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Production of volatile compounds and lipopeptides as antagonistic mechanisms of two *Bacillus* strains towards phytopathogenic fungi

Producción de compuestos volátiles y lipopéptidos como mecanismos antagonistas de dos cepas de *Bacillus* hacia hongos fitopatógenos

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Abstract

Phytopathogenic fungi are one of the main causes of diseases that affect agricultural production. For their control, in recent years, biological alternatives have been developed, such as the use of antagonistic microorganisms that produce inhibitory molecules towards these fungi, exerting a biocontrol effect. In the present study, *Bacillus licheniformis* Q19 and *Bacillus subtilis* Q20 strains were characterized for their ability to inhibit *in vitro* the mycelial growth of *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotium rolfsii*, *Colletotrichum gloesporoides* and *Phytophthora* spp. The results of dual cultures show that only Q20 inhibited the pathogens in a range from 33.3 to 50.6 %, being *A. alternata* who presented the greatest inhibition. A positive test for hemolysis, which is related to the lipopeptide production, indicates that these molecules could probably be involved in the fungal inhibition. Later, assays in plates overlapping, where the study microorganisms are not in the same culture médium, showed that Q19 and Q20 produce volatile compunds, capable of inhibiting *A. alternata* and *S. rolfsii* by 72.4 and 56.3 %, respectively. In conclusion, Q19 and Q20 produce lipopeptides and/or volatile compounds with activity against phythopathogenic fungi as biocontrol mechanisms.

Microbial antagonism, Hemolysis, Mycelium

Resumen

Los hongos fitopatógenos, son uno de los principales causantes de enfermedades que afectan la producción agrícola. Para su control, en los últimos años, se han desarrollado alternativas biológicas como es el uso de microorganismo antagonistas que producen moléculas inhibitorias hacia estos hongos, ejerciendo un efecto biocontrol. En el presente estudio, se caracterizaron las cepas de *Bacillus licheniformis* Q19 y *Bacillus subtilis* Q20 en su capacidad para inhibir *in vitro*, el crecimiento micelial de *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotium rolfsii*, *Colletotrichum gloesporoides* y *Phytophthora* spp. Los resultados de cultivos duales, muestran que solo Q20 inhibió los patógenos en un rango del 33.3 al 50.6 %, siendo *A. alternata* el que presentó la mayor inhibición. Una prueba positiva de hemólisis, la cual está relacionada con la producción de lipopéptidos, indica que probablemente estas moléculas pudieran estar involucradas en la inhibición. Después, ensayos en placas superpuesta, donde los microorganismos de estudio no se encuentran en el mismo medio de cultivo, mostraron que Q19 y Q20 producen compuestos volátiles, capaces de inhibir a *A. alternata* y *S. rolfsii* en un 72.4 y 56.3 %, respectivamente. En conclusión, Q19 y Q20 producen lipopéptidos y/o compuestos volátiles con actividad hacia hongos fitopatógenos como mecanismos de biocontrol.

Antagonismo microbiano, Hemólisis, Micelio

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Introduction

Agricultural production in Mexico has been increasing from the 1980s to the present (Baldivia & Ibarra, 2017; SIAP, 2021). However, the phytosanitary problem has always been present, in fact, between 20 and 30% of the annual product from agriculture is affected by pests and/or diseases, where fungi, bacteria, nematodes, as well as viruses and insects are involved (Villarreal *et al*., 2018).

In most cases, postharvest diseases are the limiting factor in fruit and vegetable storage (Benkeblia *et al*., 2011). Mostly, bacterial and fungal species are responsible for the losses of fruits and vegetables in the postharvest period, where the growth of the phytopathogen on the fruit leads to rotting and mycotoxin production by fungal species (Yahaya & Mardiyya, 2019).

For the control of plant pathogenic fungi, broad-spectrum chemical fungicides have conventionally been used to eradicate a large number of pathogenic species. Consequently, the indiscriminate use of these compounds has been negatively affecting soils, ecosystems and human health (Sułowicz & Piotrowska, 2016).

Therefore, the use of sustainable alternatives such as biological control microorganisms or, alternatively, metabolites to mitigate diseases in agricultural crops has been implemented since the last century with great results (Köhl *et al*., 2019).

Bacillus is a bacterial genus that has demonstrated a great antagonistic potential towards phytopathogenic microorganisms, due to its ability to produce antimicrobial compounds including enzymes, antibiotics, lipopeptides and volatile organic compounds (VOCs) (Steinborn *et al*., 2005; Vlot & Rosenkranz, 2022). Among the most studied species with major relevance in agriculture are *B. licheniformis, B. cereus, B. subtilis* and B. *pumilis.*

For example, inhibition of mycelial growth and spore production of plant pathogenic fungi such as *F. oxysporum* by VOCs produced by *B. amyloliquefaciens* has been observed (Yuan *et al*., 2012).

Likewise, Baysal *et al*. (2013), Chaurasia *et al*. (2005) and Yuan *et al*. (2012) reported antagonistic behaviour of VOCs synthesised by *Bacillus* species on common plant pathogen species belonging to the genera *Alternaria, Fusarium, Paecilomyces, Pythium, Rhizoctonia, Aspergillus, Geotrichum, Sclerotinia, Botrytis, Verticillium* and *Colletotrichum.*

In addition, they are also known to produce lipopeptide compounds, which have been shown to antagonise mycelial growth of disease-causing fungi in fruit (Ongena & Jacques, 2008; Rodríguez-Chávez *et al*., 2019; Zhang *et al*., 2022).

On the other hand, the great metabolic diversity among strains of the same species makes it increasingly relevant to search for strains in order to identify new molecules capable of antagonising the growth of phytopathogenic fungi.

Therefore, the aim of the present work was to characterise two *Bacillus* strains in their antagonistic capacity towards phytopathogenic fungi by means of in vitro assays to determine the production of VOCs and/or diffusible lipopeptides in the culture medium.

Methodology to be developed

Biological material

The study strains *B. licheniformis* Q19 and *B. subtilis* Q20 are part of the collection of the company SINQUIMICA, which is dedicated to the commercialisation of biological products. These strains were partially identified by API tests and their biocontrol activity is unknown. The plant pathogenic fungi *Rhizoctonia solani* AG4, *Fusarium oxysporum* FOX13, *Sclerotium rolfsii*, *Colletotrichum gloesporoides* and *Phytophthora spp*. are also part of the SINQUIMICA collection, which are isolated from different regions of the country.

During the experimental work, the bacterial strains were grown on TSA medium and preserved on cellulose filter paper at -20°C. While the fungal strains were subcultured for maintenance on PDA agar in a slant tube and preserved in glycerol at -20°C.

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Dual antagonism assays

The dual assays allow to determine the production of diffusible compounds through the medium generating an inhibition halo. For these tests, which were carried out in triplicate, Petri dishes (90 × 15 mm) with PDA were used. A disc (5 mm in diameter) with active mycelium of the plant pathogenic fungus was placed in the centre of the plate and the antagonistic *Bacillus* strain was inoculated 10 mm from the end of the plate by streaking (Shrivastava *et al*., 2017). It should be noted that, due to the doubling time of the bacteria, the bacteria were inoculated 24 h after inoculation of the fungus to allow growth of the fungus.

As a control, PDA plates were used where only the phytopathogenic fungus was seeded. Afterwards, the plates were incubated at 28 °C until the mycelium covered the entire surface of the medium on the control plates, determining the percentage inhibition of radial growth (% ICR) of the fungus and the inhibition halos generated by the bacterial inoculum (Ezziyyani *et al*., 2004).

Detection of lipopeptide production capacity

The bacterial strains were subjected to a haemolysis test on blood agar plates, as strains with lipopeptide production capacity are able to lyse erythrocytes. For this, a fresh culture colony on TSA agar was seeded on blood agar plates, which were incubated 48 h at 37°C, the formation of clearing halos around the colonies was taken as positive for lipopeptide production (Zhao *et al*., 2014).

Confrontation assays on inverted plates

To assess the impact of volatile organic compounds (VOCs) produced by bacterial strains on mycelial growth of plant pathogenic fungi, indirect confrontation assays were carried out on overlapping plates (Abdallah *et al.*, 2018). For this assay, three replicates were carried out for each of the bacterial strains and controls were set up where bacterial inoculation was excepted only growing the plant pathogenic fungus, the completion of the assay was determined when the mycelium covered the entire surface of the medium on the control plates, determining the percentage of inhibition of radial growth of the fungus and the inhibition halos generated by the bacterial inoculum (Ezziyyani *et al*., 2004).

Thus, discs (5 mm in diameter) of active mycelium of the phytopathogenic fungus were seeded in the centre of Petri dishes (90 × 15 mm) with PDA medium and incubated at 28°C for 24h. At the same time, the inoculum of the antagonistic strains was prepared by growing them in Falcon tubes (50 mL, polypropylene) with 20 mL of TSA broth for 24 hours at 35°C and 150 rpm. Upon completion, the bacterial pellet was recovered by centrifugation at 4000 rpm for 10 min, the resulting pellet was washed and resuspended in 10 mL of sterile water to quantify the cell density in a Neubuer chamber. The density was adjusted to $1x10^7$ cell/mL and 150 µL aliquots were inoculated onto TSA plates by extension seeding. The TSA plates were then covered with the PDA plates containing the 24 h fungal growth and Parafilm was used to bind them together, after which they were incubated at 28°C.

Results

Dual antagonism assays

For the exploration of biocontrol effects through compound diffusion by *Bacillus* spp. strains on the different plant pathogenic fungi, the inhibition of radial growth was evaluated (Figure 1). The results showed that strain Q20 reflected better inhibition percentages against most of the phytopathogenic fungi than strain Q19, highlighting the higher antagonism towards A. alternata (Table 1).

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Mushrooms	*Bacillus* Q19	*Bacillus* Q20
F. oxysporum	6.4 ± 1.9*	41.0 ± 1.3
A. alternata	24.6 ± 3.6	50.6 ± 4.7
C. gloesporoides	4.5 ± 1.6	39.1 ± 2.5
R. solani	7.6 ± 2.9	33.3 ± 3.3
Phytopthora spp.	9.6 ± 1.0	39.1 ± 0.8
S. rolfsii	4.5 ± 3.0	42.2 ± 1.7

* Values are the average of three independent tests. ± Standard deviation.

Table 1 Inhibition percentages of *Bacillus* strains on mycelial growth of plant pathogenic fungi in the dual assay

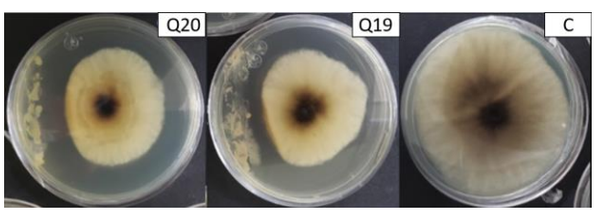

Figure 1 Inhibition of mycelial growth of *A. alternata* in dual antagonism assays with *Bacillus* Q20 and *Bacillus* Q19 strains, control (C).

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Lipopeptide production and haemolysis

By this assay, in both *Bacillus* strains, it was possible to detect the production of medium-diffusible compounds with haemolytic capabilities (Figure 2). Although an association between haemolytic activity and lipopeptide production has been reported, further studies are needed to confirm this (Płaza *et al*., 2015; Sarwar *et al*., 2018).

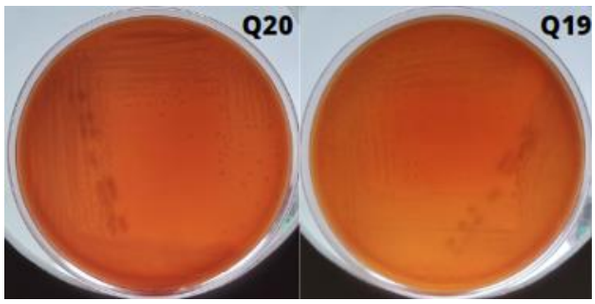

Figure 2 Haemolysis on blood agar plates caused by strains Q20 and Q19, after 24 hours incubation at 37°C.

The inhibitory behaviour of *B. subtilis* and *B. licheniformis* strains against the study pathogens, due to the probable production of lipopeptides, is in agreement with reports by different authors (Qi *et al*., 2010; Romano *et al*., 2013; Ruiz *et al*., 2014; Wang *et al*., 2020). Chaurasia *et al*. (2005) report 66.1 to 71.7 % inhibitory activities of *B. subtilis* (NRRL B-30408) against *A. alternata* and *F. oxysporum*. In addition, Dimkić *et al*. (2013) reported mycelial inhibition towards strains of the genera *Monillinia*, *Penicillium*, *Fusarium*, *Colletotricum* and *Alternaria* through the action of surfactin-like lipopeptides and iturins produced by B. *licheniformis* SS-12.6 and B. *amyloliquefaciens* SS-13.1.

Regarding the mechanism of action of lipopeptide compounds, it is known that particularly, cell wall and fungal cell membrane metabolism are the main processes affected by lipopeptide stress (Zhang *et al*., 2022) ultimately culminating in the induction of apoptosis (Qi *et al*., 2010) or cell lysis (Yu *et al*., 2019). Similarly, the mechanism of action of lipopeptides is known to differ depending on both their peptide and lipid composition (Nasir & Besson, 2012; Yu *et al*., 2019; Zhang *et al*., 2022).

Reversed plate confrontation assays

The percentage mycelial inhibition of plant pathogenic fungi, resulting from the VOCs produced by the study strains, are shown in Table 2. The results indicate that Q19 was able to antagonise mostly *A. alternata* and *S. rolfsii.* While Q20 failed to inhibit *A. alternata* but showed a remarkable ability to inhibit *S. rolfsii* (Figure 3). However, the percentage of inhibition obtained by application of the bacterial treatments to *F. oxysporum* and *R. solai* was the lowest, being less than 20 %.

Mushrooms	*Bacillus* Q19	*Bacillus* Q20
F. oxysporum	10.3 ± 1.9*	18.1 ± 3.5
A. alternata	72.4 ± 3.2	10.8 ± 6.9
C. gloesporoides	31.6 ± 4.8	39.0 ± 5.2
R. solani	2.4 ± 1.0	3.0 ± 1.9
Phytopthora spp.	32.7 ± 1.5	36.2 ± 2.5
S. rolfsii	47.7 ± 4.8	56.3 ± 5.2

* Values are the average of three independent tests. ± Standard deviation.

Table 2 Percentage inhibition of mycelial growth of phytopathogenic fungi by VOC production of *Bacillus* strains in overlapping plate tests

The inhibitory and antifungal activity of *B. subtilis* strains against fungi of agricultural interest by volatile compounds has been reported previously, Chaurasia *et al*. (2005) show 60-65% inhibitory activities of *B. subtilis* (NRRL B-30408) on *A. alternata* and *F. oxysporum*. Furthermore, Zhao *et al*. (2019) reported the inhibitory capacity of agriculturally important fungi by *B. subtilis* CF-3 where (S)-1-octanol, benzoic acid, 2,4-di-ter¬-butylthiophenol, benzaldehyde and benzothiazole were the key compounds for the inhibition of *C. gloesporoides* and *M. fructicola.*

In contrast to lipopeptides, the mechanism of inhibition of *Bacillus* spp. VOCs is unclear, however, it is known that they can inhibit conidial germination and morphologically affect the hyphae of pathogenic fungi (Zhang *et al*., 2020), it has also been reported that VOCs from *B. subtilis* modify the regulation of gene expression related to cell membrane fluidity, cell wall integrity, energy metabolism and cell wall degrading enzyme production (Wang *et al*., 2021).

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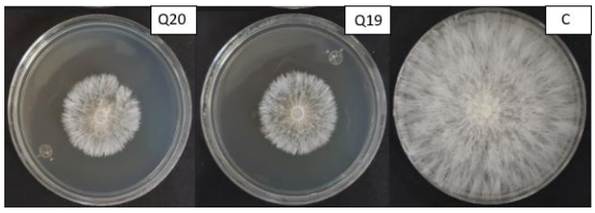

Figure 3. Inhibition of mycelial growth of *S. rolfsii* by strains Q20 and Q19 in overlapping plate assays, control (C).

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Conclusions

The study strains *B. licheniformis* Q19 and *B. subtilis* Q20 show in vitro biocontrol activity on phytopathogenic fungi affecting fruit and vegetable crops. *Alternaria alternata* was the fungus that was mostly inhibited by the production of lipopeptides from Q20 and by volatile compounds produced by Q19.

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