



# Extremophilic bacteria restrict the growth of *Macrophomina phaseolina* by combined secretion of polyamines and lytic enzymes

Ana P. Santos<sup>a</sup>, Luciana Nieva Muratore<sup>a</sup>, Anna Solé-Gil<sup>b</sup>, María E. Farías<sup>a</sup>,  
Alejandro Ferrando<sup>b</sup>, Miguel A. Blázquez<sup>b</sup>, Carolina Belfiore<sup>a,\*</sup>

<sup>a</sup> Laboratorio de Investigaciones Microbiológicas de Lagunas Andinas (LIMLA), Planta Piloto de Procesos Industriales Microbiológicos (PROIMI-CONICET), Avenida Belgrano y Pasaje Caseros, Tucumán, Argentina

<sup>b</sup> Instituto de Biología Molecular y Celular de Plantas (IBMCP), Consejo Superior de Investigaciones Científicas, Universidad Politécnica de Valencia (CSIC-UPV), Avenida de los naranjos s/n, 46022 Valencia, Spain

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## ABSTRACT

Extremophilic microorganisms were screened as biocontrol agents against two strains of *Macrophomina phaseolina* (Mp02 and 06). *Stenotrophomonas* sp. AG3 and *Exiguobacterium* sp. S58 exhibited a potential *in vitro* antifungal effect on Mp02 growth, corresponding to 52.2% and 40.7% inhibition, respectively. This effect was confirmed by scanning electron microscopy, where images revealed marked morphological alterations in fungus hyphae. The bacteria were found to secrete lytic enzymes and polyamines. *Exiguobacterium* sp. S56a was the only strain able to reduce the growth of the two strains of *M. phaseolina* through their supernatant. Antifungal supernatant activity was correlated with the ability of bacteria to synthesize and excrete putrescine, and the exogenous application of this polyamine to the medium phenocopied the bacterial antifungal effects. We propose that the combined secretion of putrescine, spermidine, and lytic enzymes by extremophilic microorganism predispose these microorganisms to reduce the disease severity occasioned by *M. phaseolina* in soybean seedlings.

## 1. Introduction

*Macrophomina phaseolina* (Tassi) Goid is a cosmopolitan soil-borne phytopathogenic fungus that infects more than 500 different plant species worldwide [1]. *M. phaseolina* represents a devastating pathogen for soybean (*Glycine max* L.) that causes charcoal rot disease and is responsible for annual crop yield losses and seed quality deterioration [2]. Nowadays fungal plant diseases represent one of the main constraints in agricultural production. Therefore, there is an increasing need to control fungal diseases to prevent crop yield reduction and ensure constant food supply for a growing world population.

The survival strategies developed by extremophilic microorganisms to adapt to adverse environmental conditions make them useful in different agricultural practices [3] and even in many biotechnological applications [4]. Some strategies are linked to the production of enzymes and other secondary metabolites that play a crucial role in the biological control of pathogens [4]. Particularly, cold-adapted bacteria are considered as a novel source of natural antibiotics for their abilities to reduce the presence of other competitive microorganisms [5,6].

Moreover, a chitinase cold-active enzyme produced by *Pseudomonas* sp. isolated from marine sediments showed significant inhibition of fungi *Verticillium dahlia* and *Fusarium oxysporum* f. sp. *cucumerinum* which can cause cotton wilt and cucumber blight, respectively [7]. Consequently, microorganisms capable of coping with adverse conditions could be tested as efficient biocontrol agents (BCAs).

The use of plant growth-promoting bacteria (PGPB) as BCAs is a natural, safe, and productive alternative to manage pathogen infection [8], and understanding their mechanism of action is essential to achieve optimum disease control. BCAs generally control plant diseases through the following mechanisms: competition for resources (nutrients and host sites), induced systemic resistance, and antifungal metabolite production [9]. However, the mechanism of action of some microbial antagonists against pathogen populations is not entirely clear [10].

Polyamines (PAs) are small aliphatic polycation compounds present in all eukaryotes, which are involved in many physiological processes and play a crucial role in the regulation of cellular metabolism. PAs could also contribute to plant acclimation to stressful environmental conditions by increasing the resistance of plants to lesions, salinity,

\* Corresponding author.

E-mail address: [carobelfiore@hotmail.com](mailto:carobelfiore@hotmail.com) (C. Belfiore).

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drought, temperature, and infections by pathogens [11]. Therefore, most studies have focused on the impact of polyamines on plant growth, stress responses, and disease [12], but only a few of them have focused on the polyamines produced by PGPB and their antagonistic activity [13,14].

The aims of this work were to study the inhibitory effect of extremophilic microorganisms isolated from Andean soil [15] and characterize their antifungal activities against two strains of *M. phaseolina* Tassi (Goid) (Mp 02 and 06). We assessed the ability of the bacteria to produce and secrete metabolites, such as polyamines and lytic enzymes, which could be involved in the *M. phaseolina*-dependent growth inhibition of soybean. Finally, we studied the structural damage induced in the phytopathogen structures by scanning electron microscopy (SEM).

## 2. Material and methods

### 2.1. Fungus and culture conditions

The *M. phaseolina* Tassi (Goid) used in this study was kindly provided by Laboratorio de Suelos, Aguas y Fertilizantes, EEA-INTA, Salta, Argentina. Two strains of *M. Phaseolina* (Mp 02 and 06) were cultured in potato dextrose agar (PDA) at  $28 \pm 2$  °C for 7 days. The strains were deposited in PROIMI culture collection (WDCM 587) with the strain numbers PROIMI100150 (Mp 02) and PROIMI100151 (Mp 06).

### 2.2. Bacterial strains and growth conditions

Bacterial isolates from extreme environments in the Andean soil [15, 16], such as *Stenotrophomonas* sp. AG3, *Exiguobacterium* sp. S56a and S58 (Genbank accession numbers: MW442973; MW442976 and MW442975) were used in this study. All the strains were deposited in PROIMI culture collection (WDCM 587) with the strain numbers: PROIMI100153, PROIMI100154 and PROIMI100155, respectively. The bacterial strains were cultured in a Luria-Bertani (LB) medium at  $28 \pm 2$  °C and 200 rpm, for 4 and 12 h, to reach exponential ( $10^5$  CFU/mL) and stationary ( $10^9$  CFU/mL) growth phases, respectively. The bacteria were centrifuged at  $10,000 \times g$  at 4 °C for 10 min. The cell pellets (CP) and cell-free culture supernatants (CFS) of each growth phase were collected and kept on ice for further analysis.

### 2.3. Quantification of polyamines produced by extremophilic microorganisms

The polyamine extraction and quantification of the bacterial cell pellet (CP) and supernatant (CFS) samples were carried out according to Solé-Gil et al. [17], with some modifications. The bacterial cell pellets were resuspended in 1000 µL of distilled water, treated with 20 µL of lysozyme (10 mg/mL), and kept on ice for 10 min. Then, each bacterial cell was sonicated to disruption for 30 s on ice ten times and centrifuged at  $10,000 \times g$  for 10 min to pellet debris. Lysates of sonicated CP and 500 µL aliquot of the CFS were treated with 2.5 mL of perchloric acid 5% (w/v) and followed by the addition of 500 µL of the internal standard (1, 6-Hexanediamine 1 mM, HD). The samples were centrifuged at  $15,000 \times g$  for 20 min and the supernatants stored at 4 °C for 30 min. Then 3 mL of each supernatant was neutralized with 2 mL of 2 M sodium hydroxide and followed by the addition of 10 µL of benzoyl chloride. The samples were vortexed and incubated for 20 min at room temperature. Finally, 4 mL of saturated sodium chloride solution was added to each sample. Benzoyl-amides were extracted in 2 mL of diethyl ether and the organic phases were dried using  $N_2$  and resuspended with 150 µL of methanol. The resuspended samples were filtered through a 0.22 µm syringe filter, and the HPLC chromatograms were produced by injecting 30 µL of the samples onto a Luna C18(2) (Phenomenex) column. The elution was conducted at room temperature, the flow rate was 1 mL  $min^{-1}$ , and the UV detector of the system was set at 254 nm. Polyamine

measurements were done in triplicate.

### 2.4. Cell wall-degrading enzymes (CWDEs)

Extremophilic bacteria were screened for the production of three CWDEs:  $\beta$ -1,4-glucanase, cellulase, and chitinase.  $\beta$ -1,4-glucanase and cellulase activities were estimated in minimal medium [18,19] with barley flour and carboxymethylcellulose as the sole carbon source, respectively. The plates were inoculated with overnight bacterial cultures and incubated at  $28 \pm 2$  °C for 48 h. The formation of translucent halos around the colonies confirmed the ability of the bacteria to use the substrates as the sole carbon source. To visualize the halos for the cellulose activity, the plates were flooded with an aqueous solution of congo red 1% (w/v) for 30 min and washed with 1 M NaCl [20].

The chitinase production was evaluated in minimal medium [21] containing chitin as the sole carbon source. Colloidal chitin was prepared by dissolving 10 g of crab shell chitin (Sigma-Aldrich) in 150 mL of concentrated HCl [22]. The plates were inoculated ( $10^9$  CFU/mL) and incubated at  $28 \pm 2$  °C for 8 days. The chitinolytic activity was evidenced through the formation of clear halos around the colonies.

The enzymatic activity indexes (EI) were calculated for the three CWDEs by measuring the clearance zone and using the following expression:

$$EI = \text{diameter of hydrolysis zone} / \text{colony diameter} \quad (1)$$

Each experiment was performed in triplicate.

### 2.5. Organic acid production

The bacteria were cultured in MM9 agar medium [23] with methyl red as an indicator at  $28 \pm 2$  °C for 24 h, and the pink zones around the colonies indicated the ability to produce organic acid. All isolates were tested in triplicate.

### 2.6. HCN determination

To stimulate the production of HCN, overnight bacterial cultures were streaked in Luria-Bertani (LB) plates supplied with 4.4 g/L glycine. A sterile filter paper was saturated in 0.5% (w/v) picric acid and 1% (w/v)  $Na_2CO_3$  solution and fixed to the underside of Petri dishes [24]. The plates were prepared in triplicate, sealed with parafilm and incubated at  $28 \pm 2$  °C for 48 h. The color change of the filter paper from yellow to brown was recorded as an indication of cyanogenic production.

### 2.7. Antifungal activity

Three independent experiments were performed for this screening step, with three replicates per strain.

#### 2.7.1. Effects of bacterial antagonists

Extremophilic bacteria were tested for their ability to inhibit the growth of two strains of *M. phaseolina* (Mp 02 and 06) by employing a dual culture assay [25] on PDA plates. Bacterial cell suspension ( $10^9$  CFU/mL) grown previously in LB medium was streaked in a straight line on one side of the Petri dishes, leaving 1 cm from the margin. Once the moisture from the inoculum was absorbed by the culture medium, a mycelial disk (6 mm) of the phytopathogen was placed at a distance of 70 mm from the bacterial culture. Plates with sterile distilled water (instead of bacterial suspension) and antagonist fungus served as control. The plates were incubated at  $28 \pm 2$  °C in the dark, and the percentage of inhibition (PI) was registered every day for 105 h. The PI was calculated using Shrivastava et al. [1] equation, as follows:

$$\%PI = (C - T) / C \times 100 \quad (2)$$

where 'C' is the colony growth of *M. phaseolina* in control plates, and T is

the colony growth of the pathogen in dual-culture plates.

### 2.7.2. Effects of cell-free culture filtrate

The antifungal properties of the CFS were tested against *M. phaseolina*. Overnight bacterial suspensions ( $10^9$  CFU/mL) were centrifuged at  $10,000 \times g$  at  $4^\circ\text{C}$  for 10 min. The supernatants were filtered through a  $0.22 \mu\text{m}$  syringe filter, and  $100 \mu\text{L}$  of each was placed in PDA plates. The plates were inoculated with a 6-mm disk of *M. phaseolina* placed in the center of the plate and further incubated at  $28 \pm 2^\circ\text{C}$  for 72 h, in the dark. The PI of the pathogen was scored every day and calculated according to Eq. (2) by comparing the growth of the fungus in control plates, where the supernatants were replaced by sterile distilled water.

### 2.7.3. Production of volatile compounds

Antagonism due to volatile compounds was evaluated by preparing half of a Petri plate containing LB medium inoculated with  $100 \mu\text{L}$  of bacterial suspension ( $10^9$  CFU/mL) side down and the other half with PDA medium with a 6-mm disk of the fungi side up. The plates were sealed with parafilm and incubated at  $28 \pm 2^\circ\text{C}$  for 72 h, in the dark. Controls were performed with half the PDA plate and sterile distilled water. The inhibition percentage was calculated by Eq. (2).

### 2.7.4. Imaging with scanning electron microscopy (SEM)

The mycelia samples corresponding to the interaction regions of the dual-culture test and the cell-free culture filtrate were examined by SEM. The samples were fixed in Karnovsky buffer (1.7% glutaraldehyde and 1.5% paraformaldehyde), pH 7.2, for 24 h. Then, the samples were dehydrated successively with ethanol (30%, 50%, 70%, 90%, and 100%) for 10 min. After the dehydration process, the samples were washed twice with acetone (100%) for 10 min. Complete dehydration of samples was carried out by the critical point technique using Denton Vacuum equipment (model DCP-1). Subsequently, the samples were placed on an aluminum support with conductive double-sided carbon adhesive tape on its surface and coated with gold by Ion Sputter (JEOL® model JFC-1100). Finally, the electron microscope visualization was performed using a ZEISS SUPRA 55 VP scanning from CISME (Centro de Investigaciones y Servicios de Microscopía Electrónica). Fungal colony of *M. phaseolina* isolates grown on PDA plates with distilled sterile water (instead of bacterial suspension) were used as a control.

### 2.8. Effects of exogenous polyamines application on *M. phaseolina* growth

PDA plates were supplemented with polyamine (putrescine, cadaverine, spermidine, spermine, and thermospermine) standards at different concentrations: 10, 50, 100, 500, and  $1000 \mu\text{M}$ . Then, a 6-mm disk of the fungi was placed in the center of the PDA plates and incubated at  $28 \pm 2^\circ\text{C}$  for 72 h. After the incubation, the inhibition percentage of fungal growth was analyzed and compared with control plates (PDA medium) according to Eq. (2). The experiments were carried out in triplicate with three independent trails.

### 2.9. Effect of extremophilic bacteria on growth inhibition caused by *M. phaseolina* in germinated soybean seeds

Extremophiles cultures and their supernatants were used to analyze their effects on soybean growth damage caused by *M. phaseolina* according to Simonetti et al. [26] protocol with some modifications. Soybean seeds (BAYER CZ 6806 IPRO) were previous sterilized with ethanol 70% for 15 min, rinsed with sterile distilled water and drained on a clean bench. Then, the seeds were mixed with bacterial suspension ( $10^9$  CFU/mL) and sterile-filtered supernatants at a dosage of 5 mL/kg of seeds for 15 min at 200 rpm. Sterile distilled water was used instead of bacterial suspension as a control. Finally, six seeds were placed in PDA plates completely covered by *M. phaseolina* (02 and 06) growth and incubated at  $28 \pm 2^\circ\text{C}$  for 5 days. The experiment was carried out in

triplicate and PDA plates without the phytopathogen were used as uninoculated controls. The severity index was evaluated according to Simonetti et al. [26] scale only in the plates previously incubated with the fungus. The disease severity assessment was estimated at 0: healthy seed; 1: seed teguments invaded by mycelium; 2: seed teguments and radicle invaded by mycelium; 3: seed teguments, radicle and interior of the seed invaded by mycelium; 4: seed teguments invaded by mycelium and sclerotia, without internal mycelium; 5: seed teguments invaded by mycelium and sclerotia with the presence of internal mycelium; 6: seed teguments, radicle and/or interior invaded by mycelium and sclerotia and 7: seed not germinated and completely colonized by the fungus.

### 2.10. Statistical analysis

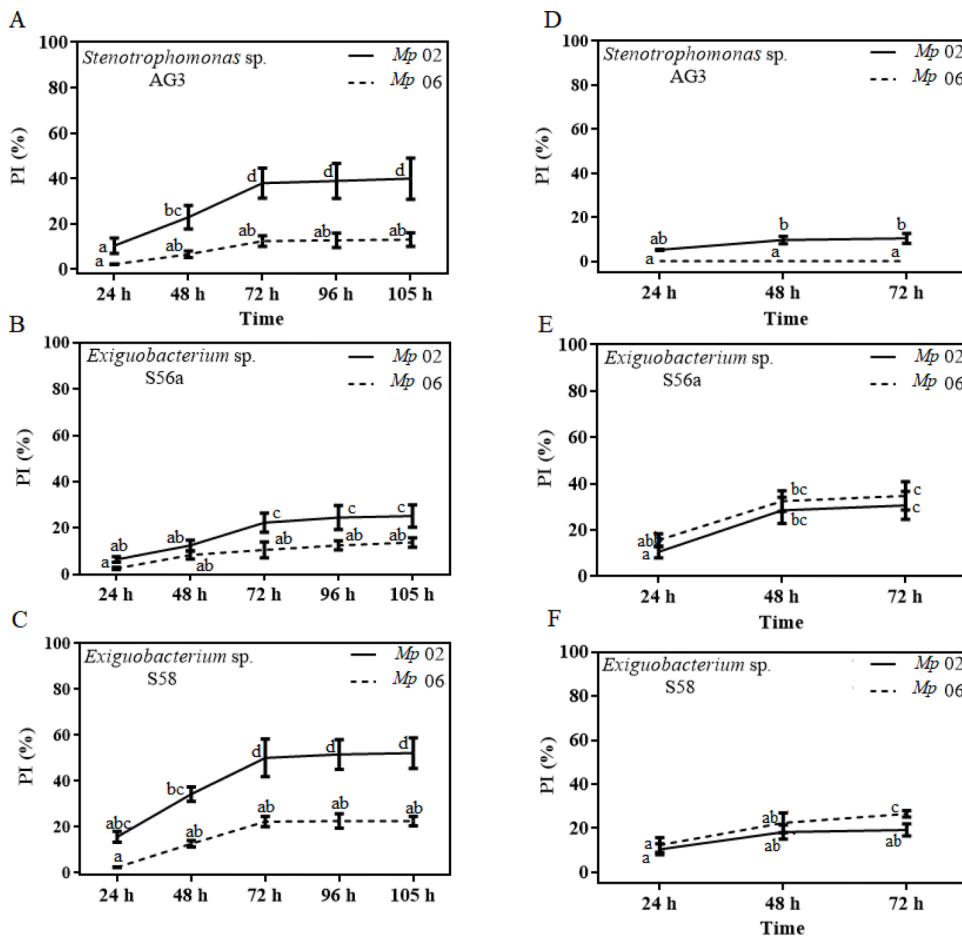
To analyze the inhibitory activities of the bacteria, the data were subjected to analysis of variance (ANOVA) using GraphPad Prism 7.0 software. Treatment means were analysed by Tukey's HSD test, and the level of significance was set at  $p < 0.05$ . The assays were performed in triplicate.

## 3. Results

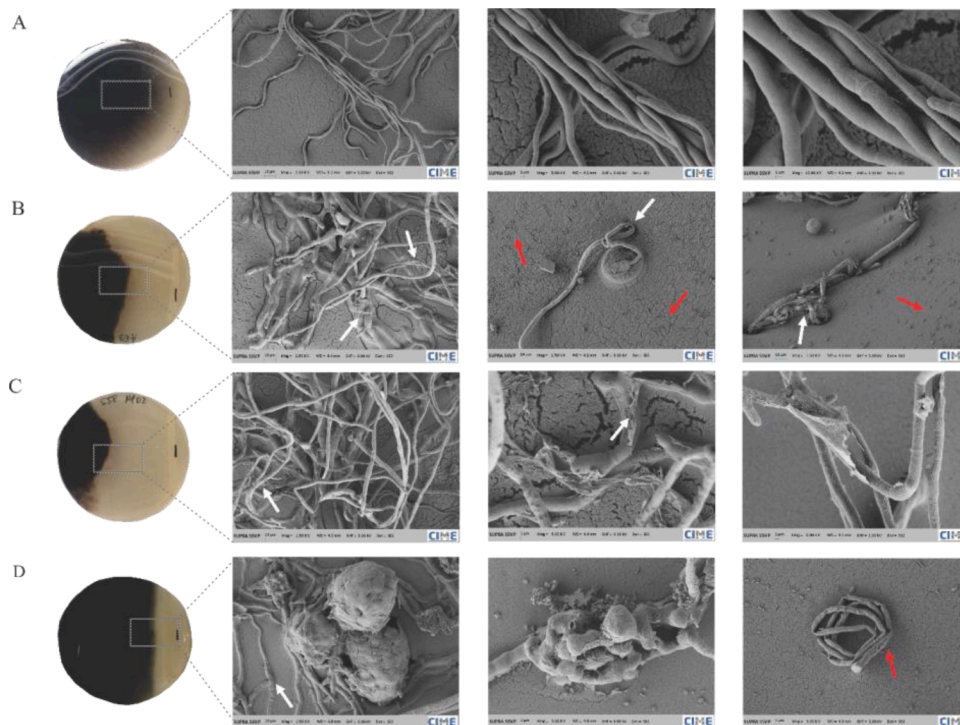
### 3.1. Biocontrol activities of extremophile bacteria

As a proof of concept for the use of extremophilic bacteria as potential BCAs, we tested the ability of three previously characterized strains [16] to restrict the growth of two different isolates of the fungus *M. phaseolina* (*Mp* 02 and *Mp* 06) obtained from soybean roots in northwestern Salta province (Argentina). In dual-culture assays, the fungus *Mp* 02 was the most inhibited by the three bacterial strains: *Exiguobacterium* sp. S58 (Fig. 1C), *Stenotrophomonas* sp. AG3 (Fig. 1A), and *Exiguobacterium* sp. S56a (Fig. 1B). The inhibition became evident at 72 h of incubation, reaching values of 52.2%, 40.7%, and 25.2% inhibition at 120 h, respectively. SEM studies confirmed the growth impairment and the damage induced in *M. phaseolina* individuals by the three bacterial isolates (Fig. 2). The hyphae cultured without extremophilic bacteria were regular in shape and had a healthy appearance (Fig. 2A). However, when *Mp* 02 was exposed to *Stenotrophomonas* sp. AG3 and *Exiguobacterium* sp. S58, morphological changes in fungal hyphae were observed. The hyphae surface became disintegrated and ruptured by the presence of the bacteria (Fig. 2B and C). In addition, *Exiguobacterium* sp. S56a also caused marked deformations in *Mp* 02 fungal structures, leading to the detachment of the hyphae from the surface and the accumulation of debris (Fig. 2D). The biocontrol activity of the bacteria on the *Mp* 06 strain was very low, compared to the *Mp* 02 strain (Fig. 1A, B, and C).

Cell-free supernatants also displayed antifungal properties after 72 h (Fig. 1), which did not increase after longer incubation periods. *Stenotrophomonas* sp. AG3 cell-free culture filtrates inhibited the *Mp* 02 strain growth only by 10.5% (Fig. 1D). SEM images showed slight alterations in the structural architecture of *Mp* 02 hyphae after incubation with cell-free supernatant of *Stenotrophomonas* sp. AG3 (Fig. 3B); however, there also appeared to be some healthy-looking hyphae. Similarly, the inhibitory effect of *Exiguobacterium* sp. S58 cell-free culture filtrates decreased by half against *Mp* 02 (19.3%), compared to bacterial cultivation (Fig. 1F). However, exposure to bacterial supernatant resulted in pronounced distortions and desiccation of fungal hyphae (Fig. 3C), compared to the control (Fig. 3A), where the hyphae were homogeneous and cylindrical in shape. The highest inhibition percentage was displayed by *Exiguobacterium* sp. S58 against *Mp* 06, restricting their growth up to 26.6% (Fig. 1F). *Exiguobacterium* sp. S56a was the only bacterial isolate whose cell-free supernatant was able to restrict the growth of the two fungal strains, *Mp* 02 and 06, to 30.6% and 34.8%, respectively (Fig. 1E). When *Mp* 02 hyphae were examined by SEM after the incubation with the *Exiguobacterium* sp. S56a supernatant, disruptions of hyphal structures were observed since the hyphae were twisted

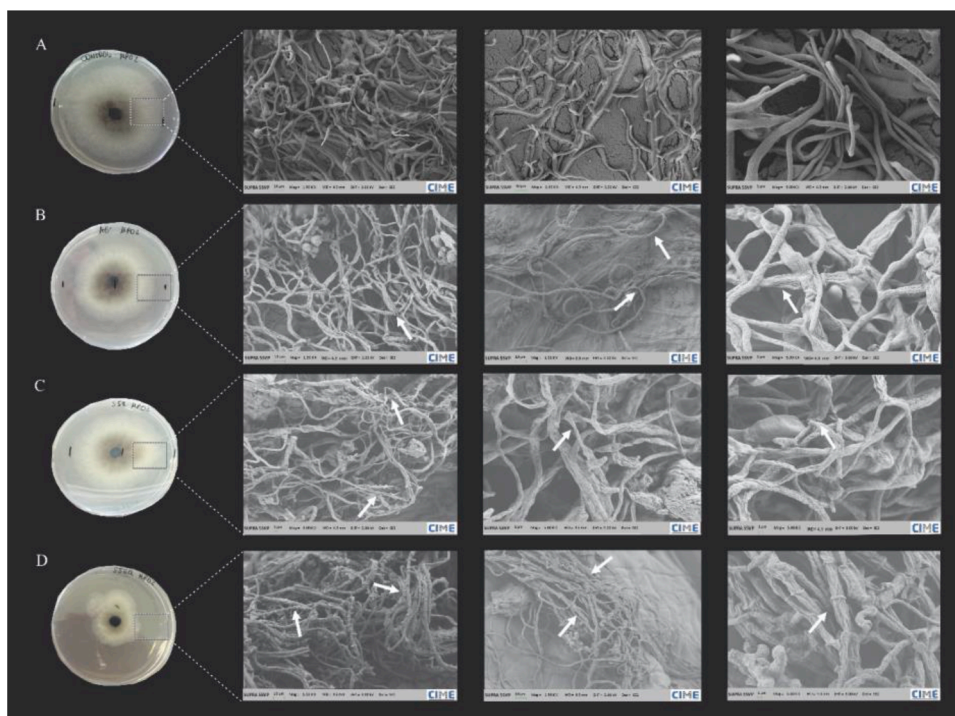


**Fig. 1.** Antagonistic activity of isolates against two strains of *M. phaseolina* on potato dextrose agar. The fungus was co-cultivated with *Stenotrophomonas* sp. AG3 (A), *Exiguobacterium* sp. S56a (B) and *Exiguobacterium* sp. S58 (C); or exposed to the supernatants of the corresponding bacteria (D-F). The mycelial growth inhibition percentage (IP) was scored at the indicated time points. Data represent the mean percentage of fungal inhibition  $\pm$  standard error. Values with different letters show significant differences ( $p < 0.05$ ) according to Tukey's HSD test.



**Fig. 2.** SEM analysis of *Mp 02* mycelium in dual-culture plates. Multiple images of fungal appearance were examined following 105 h exposure to a mock solution (A), *Stenotrophomonas* sp. AG3 (B), *Exiguobacterium* sp. S58 (C) and *Exiguobacterium* sp. S56a (D). White arrows indicate hyphal damage, and red arrows point to visible bacteria. Scale bars, 1 and 10  $\mu\text{m}$ .





**Fig. 3.** SEM analysis of *Mp* 02 mycelium in cell-free culture filtrate plates. Multiple images of fungal appearance were examined following 72 h exposure to a mock solution (A), *Stenotrophomonas* sp. AG3 (B), *Exiguobacterium* sp. S58 (C) and *Exiguobacterium* sp. S56a (D) supernatants. Scale bars, 1 and 10  $\mu\text{m}$ .

and shriveled (Fig. 3D).

Given that the inhibition of hyphal growth by extremophilic bacteria could not be attributed to the production of volatile compounds, since only *Stenotrophomonas* sp. AG3 showed a low activity against *Mp* 02 of 10.5% (results not shown), we decided to investigate two alternative mechanisms: the production of polyamines as antifungal agents and the production of cell-wall degrading enzymes.

### 3.2. Bacterial polyamine content and secretion

The main polyamines found in bacteria are putrescine (Put), cadaverine (Cad), spermidine (Spd), and spermine (Spm) [27]. However, unusual polyamines such as thermospermine (Tspm) are typical of extreme thermophilic microorganisms [28]. Therefore, the levels of the five polyamines were assessed in the bacterial cells and supernatants by HPLC analysis at the exponential and stationary growth phases (Fig. S1). The strains *Stenotrophomonas* sp. AG3 (Fig. 4A) and *Exiguobacterium* sp. S58 (Fig. 4C) were able to synthesize and secrete to the medium the five polyamines under study during exponential growth, with a maximum yield for Spd and lower levels of Spm and Tspm. Similar results were obtained when the bacterial population reached the stationary phase (Fig. 4B, and D). In the case of *Exiguobacterium* sp. S56a, although no extracellular polyamines were detected in the exponential growth phase (Fig. 4E), the five polyamines were present in the supernatant when the bacteria entered the stationary growth phase with the highest secretion of Put and Tspm (Fig. 4F).

Polyamines were also detected intracellularly in *Stenotrophomonas* sp. AG3 and *Exiguobacterium* sp. S58 and S56a, but the concentrations were considerably higher only in the strain *Exiguobacterium* sp. S56a in the exponential phase (Fig. 4E), probably because these polyamines were not secreted.

### 3.3. Exogenous polyamine application inhibits the growth of the phytopathogen *M. phaseolina*

The observation that the bacterial isolates secreted polyamines to the

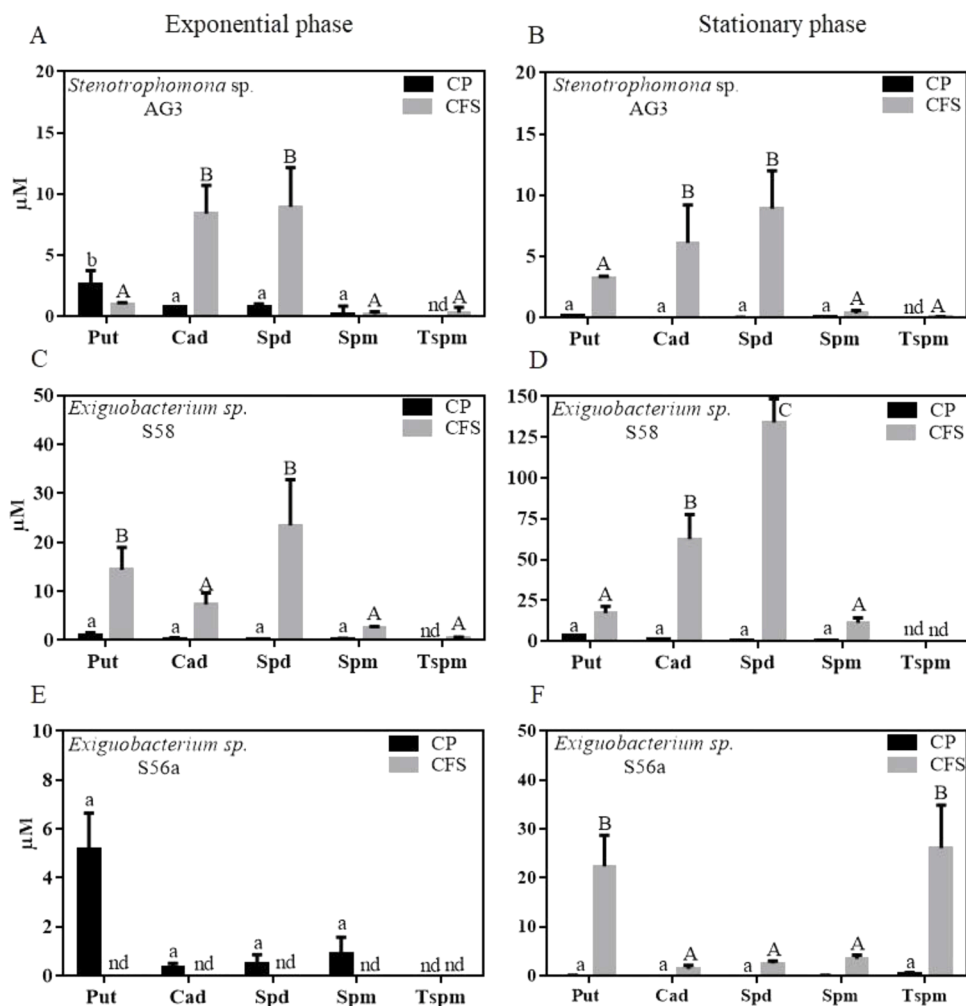
medium—which showed antifungal properties—prompted us to investigate whether exogenous polyamine application could be harmful for fungal growth. We assessed the effect of different concentrations of polyamines on *M. phaseolina* growth. *In vitro* experiments showed that three polyamines: Put, Spd, and Spm significantly reduced the growth of *M. phaseolina* with a dose-effect (Fig. 5). Put demonstrated the strongest antifungal activity at 10  $\mu\text{M}$  concentrations (Fig. 5A). This polyamine inhibited *Mp* 02 and 06 fungal growth up to 30.7% and 41.7%, respectively. Higher concentrations of Spd (50  $\mu\text{M}$ ) were necessary to reduce *Mp* 02 growth to 23.6% (Fig. 5B) while concentrations between 50 and 10  $\mu\text{M}$  inhibited *Mp* 06 to 27.6% (Fig. 5B). Although Spm antifungal activity was lower, the compound restricted *Mp* 02 and 06 growth up to 15.7%, and 15.2%, respectively, at 10  $\mu\text{M}$  concentration (Fig. 5C). By contrast, Cad and Tspm did not significantly inhibit the growth of *M. phaseolina* at the concentrations evaluated (Fig. 5D and E).

### 3.4. Characterization of plant growth promotion traits and CWDEs

Secretion of cell wall-degrading enzymes produced by PGPB can lead to the breakdown of the cell wall of phytopathogens resulting in cell death. Therefore, we tested the ability of the bacteria to secrete lytic enzymes. Our results demonstrated that *Exiguobacterium* sp. S56a and S58 were able to produce three important CWDEs:  $\beta$ -1,4-glucanase, cellulase, and chitinase (Table 1), while *Stenotrophomonas* sp. AG3 showed only chitinolytic activity (EI =  $2.1 \pm 0.3$ ). Among the other PGP properties evaluated, the extremophilic microorganisms showed no capacity to produce HCN and organic acids (Table 1).

### 3.5. Effect of extremophilic bacteria on the growth inhibition of germinated soybean seeds caused by *M. phaseolina*

There was a correlation between biocontrol activities displayed by extremophilic bacteria in PDA plates and their abilities to reduce the damage caused by *M. phaseolina* on germinating soybean seeds. The damage severity of *Mp* 02 on soybean seedlings (Fig. 6A) was significantly reduced by all treatments with exception of *Stenotrophomonas* sp.



**Fig. 4.** Bacterial polyamine production detected in cell pellet (CP) and cell-free culture filtrates (CFS) corresponding to exponential (A, C, and E) and stationary (B, D, and F) growth phases. Data represent the mean  $\pm$  standard error of three independent biological replicates. Values with same letters did not differ significantly according to Tukey's HSD test with  $p < 0.05$ . Lowercase letters on the bar compare each polyamine production detected in CP. Uppercase letters compare each bacterial polyamine secreted to the CFS. nd; no detection.

AG3 cell-free supernatants where the damage occasioned by the fungus was similar to non-inoculated controls (Fig. 6C). *Exiguobacterium* sp. S56a supernatants were the most effective in controlling disease severity of *Mp* 02 (Fig. 6C) and 06 compared to the others cell-free supernatants (Fig. 6A and B).

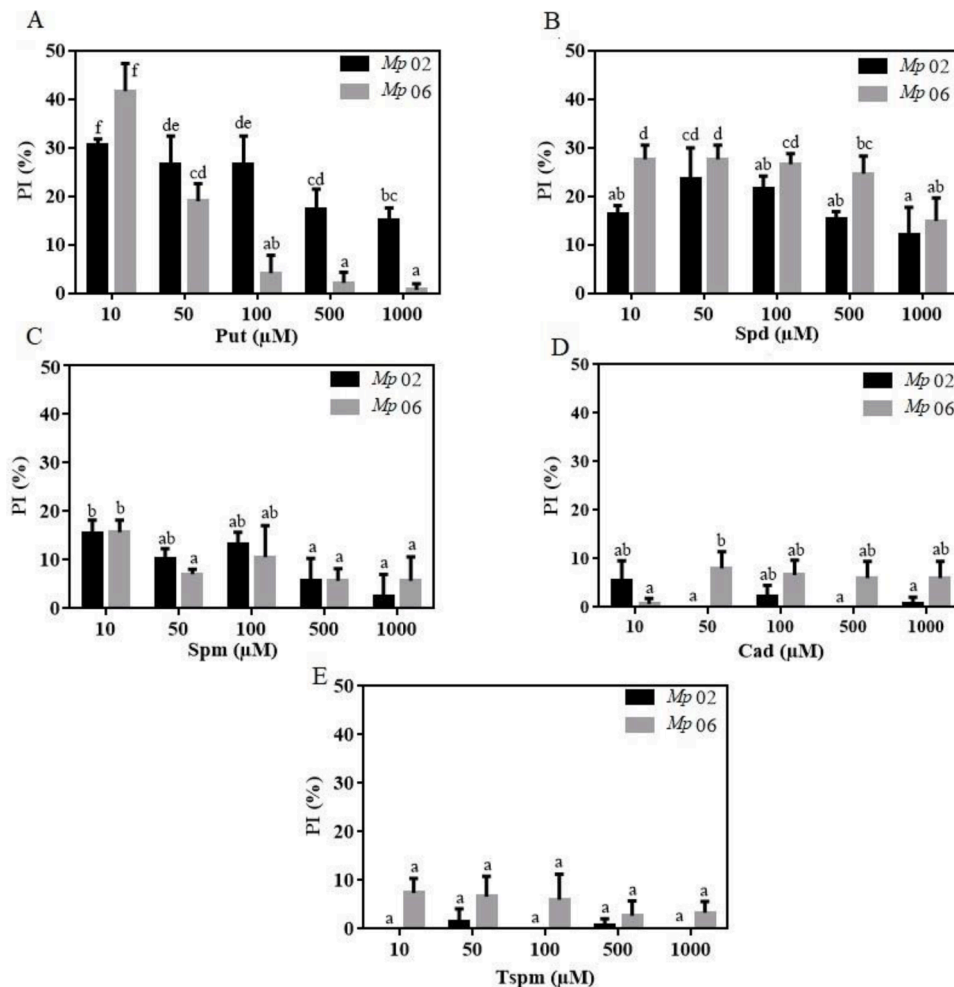
#### 4. Discussion

The adverse effects of fungicides on the environment and their continuous use against the same pathogen—leading to the development of resistant strains [29]—have motivated the search for safer and cost-effective alternatives that enhance phytopathogen control. The use of PGPB plays a crucial role in the biocontrol of plant diseases, and these bacteria are considered a promising alternative to fungicides as they are harmless to the environment. The present study demonstrates that bacterial strains of the genera *Exiguobacterium* and *Stenotrophomonas*, isolated from extreme environments (Andean soil), could be used as potential BACs to reduce the damage caused by *M. phaseolina* infections. Our research expands the BACs portfolio, since the most promising species hosting biocontrol agents for *M. phaseolina* have been reported to be *Trichoderma*, *Aspergillus*, *Penicillium*, *Bacillus* and *Pseudomonas* [30].

At present, little is known about the interaction between extremophilic microorganisms and *M. phaseolina*; an understanding of this interaction is crucial for the development of extremophiles as effective BACs. Our results showed that the antifungal effects might have been due to one or more antifungal compounds produced by bacteria. Cell wall-degrading enzymes seemed to be one of the mechanisms

responsible for *Exiguobacterium* sp. S58 and S56a antagonism, since protease [16], chitinase, glucanase, and cellulase activities were detected, and the damage caused by these bacteria to *M. phaseolina* hyphae provide further support. The microbial lytic enzymes have the potential to inhibit phytopathogens by hydrolyzing the fungal cell wall components [31]. They disrupt the fungal growth by their lytic action on cell walls, hyphal tips, and germ tubes [32] and partial swelling in the hyphae and at the hyphal tip, leading to hyphal curling or bursting of the hyphal tip [8]. Although *Stenotrophomonas* sp. AG3 showed only protease [16] and chitinase activities, our SEM analysis suggests that the action of these lytic enzymes could be responsible for the highly effective damage on *Mp* 02, by inducing their cell wall degradation. *Stenotrophomonas* sp. AG3 chitinolytic properties are considered strong for having a chitinolytic index above 2 [33].

Notably, the inhibitory effects of *Exiguobacterium* sp. S56a were independent of the *M. phaseolina* strains evaluated, since this extremophilic bacterium was able to affect, in different percentages, the growth of the two phytopathogen strains (*Mp* 02 and 06). As we described, the cell-free culture filtrates of *Exiguobacterium* sp. S56a showed antifungal properties able to protect from damage caused by *M. phaseolina* on germinated soybean seeds (Fig. 6). These properties may be related to the ability of the bacteria to synthesize and secrete to the supernatant polyamines, such as Put. Polyamines have been reported to stabilize or destabilize the DNA via binding interactions [11]. Particularly, a synthetic analogue of Put demonstrated antifungal and fungicidal properties against a plant pathogenic fungus (*Pyronophora avenae*) by decreasing the methylation level of the pathogen's DNA through



**Fig. 5.** The influence of exogenously applied polyamines at different concentrations on *Mp* 02 and 06 growth. Inhibition percentage caused by: (A) Put, (B) Spd, (C) Spm, (D) Cad, and (E) Tspm. The data represent the mean  $\pm$  standard deviations from three independent experiments. Values with different letters show significant differences ( $p < 0.05$ ) according to Tukey's HSD test.

**Table 1**

PGP properties and cell wall degrading enzymes of extremophilic bacteria.

Cell wall degrading enzymes (CWDEs)	Cell wall degrading enzymes (CWDEs)				
	$\beta$ -1,4-glucanase	Cellulase	Chitinase	Organic acid production	HCN Production
<i>Stenotrophomonas</i> sp. AG3	nd	nd	2.1 $\pm$ 0.3	nd	nd
<i>Exiguobacterium</i> sp. S56a	2.3 $\pm$ 0.3	1.3 $\pm$ 0.2	1.4 $\pm$ 0.2	nd	nd
<i>Exiguobacterium</i> sp. S58	1.7 $\pm$ 0.2	0.9 $\pm$ 0.1	1.9 $\pm$ 0.3	nd	nd

The values represent the EI averages  $\pm$  standard error of at least three independent experiments.

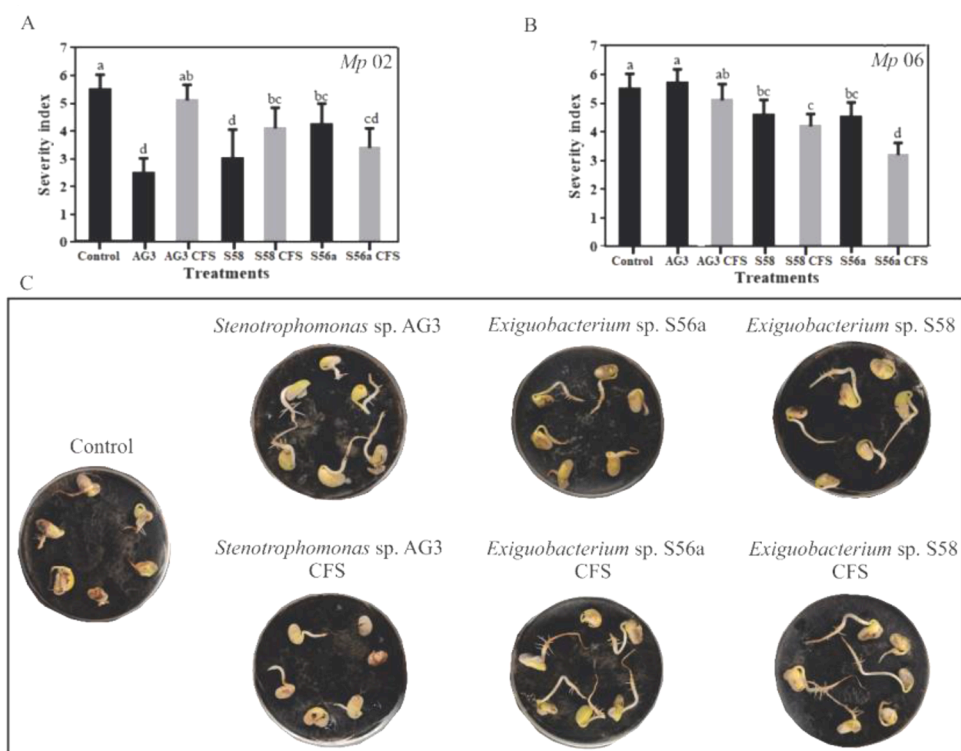
nd = no detection.

inhibition of cysteine DNA methylase [34]. Our experiments showed that exogenous application of Put at a low dosage (10  $\mu$ M) reduces the growth of two phytopathogens within the range of 30.7 and 41.7%. While applying higher doses of Put (50–1000  $\mu$ M), the inhibitory effect began to decrease (Fig. 5A). Therefore, Put secretion by *Exiguobacterium* sp. S56a (22.3  $\mu$ M) seems to be another strategy of the bacteria against *M. phaseolina*.

Previous studies also demonstrated that some Spd analogues reduced infection of barley, bean, and apple seedlings caused by a variety of powdery mildew fungi [35]. Although the role of Spd as an antifungal compound is poorly understood, recent research showed that Spd was required for bacterial antifungal activity and heat-stable antifungal factor (HSAF) production [14]. Our results also suggest that Spd participates in the *Exiguobacterium* sp. S58 supernatant antifungal activity. Spd was the major polyamine detected in *Exiguobacterium* sp. S58

cell-free culture filtrate and possibly the one responsible for the growth inhibition of *Mp* 02 and 06 fungi. This was evidenced by exogenous Spd application since the concentrations needed to restrict the growth of *Mp* 02 and *Mp* 06 (Fig. 5B) were within the range of those produced by *Exiguobacterium* sp. S58 (134.1  $\mu$ M). Although *Stenotrophomonas* sp. AG3 also synthesizes and secretes Spd to the medium, its concentrations were not high enough (8.2  $\mu$ M) to produce a noticeable inhibitory effect in *Mp* 02 and *Mp* 06 growth (Fig. 1D).

The results suggest that the combined effects of different metabolites secreted through the growth phases of *Stenotrophomonas* sp. AG3 and *Exiguobacterium* sp. S58 could predispose the bacteria to gain a competitive advantage as antagonist. This was evidenced in dual-culture assays where the cell cultures showed a greater inhibition effect than the supernatant, possibly due to the ability of the bacteria to keep on synthesizing lytic enzymes and polyamines. The cell wall-degrading



**Fig. 6.** *In vitro* biocontrol effects against *M. phaseolina* in germinating soybean seeds. Severity index of (A) *Mp 02*, and (B) *Mp 06* in 5-day-old soybean roots treated with bacteria and cell-free supernatants (CFS). Data represent the mean  $\pm$  standard error ( $n = 3$ ). Different letters indicate significant differences among treatments according to Tukey's HSD test ( $p < 0.05$ ). (C) Different treatment of soybean seeds germinated in plates in the presence of a 7-day-old colony of *Mp 02* on PDA medium.

enzymes produced by *Stenotrophomonas* sp. AG3 and *Exiguobacterium* sp. S58 were able to permeabilize and degrade the fungal cell wall, and the greater permeability of the cell wall possibly facilitated the entry of antifungal compounds, such as polyamines. *Trichoderma* spp. developed a similar and efficient mechanism of action to kill their fungal prey by applying their lytic enzyme secretion abilities, which allowed the entry of the toxic metabolites that cause the infection of fungal structures [36]. Furthermore, the fungal antagonistic effects of *Stenotrophomonas* sp. AG3 and *Exiguobacterium* sp. S58 were confirmed *in planta* when the cell cultures significantly reduced the disease severity of *Mp 02* on soybean seedlings (Fig. 6A and F).

## 5. Conclusion

The results show that extremophilic microorganisms from Andean soil displayed useful characteristics as potential biocontrol agents against *M. phaseolina*, which are manifested by marked morphological distortions on fungal hyphae. Our studies expand the understanding of the molecular mechanisms of barely explored genera, such as *Stenotrophomonas* sp. and *Exiguobacterium*, in biocontrol of soybean charcoal root rot disease. These findings promote future research aimed at investigating the use of extremophilic microorganisms in crop management practices in order to provide an efficient alternative to control *M. phaseolina* infections.

## CRediT authorship contribution statement

**Ana P. Santos:** Methodology, Investigation, Conceptualization, Formal analysis. **Luciana Nieva Muratore:** Methodology, Investigation. **Anna Solé-Gil:** Methodology, Investigation. **María E. Farías:** Resources, Supervision. **Alejandro Ferrando:** Conceptualization, Formal analysis, Resources, Supervision. **Miguel A. Blázquez:** Conceptualization, Formal analysis, Resources, Supervision. **Carolina Belfiore:** Investigation, Conceptualization, Formal analysis, Resources, Supervision.

## Declaration Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.btre.2021.e00674](https://doi.org/10.1016/j.btre.2021.e00674).

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