 2012) , using the following equation:

$$\boldsymbol{Tc} = \frac{crecimiento\ final - crecimiento\ inicial}{tiempo\ final - tiempo\ inicial} \qquad (2)$$

Calculation of the percentage of sporulation inhibition

The percentage of sporulation inhibition was calculated once the fungal control reached its maximum growth (Rodrigues et al., 2010; Velasquez Gurrola, 2005) .

Morphological characteristics

Among the macroscopic characteristics, the color of the colonies was evaluated(Pantone® USA | Pantone Color Systems - Introduction, n.d.) , as well as the characteristics of the mycelium, as described by various authors depending on the fungus. To determine the growth rate, the scale described in Table 2 was used (Hernandez Romero, 2018) .

Table2 . Fungal growth scale

Fast	Between one and two weeks
Moderate	Between two and three weeks
Slow	Between three and four weeks

Experimental specifications

The treatments were carried out using phytopathogenic fungi and beneficial fungi, as shown in Table 3.

Table3 . *Treatments*

Code	Description	Code	Description
T1	*Bacillus subtilis* F Cohn + *Fusarium oxysporum*	PG01	*Bacillus subtilis* F Cohn + *A. oligospora*
T2	*Bacillus subtilis* F Cohn + *Aspergillus* sp.	CH02	*Bacillus subtilis* F Cohn + *A. oligospora*
T3	*Bacillus subtilis* F Cohn + *Colletotrichum* spp.	CH01	*Bacillus subtilis* F Cohn + *A. musiformis*
T4	*Bacillus subtilis* F Cohn + *Penicillium* spp.	PG02	*Bacillus subtilis* F Cohn + *A. conoides*
T5	*Bacillus subtilis* F Cohn + *Neopestalotiopsis* sp.	A21	*Bacillus subtilis* F Cohn + *B. bassiana*
T6	*Bacillus subtilis* F Cohn + *Alternaria grandis*	A13	*Bacillus subtilis* F Cohn + *M. anisopliae*
T7	*Bacillus subtilis* F Cohn + *Moniliophthora roreri*	Th01	*Bacillus subtilis* F Cohn + *T. harzianum*
T8	Controls	T8	Controls

Results

Dual method

In the dual method, the variable indicating the percentage of mycelial growth inhibition in beneficial fungi shows that A. musiformis exhibits the highest percentage of inhibition, with 49.95% vertical inhibition and 47.7% horizontal inhibition, with a growth rate of 6.45 mm/day. The growth rate of the genus Arthrobotrys sp. is 5 mm/day (Castillo Ávila & Medina Medina, 2014) , while T. harzianum showed 0% vertical inhibition and 15.07% horizontal inhibition, with a growth rate of 11.8 mm/day.

Meanwhile, among the phytopathogenic fungi, Moniliophthora roreri achieved the highest percentage with 61.85% vertical inhibition and 27.43% horizontal inhibition, with vertical reductions in its growth rate of 4.2 mm/day (Suárez Contreras & Rangel Riaño, 2013) . Bacillus subtilis, through its antibiosis action, is effective in inhibiting phytopathogenic fungi (Caulier et al., 2019) , and the variety of metabolites generated by Bacillus subtilis, such as antibiotics and lipopeptides (Villarreal-Delgado et al., 2018) , may have antifungal effects. The other fungi obtained percentages above 20% (vertical) and below 15% (horizontal).

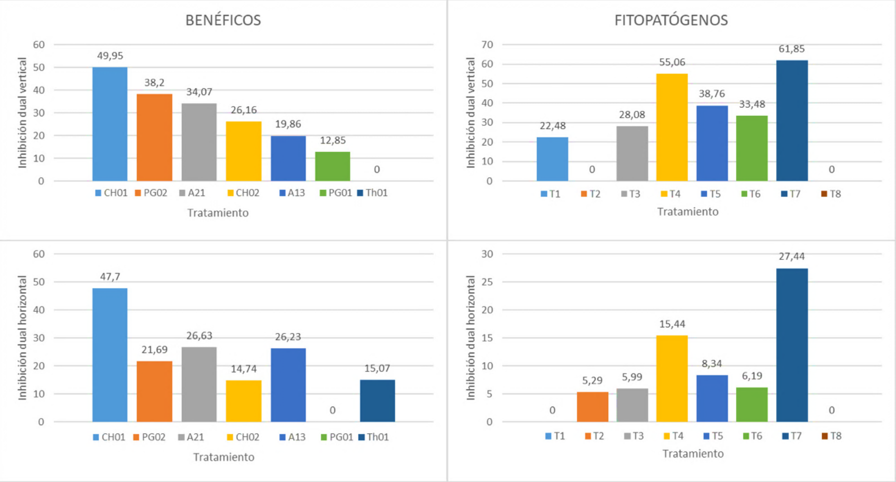

Figure1 . Percentage of inhibition of beneficial and phytopathogenic fungi against B. subtilis using the dual method.

Volatile Compounds Method

In the volatile compounds method (0 hours), the treatment with the highest percentage of inhibition of mycelial growth in beneficial fungi was A. conoides, with 36.6% vertical inhibition and 59.31% horizontal inhibition, with a growth rate of 5.45 mm/day. The treatment with the lowest inhibition was treatment A13 corresponding to M. anisopliae, with 0% vertical inhibition and 22.3% horizontal inhibition, exhibiting a growth rate of 5.85 mm/day; the growth rate for M. anisopliae is an average of 10 to 20 mm/day (Padilla-Melo et al., 2000) .

Regarding phytopathogenic fungi, Moniliophthora roreri achieved the highest percentage of inhibition in both vertical and horizontal directions, exceeding 40%, with a reduction in growth rate of 3.33 to 3.37 mm/day, which is lower than in previous studies (with inhibition rates exceeding 62.5%. Followed by Neopestalotiopsis sp., which showed an inhibition percentage of 20–35%, with a reduction in its growth rate from 4.10 to 4.30 mm/day, due to the emission of volatile compounds by Bacillus spp. strains, (Tahir et al., 2017) . Some of the VOCs that have demonstrated microbial activity include benzothiazole, benzaldehyde, phenylacetaldehyde, and 2,3-butanediol (Pedraza et al., n.d.) . The other fungi obtained inhibition percentages below 25%.

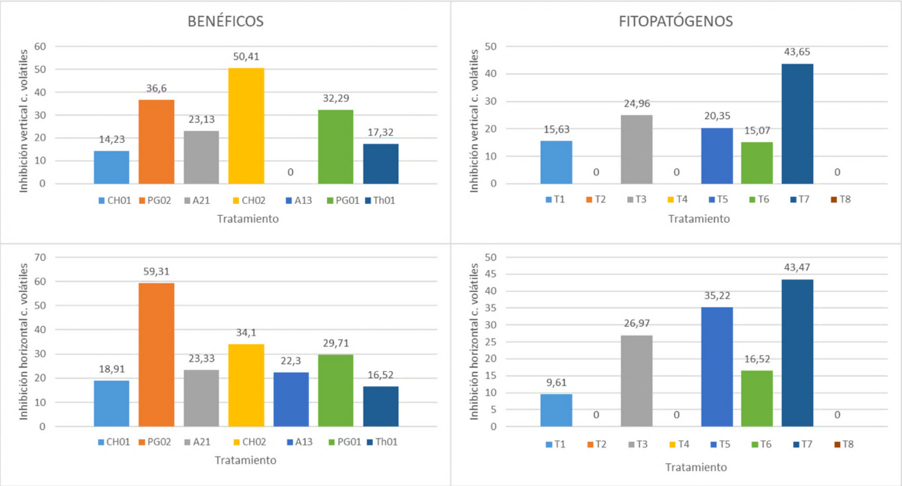

Figure 2. Percentage of inhibition of beneficial and phytopathogenic fungi against B. subtilis using the volatile compound method at 0 hours post-exposure.

Volatile compound method (2nd growth stage)

Using this method on beneficial fungi, B. bassiana showed the highest inhibition, with 37.78% vertical inhibition and 40.07% horizontal inhibition, exhibiting a growth rate of 2.35 mm/day, similar to the growth rate of 60 mm/day for B. bassiana reported in previous studies

(. The treatment with the lowest inhibition percentage was T. harzianum, with 0.99% vertical inhibition and 1.97% horizontal inhibition, exhibiting a growth rate of 1.9 mm/day.

Against the phytopathogenic fungi, Colletrotichum spp., it had the highest inhibition percentage at 31% and a reduction in growth rate of 0.8 mm/day. However, the other treatments obtained lower values in both vertical and horizontal directions, with inhibition percentages below 22% and reductions in growth rate of up to 1.8 mm/day. Once the fungus is fully developed, it is more difficult to control it using microorganisms such as Bacillus subtilis and . It is important to note that Aspergillus spp. showed no inhibition by any method due to its ability to produce mycotoxins, which leads to greater resistance to control by microorganisms (Martínez Padrón et al., 2013) .

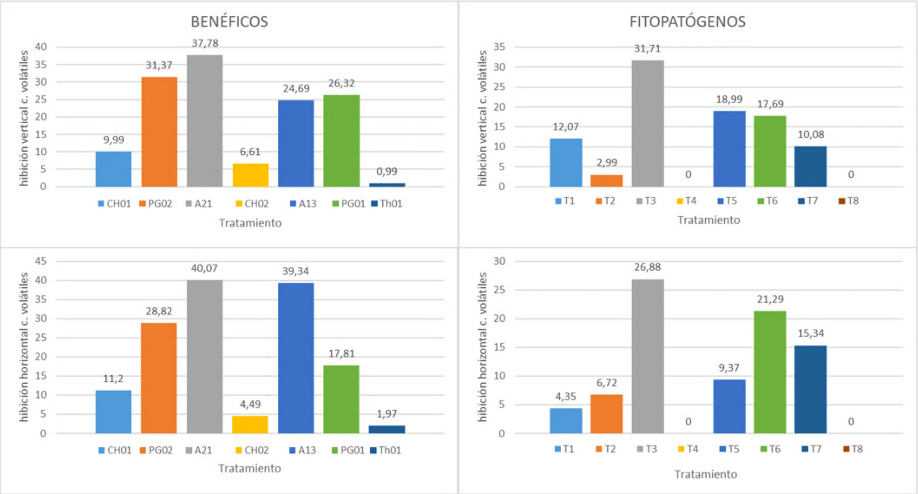

Figure 3. Percentage of inhibition of beneficial and phytopathogenic fungi against B. subtilis using the volatile compounds method 96 hours after exposure.

Percentage of inhibition of fungal sporulation

Bacillus subtilis possesses antimicrobial activity capable of combating spores from various types of microorganisms, such as bacteria and fungi (Ruiz-Sánchez et al., 2016) . Likewise, it has been demonstrated to possess an exceptional ability to inhibit the growth and sporulation of various phytopathogenic fungi, both in vitro and in vivo.

Figure 4 shows the percentage of spore formation inhibition in beneficial fungi; the highest inhibition was found for CH02, corresponding to A. oligospora, with a percentage of 89.32%, and PG02, corresponding to A. conoides, with a percentage of 87.42%. On the other hand, the lowest inhibition percentages were observed for M. anisopliae, at 12.78%, and T. harzianum, at 7.34%.

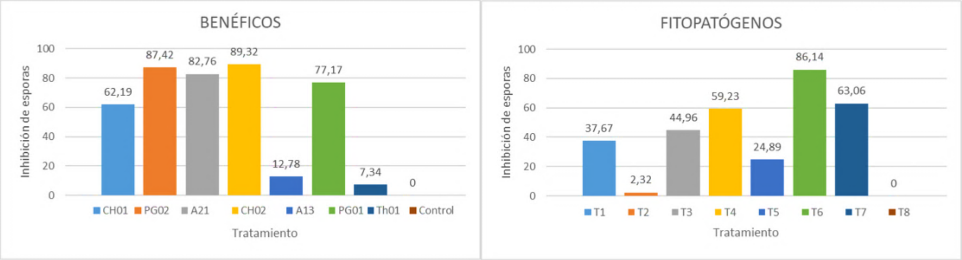

Figure 4. Percentage of spore formation inhibition by beneficial and phytopathogenic fungi.

Regarding phytopathogenic fungi, Alternaria grandis showed the highest percentage at 86.14%, due to the difficulty of sporulation for this fungus (Woudenberg et al., 2014) ; followed by Moniliophthora roreri, which obtained 63.05%, consistent with results showing sporulation inhibition values exceeding 60.85% (Vera Loor et al., 2021) .

Furthermore, the production of hydrolytic enzymes degrades the cytoplasmic membrane of filamentous fungi, thereby reducing fungal sporulation. Similarly, research indicates that against M. roreri and Fusarium oxysporum f. sp. Lycopersici, the presence of Bacillus subtilis was observed to inhibit spores with an effectiveness of over 90% (Guato-Molina et al., 2019) .

In the case of phytopathogenic microorganisms, changes in morphological characteristics occurred in most treatments with Bacillus subtilis, with the exception of Aspergillus spp. In the other treatments, the changes were noticeable, whether in color, growth pattern, or similarly, in the mycelium; this is due to the production of lytic enzymes that aid in the degradation of the main polysaccharides of the fungal cell wall through the hydrolysis of their glycosidic bonds (Caulier et al., 2019) . Similarly, the production of lipopeptides causes the formation of pores and, consequently, an osmotic imbalance in the cytoplasmic membrane (Villarreal-Delgado et al., 2018) .

Iturine and fengicin are compounds with strong biocontrol properties, as they are capable of inhibiting the action of a wide variety of plant pathogens (Ragazzo-Sánchez et al., 2011) . On the other hand, surfactin alone is not capable of inhibiting fungal growth, but it has been shown to have a synergistic effect with the antifungal activity of iturine A.

It has been demonstrated that Trichoderma spp. is capable of synthesizing secondary metabolites involved in the production of volatile compounds with antimicrobial properties (Hernández-Melchor et al., 2019) . Among these compounds, tetracyclic diterpenes such as harziandione, sesquiterpenes such as trichothecenes, trichodermin, and harzianum A, and the triterpene viridine stand out.

Conclusions

Bacillus subtilis F. Cohn causes a decrease in growth in the fungi *A. oligospora* (CH02), *A. musiformis* (CH01), *A. conoides* (PG02), and *B. bassiana* (A21), with mycelial growth inhibition exceeding 50%, affecting the growth rate. *Moniliophthora roreri* had the highest inhibition percentage, with 61.85% vertical and 27.43% horizontal inhibition in the dual method, and over 40% in volatile tests. The *Aspergillus* spp. strain, *A. oligospora* (PG01), *M. anisopliae* (A13), and *T. harzianum* (Th01) showed the lowest inhibition percentage in all methods used.

Alternaria grandis, *Moniliophthora roreri*, *Penicillium* spp., *A. oligospora* (PG01), *A. oligospora* (CH02), *A. musiformis* (CH01), *A. conoides* (PG02), and *B. bassiana* (A21) had the highest spore formation inhibition percentages, exceeding 50%. In contrast, the fungi *Neopestalotiopsis* sp., *Aspergillus* spp., *M. anisopliae* (A13), and *T. harzianum* (Th01) showed percentages below 25%.

Through this study, the morphological characteristics of each of the phytopathogenic fungi were determined, concluding that *Bacillus subtilis* F. Cohn causes changes in both macroscopic and microscopic characteristics due to the wide range of substances and compounds it is capable of producing.

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e-ISSN:2706-6800
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