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Article

Growth promotion function of Bacillus sp. strains isolated from salt-pan rhizosphere and their biocontrol potential against *Macrophomina phaseolina*

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Abstract: In the last decades, intensive crop management has involved excessive use of pesticides or fertilizers, compromising environmental integrity and public health. Accordingly, there has been worldwide pressure to find an eco-friendly and safe strategy to ensure agricultural productivity. Recently, Plant Growth-Promoting (PGP) rhizobacteria are receiving increasing attention as suitable biocontrol agents against agricultural pests. In the present study, 22 spore-forming bacteria were selected among a salt-pan rhizobacteria collection for their PGP traits and their antagonistic activity against the plant pathogen fungus *Macrophomina phaseolina*. Based on the higher antifungal activity, strain RHFS10, identified as *Bacillus vallismortis*, was furtherly examined and cell-free supernatants assays, column purification, and tandem mass spectrometry employed to purify and preliminarily identify the antifungal metabolites. Interestingly, the minimum inhibitory concentration assessed for the fractions active against *M. phaseolina*, resulted 10 times lower and more stable than the one estimated for the commercial fungicide pentachloronitrobenzene. These results suggest the use of *B. vallismortis* strain RHFS10 as a potential Plant Growth Promoting Rhizobacteria to efficiently control phytopathogenic fungus *M. phaseolina*, in alternative to chemical pesticides.

Keywords: plant-growth-promoting bacteria, spore-forming bacteria, *Bacillus vallismortis*, *Macrophomina phaseolina*, phenotypic and genotypic characterization, Biocontrol agents

1. Introduction

In the last century, the increase in world population was three times greater than during the whole history of humanity. To cope with the rising request for nutrients, like wheat and rice, current agricultural practices are based on the wide use of chemical fertilizer and pesticides. As result, agrochemical multinationals have gradually acquired the control of global food production and push to agriculture increasingly diverging from the traditional one [1]. Besides, the extensive use of synthetic agrochemicals has generated heavy environmental pollution and serious risk for human and animal health due to their translocation along the food chain [1, 2]. The massive use of pesticides has also led to a gradual loss of protection efficiency, due to new resistances acquired by pests, with a continuous increase of the pesticide dosage [2, 3]. A sustainable and safe strategy to ensure crop production is to substitute agrochemicals with Plant Growth-Promoting

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Rhizobacteria (PGPR), as agents stimulating plant growth and health [3, 4, 5]. These beneficial microbes not only play an important role in increasing soil fertility but also enhance the growth and vigour of the plants: PGPRs, colonizing the roots, may enhance nutrient uptake by nitrogen fixation or P solubilization [4], reduce abiotic stresses by biofilm production [5] or regulate plant hormone production [4]. Emerging evidence has shown that rich microflora of the rhizosphere can reduce plant disease through several antagonistic mechanisms as competition, the production of cell wall-degrading enzymes, (e.g. chitinase, glucanase, and protease) [6], volatile compounds and siderophores [7], antibiosis or the induction of plants' systemic resistance [8]. Replacing agrochemicals with the application of PGPRs may have a wide both economic and environmental impact, including relevant benefits as rising yields, reduction or elimination of chemical residues, limited or no development of resistance by pests and pathogens, employment of agricultural raw materials, and a low risk to non-target organisms, including pollinators. For this reason, intensive research on this group of microorganisms has been taking over, to develop new biofertilizers and biocontrol agents.
In this contest, *Bacillus* genera include several endophytic bacteria species and plant growth promoting (PGP) features have been associated with different strains [9, 10]. In addition to the benefits shared with other PGPR, *Bacillus* spp. are suitable as biofertilizer and/or bioagent because: i) their application has little, if any, effect on the composition of the soil microbial communities, being common members of the plant root microflora [11]; ii) they produce a wide range of biologically active secondary metabolites able to inhibit the growth of plant pathogens [12]; iii) these bacteria may form endospores, which can survive to high temperature and dehydration, making easier the formulation of a commercial product [13].

This manuscript describes the screening of 22 Bacillus strains isolated from samples of the rhizosphere of Juniperus sabina collected from the National Park of Ses Salines d'Eivissa, Formentera (Spain), focused to find a PGPR strain with antagonistic activity against phytopathogenic fungus *Macrophamina phaseolina*. *M. phaseolina* (Tassi) Goid is one of the most important fungal plant pathogens infecting more than 500 cultivated and wild plant species, causing charcoal rot and surviving for up to 15 years in the soil as a saprophyte [14]. The fungus induces heavy damages in agrarian plants with a high world market value, like soy, sunflower, leguminous, and corn [14]. Soybean grains, in particular, are globally utilized not only as foods but also as a substrate for feeds, fuels, and bio-based materials [15]. Thus, many efforts are made for the control of *M. phaseolina* to reduce or avoid the loss of agricultural yields and the consequent economic damage.

2. Results

2.1. Isolation and screening of Plant Growth Promoting spore-forming Rhizobacteria

Aerobic spore-forming bacteria were isolated from rhizosphere samples of *Juniperus sabina* collected in Parque Natural de Ses Salines d'Eivissa, Formentera (Spain), as described in the Material and Methods section. After a preliminary characterization based on morphology and growth properties, 22 isolates were selected (Table 1).

Table1. Preliminary characterization of spore-forming bacteria isolated from the rhizosphere of *Juniperus sabina* plants.

Isolate code	Colony colour	Colony morphology	*Anaerobic growth	pH range	Temperature range (°C)
RHFS01	Creamy-white	Rhizoid	++	4-12	15-50
RHFS02	Creamy-white	Irregular	++	4-12	25-50
RHFS03	Creamy-white	Rhizoid	+++	4-12	15-60
RHFS04	Creamy-white	Punctiform	+++	4-12	15-60
RHFS05	Creamy-white	Lobate	+	4-12	15-50
RHFS06	Creamy-white	Irregular	+++	6-12	15-60

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RHFS07	Brown	Circular	+++	4-12	15-60
RHFS08	Creamy-white	Filamentous	+	4-12	15-50
RHFS09	Creamy-white	Irregular	+++	2-12	15-50
RHFS10	White	Undulate	++	6-12	15-50
RHFS11	Creamy-white	Irregular	+++	4-12	15-60
RHFS12	Creamy-white	Circular	+++	4-12	15-60
RHFS13	Creamy-white	Circular	+++	2-12	15-60
RHFS14	Creamy-white	Circular	+	4-12	15-50
RHFS15	Creamy-white	Irregular	+++	4-12	15-60
RHFS16	Orange	Irregular	++	4-12	15-60
RHFS17	Translucent	Lobate	+++	4-12	15-50
RHFS18	Milky white	Filamentous	++	4-12	15-60
RHFS19	Milky white	Irregular	+++	4-12	15-60
RHFS20	Creamy-white	Undulate	++	4-12	15-40
RHFS22	Yellow	Translucent	+++	4-12	15-40
RHFS28	Creamy-white	Circular	++	4-12	40

*Anaerobic growth: +:low growth; ++:moderately growth ; +++:high growth

Analysis of the DNA sequence of the 16S RNA gene of the 22 strains allowed to identify all of them as belonging to the *Bacillus* genus (Table 2). A phylogenetic analysis performed by comparing the 16S sequences with those of reference bacteria is reported in Figure 1 and supports the preliminary BlastN analysis (Table 2) with a bootstrap value >0.90. All isolates belong to species commonly considered as plant growth-promoting rhizobacteria (PGPR) for their ability to colonize roots [11, 15] and produce antimicrobial molecules [12, 16].

Table 2. Bacteria identification using 16S rRNA gene sequences (1.5Kb). The details of strain identification and accession numbers are reported.

Strains code	rganism lentified	Closest type strain in e	Accession No.	(%)Sequence similarity
RHFS01	*Bacillus*	*B.licheniformis strain QT-98*	MT065812.1	98.96
RHFS02	*Bacillus*	*B.mojavensis strain YZJP308*	MN931392.1	100.00
RHFS03	*Bacillus*	*B.paralicheniformis strain B34-013*	MK063845.1	100.00
RHFS04	*Bacillus*	*B.subtilis subsp. inaquosorum strain YZYR10*	MN931257.1	100.00
RHFS05	*Bacillus*	*B.velezensis strain EMP09*	MN062933.1	100.00
RHFS06	*Bacillus*	*B.halotolerans strain FUM1*	MK093005.1	100.00
RHFS07	*Bacillus*	*B.flexus strain S6c*	MT645459.1	99.65
RHFS08	*Bacillus*	*B.licheniformis SV12*	LC422787.1	99.89
RHFS09	*Bacillus*	*B.velezensis strain XC1*	MT649755.1	99.85
RHFS10	*Bacillus*	*B.vallismortis strain CBs8*	MK290421.1	100.00
RHFS11	*Bacillus*	*B.velezensis strain GST21*	MN809529.1	100.00
RHFS12	*Bacillus*	*B.subtilis strain HR02*	MK283755.1	100.00
RHFS13	*Bacillus*	*B.tequilensis strain R-QL-48-26*	MT078639.1	100.00
RHFS14	*Bacillus*	*B.subtilis strain R47*	MH359177.1	90.19
RHFS15	*Bacillus*	*B.velezensis strain 1601*	MW242869.1	100.00

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RHFS16	*Bacillus*	*B.toyonensis strain IBB-TEB3*	MT573517.1	100.00
RHFS17	*Bacillus*	*B.amyloliquefaciens strain NO10*	MT377854.1	100.00
RHFS18	*Bacillus*	*B.amyloliquefaciens strain S8TS*	MK729078.1	100.00
RHFS19	*Bacillus*	*B.paralicheniformis strain AJVR1*	MT459810.1	100.00
RHFS20	*Bacillus*	*B.proteoliticus strain 1372*	MT573794.1	100.00
RHFS22	*Bacillus*	*B.tequilensis strain CFR01*	MT641220.1	100.00
RHFS28	*Bacillus*	*B.cereus strain F3-1-38*	KX350019.1	100.00

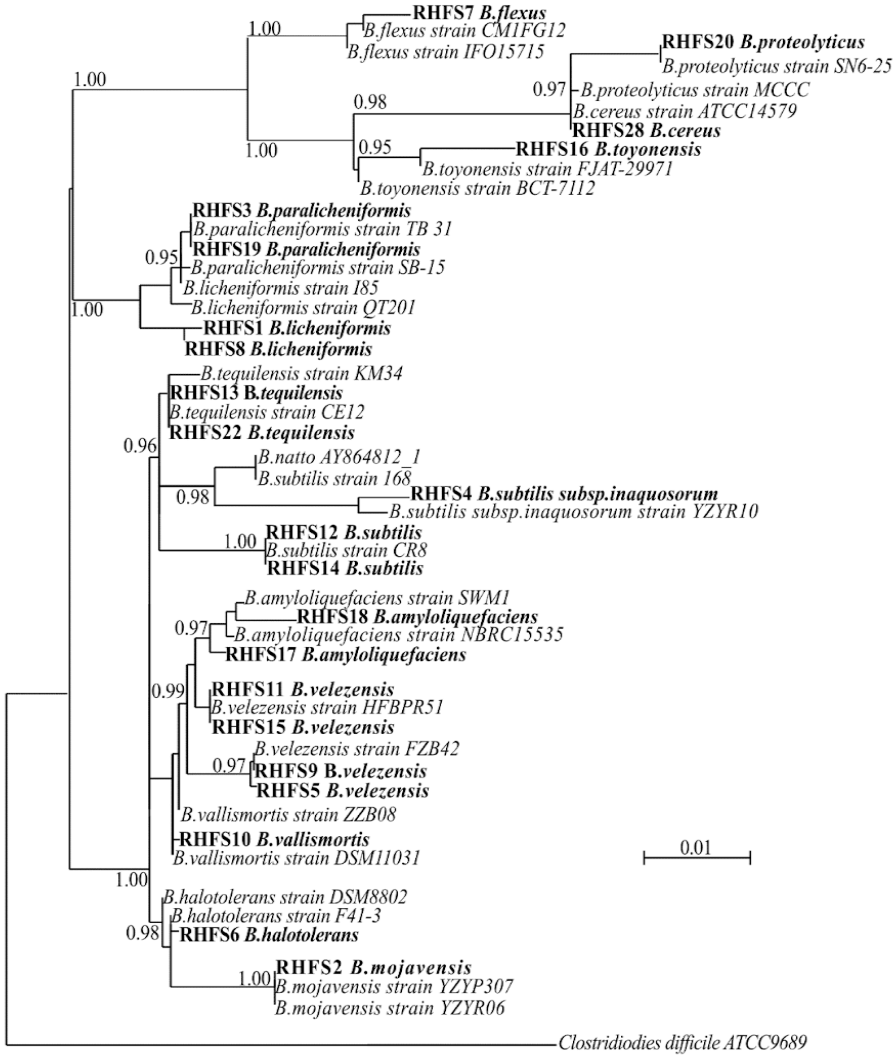

FIGURE 1. Phylogenetic tree of isolated rhizobacteria. The phylogenetic tree was constructed using the Maximum-likelihood algorithm based on 16S rRNA gene sequences. The gene sequences of the isolated bacteria were aligned to reference bacteria belonging to the same species obtained from BlastN analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches.

The selected strains were analyzed for PGP traits, by testing the presence of both fertilizing and biocontrol features. As summarized in Table 3, a high proportion was able

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to solubilize phosphate (Supplementary Figure S1), produce siderophores (Supplementary Figure S2) and efficiently scavenge H_2O_2, while only some of the strains were biosurfactant and biofilm producers and able of swarming motility.

Table 3 Summary of plant growth-promoting activities exhibited by 22 isolated rhizobacteria. +++: strong activity (formation halo ≥ 10 mm); ++: moderate activity (5mm < halo < 10 mm); +: slight activity (halo < 5mm); —: no activity.

	PLANT GROWTH PROMOTING ACTIVITIES					
Strains code	**Siderophores production**	**Phosphate solubilization**	**Biosurfactant production**	**Catalase activity**	**Biofilm**	**Swarming**
RHFS1	+	-	-	+++	-	-
RHFS2	-	++	-	-	+	+++
RHFS3	-	+	+	-	-	-
RHFS4	-	+	+++	+++	-	-
RHFS5	+	-	-	++	-	-
RHFS6	-	+	-	+++	-	-
RHFS7	-	-	-	+++	-	-
RHFS8	++	+	-	+++	-	-
RHFS9	+	-	-	++	+	+++
RHFS10	+++	++	-	+++	++	+++
RHFS11	+	+	-	++	-	-
RHFS12	-	+	-	+	+	-
RHFS13	-	-	+	++	-	-
RHFS14	-	++	+	+++	-	-
RHFS15	+	+	-	-	-	-
RHFS16	+	+	-	-	+	-
RHFS17	+	+	+	+++	-	-
RHFS18	+++	++	-	+++	+++	++
RHFS19	++	++	-	+++	+++	++
RHFS20	+	-	-	+++	-	-
RHFS22	+	+	-	-	-	++
RHFS28	-	-	-	++	-	-

Then, the potentiality as biocontrol agents of the 22 strains was tested analyzing their ability to secrete lytic enzymes [17] (Supplementary Figure S3). As shown in Table 4, the number of proteases- and xylanase- producers was the highest (over 90%) followed by amylase-, chitinase- and cellulase- producers (over 80%), whereas less than 50% were lipase-producers (45%).

Table 4. Summary hydrolytic activities exhibited by 22 rhizobacteria isolated.

	HYDROLYTIC ENZYME ACTIVITIES

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Strains code	Protease activity	Amylase activity	Lipase activity	Xylanase activity	Cellulase activity	Chitinase activity
RHFS1	+++	+++	+	++	++	+
RHFS2	+++	+++	+	++	+++	++
RHFS3	+++	+++	++	+	+++	-
RHFS4	+++	+++	-	+++	+++	++
RHFS5	+++	+++	-	+	+	++
RHFS6	+++	+++	-	+++	++	-
RHFS7	+	-	+	+++	+++	++
RHFS8	+++	+++	-	++	+	++
RHFS9	+++	+++	++	-	+++	++
RHFS10	+++	+++	++	+++	+++	++
RHFS11	+++	+++	+	+++	-	++
RHFS12	-	+++	-	+++	+++	++
RHFS13	+++	-	-	++	-	++
RHFS14	+	+	-	+++	-	+
RHFS15	+++	+++	+	++	+++	++
RHFS16	++	++	-	+++	+++	-
RHFS17	+++	++	-	+	+	++
RHFS18	+++	+++	++	+++	+++	++
RHFS19	+++	+++	++	+++	+++	+
RHFS20	+	++	-	++	++	++
RHFS22	+++	-	-	+	++	++
RHFS28	+++	+++	-	++	++	++

+++: strong activity (formation halo ≥ 10 mm); ++: moderate activity (5mm < halo < 10 mm); +: slight activity (halo < 5mm); —: no activity

2.2 Antagonistic Activity of spore-forming isolates against Fungal Plant Pathogen

The antagonistic activity of the 22 strains was examined against the phytopathogen *Macrophomina phaseolina* by dual-culture assay (Figure 2A).

Based on the size of the inhibition zone in dual culture tests, some strains were found to be highly efficient against the fungal pathogen while others had a limited or no antimicrobial activity (Figure 2b). For a more detailed analysis, the produced inhibition halos were observed under a stereomicroscope highlighting an agar-diffusible antifungal molecule production by the most active strains (Figure 2A, panel 4; Supplementary figure S4).

Of all analyzed isolates, RHF10 and RHF18 proved the higher potentiality as PGPR, since possessing traits beneficial for both plant growth, as the ability to phosphorus solubilization or siderophore production, and antagonistic ability against phytopathogen. For these reasons, both strains were selected for further experiments. Strain RHFS28, able

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to produce lytic enzymes but not showing antifungal activity, was selected as a negative control for the next experiments.

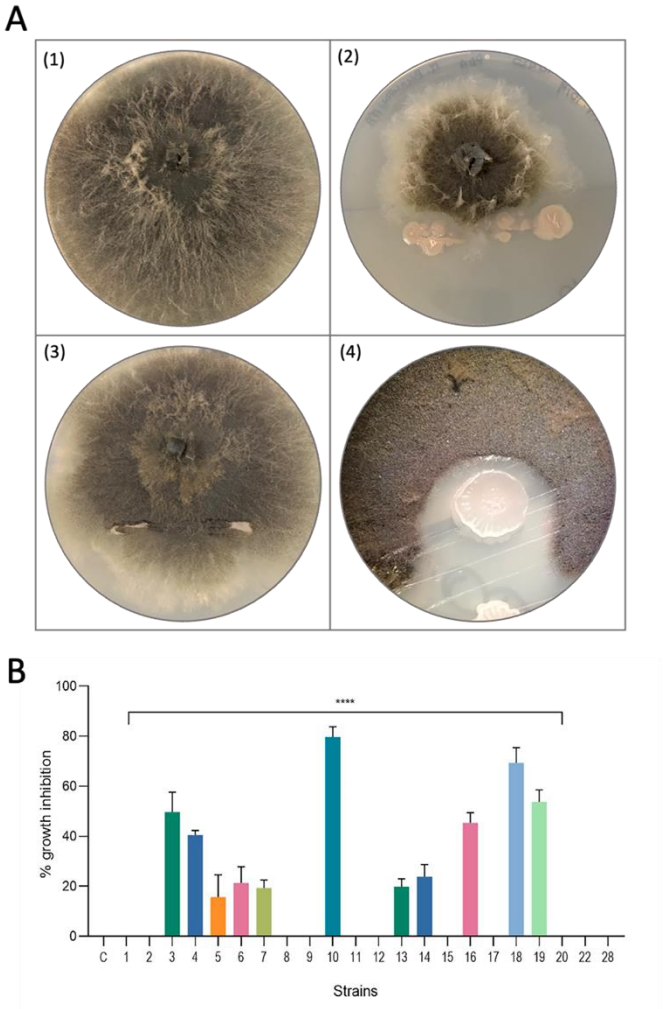

Figure 2. Antagonism assays in solid medium. **(A)** Representative photographs of dual culture assay for in vitro inhibition of mycelial growth of *Macrophomina phaseolina* by isolated strains**.** 1) *M.phaseolina* (control plate); 2) example of active strain (RHF10) against *M.phaseolina* growth; 3) example of inactive strain (RHF28) against *M.phaseolina* growth; 4) Images of interaction zone of RHFS10 strain and *M.phaseolina* acquired with a stereoscopic microscope (10x magnification). **(B**) Inhibition of fungal growth reported as the percentage reduction of the diameter of the fungal mycelia in the treated plate compared to that in the control plate. All experiments were performed in triplicate with three independent trials. Data are presented as means ± standard deviation (n = 4) compared to control *M.phaseolina* grown without bacteria. For comparative analysis of groups of data one-way ANOVA was used and *p values* are presented in the figure: ****: extremely significant <0.0001.

To assess the effect of the cell-free culture supernatants (CFSs) of RHF10 and RHF18 on mycelial growth, the CFSs at 24h, 48h, 72h and 96h were collected and tested against M. phaseolina. CFSs of strain RHFS28 and the commercial fungicide pentachloronitrobenzene (PCNB) were used as a negative and positive control of the experiments, respectively (Figure 3A). The antifungal activity increased proportionally

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with the growth time reaching a maximum after 72 hours, indicating the antifungal molecule as a secondary metabolite (Figure 3B). Based on the efficiency of inhibition, measured by the percentage of mycelial growth reduction, strain RHFS10 was chosen for further investigation.

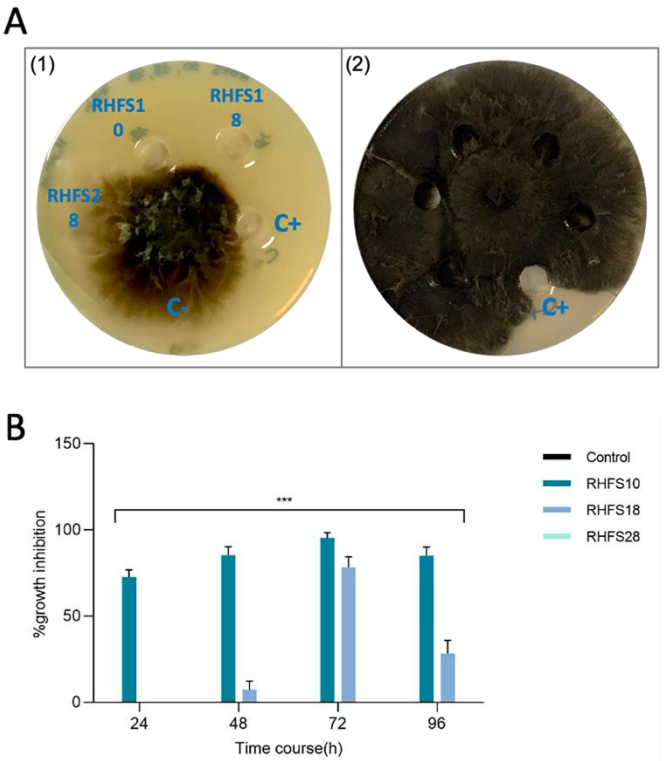

Figure 3. Antifungal activity of secreted metabolites by PGPR strains. (**A**) Effects of the CSFs from RHF10, RHFS18 and RHFS28 strains collected after 72h of growth on the mycelial growth of *M. phaseolina* (panel 1). C+: Positive control, Pentachloronitrobenzene; C-: Negative control, Toluene. All experiments were performed in triplicate with three independent trials. (**B**) Antifungal activity of the CFSs of the three strains collected from 24 to 96 hours of growth. Percentage of fungal growth inhibition was reported as the percentage reduction of the diameter of the fungal mycelia compared to control plate (panel A-2). Data are presented as means ± standard deviation (n = 3). For comparative analysis of groups of data one-way ANOVA was used and *p values* are presented in the figure: ***: extremely significant <0.001.

2.3 Characterization of antifungal metabolites

The stability of the antifungal metabolites secreted by RHFS10 was tested by incubating the CFS collected after 72 hours (72-CFSs) with different proteolytic enzymes or organic solvents and then testing for inhibition of mycelial growth.

As shown in Table 4, the 72-CFS still has a notable activity after incubation with lipase or with organic solvents but decreased under the action of Proteinase K or Pepsin.

Thermo-stability was verified incubating the 72-CFS at increasing temperatures for 1 or 3 hours. The results showed that treatments at 65 and 75°C do not affect the inhibitory effect against *M. phaseolina*, while at 85°C a reduction of the antifungal activity was observed (Figure 4).

Finally, metabolites of the 72-CFSs were extracted with Ethyl acetate at pH 2.0 and pH 7.0 and the two obtained phases were separated and tested against M.phaseolina. The results showed that the antifungal activity was mainly associated with the aqueous phase

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at pH 7.0 (data not shown). This data indicated a protein nature of the bioactive molecules in agreement with the protease sensitivity recorded in the previous tests.

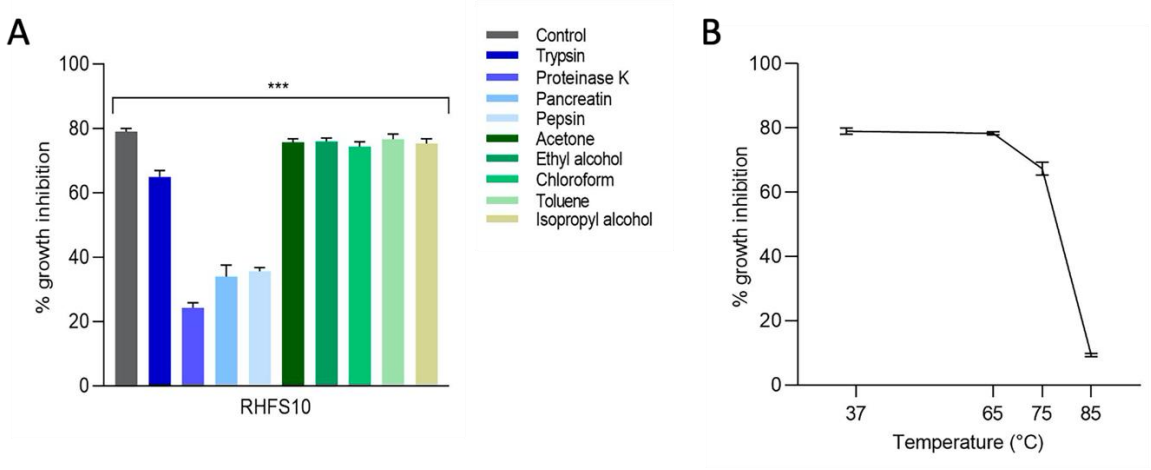

Figure 4. Stability of secreted antifungal metabolites. 72-CFS of RHFS10 was treated separately, with different enzymes and organic solvents (A) or incubated at increasing temperatures (37°C, 65°C, 75°C, and 85°C) (B) and tested against *M.phaseolina.* All data represent the average of three separate experiments. ANOVA statistical analysis is extremely significant indicated ***p<0.001.

2.4.1. Purification of antifungal metabolites

To preliminary identify the antifungal compounds released by the RHFS10 strain, 72-CFS was subjected to purification by two different-steps. First, the 72-CFS was fractionated and the obtained fractions were tested against *M.phaseolina*. As shown in Figure 5A, the antifungal activity was observed in the fraction containing compounds with a molecular weight between 10 and 50 kDa. In the second step of purification, the polypeptides present in 72-CFS were collected with ammonium sulfate, dialyzed to eliminate the polypeptides with a molecular weight lower than 10 kDa, and subjected to column chromatography. The three obtained fractions were tested against M.phaseolina and the peaks 1 and 2 showed a wide zone of inhibition while no antagonistic activity was detected for the metabolites recovered in the peak 3 (Figure 5B).

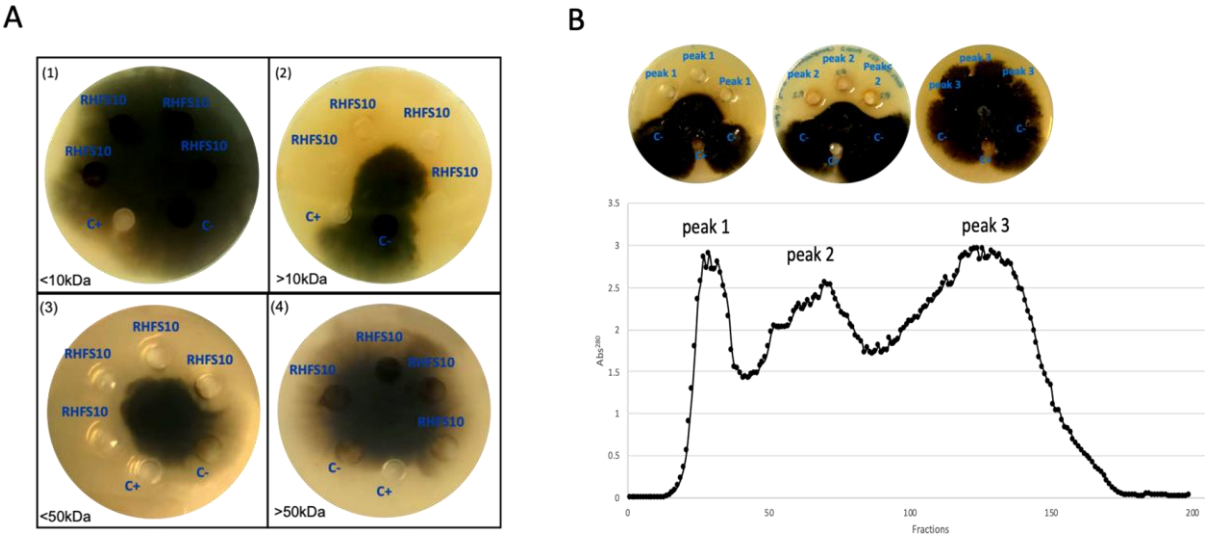

Figure 5. Antifungal activity of Cell-free supernatant fractions of RHFS10. (A) 72-CFS was size-fractionated using 10-kDa, 30-kDa and, 50-kDa cutoff spin columns, and the obtained fractions tested against *M.phaseolina*. The results obtained with fractions <10kDa

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(1), >10kDa(2), <50kDa (3) and >50kDa(4) are reported. C+: Positive control, Pentachloronitrobenzene; C-: Negative control, Toluene; RHFS10: 0.1 ml of fractionated 72-CFS. (B) Elution profile of 72-CFS after Sephadex G-50 fine column chromatography. The antagonist activity of the three recovered peaks (1mg/dot) is reported in the upper part of the panel. All data represent the average of three separate experiments. ANOVA statistical analysis is extremely significant indicated ***p<0.001.

2.4.2 Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) of the antifungal compounds present in peaks 1 and 2 was determined, incubating decreasing concentrations of peaks 1 and 2 (Figure 6 A-B) with *M.phaseolina* plugs. The antifungal efficiency of the compounds present in the peaks was compared to the commercial fungicide PCNB (Figure 6D). The results obtained after 5 days of incubation, clearly showed higher antifungal activity of peaks 1 and 2 than the fungicide PCNB. In particular, the deduced MIC for both peaks was 50µg/mL, 10 times less than that deduced for PCNB (0.5mg/mL). We also compared the stability of the antifungal activity over time. In this regard, the bioactive compounds present in peaks 1 and 2 perfectly retained their fungal growth inhibition up to 14 days, while PNCB's efficiency decreased after a week. Peak 3 confirmed its inactivity (Figure 6C).

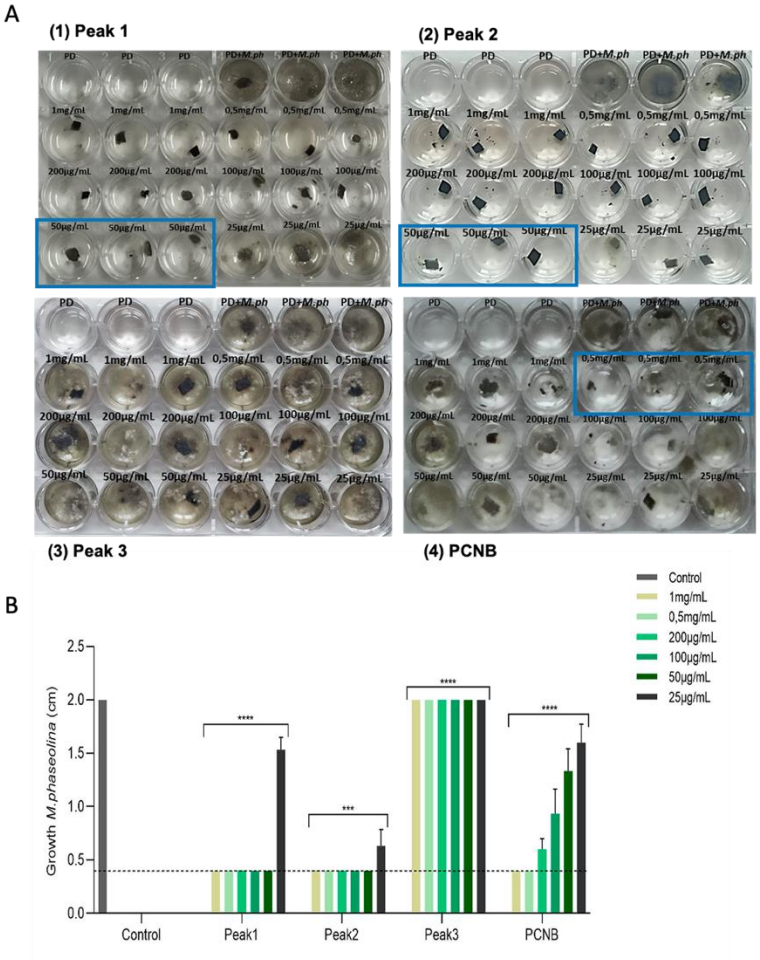

Figure 7. Minimum Inhibitory Concentrations of purified fractions of 72-CFS on fungal growth. (A) MIC of the antifungal compound present in purified fractions of 72-CFS using

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a 24-well plate assay. The commercial fungicide PCNB (panela-4) was used as a reference. The tested concentrations are indicated. Fungal plugs incubated with only PD broth (PD+ *M.ph*) and the PD alone (PD) were used as a control. The blu lines represent the MIC of the tested samples. (B) Graphical representation of the MIC assay. The dotted line indicates the starting size (mm) of *M.phaseolina* plug (4x4mm) at the beginning of the experiment. The results were obtained after 5 days of incubation at 28°C. Data are presented as means ± standard deviation (n = 3 replication for each different concentration). ANOVA statistical analysis is extremely significant indicated ****p<0.0001 and ***p<0.001.

2.4.3 Preliminary identification of bioactive compound

Finally, the three fractions were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC_MS/MS). As shown in Table 5, several protease and lytic enzymes were identified in the two antifungal active peaks. Two different forms of subtilisin-like protein were identified in peak 1, showing an apparent molecular weight of 39 kDa and 28 KDa and corresponding to the mature serine-protease and the pro-enzyme, respectively. Besides, the glucuronoxylanase XynC was also detected. Both subtilisin-like protein forms were also present in peak 2, even if with a lower concentration, together with a B-glucanase, whereas peak 3 contains a metalloprotease and an alpha-amylase. Since serine-proteases, Beta-glucanase and glucoronoxylanase were demonstrated to act as antifungal agents [18,19], our results suggested that the activity of these secreted molecules could be responsible, at least partially, of the antifungal action of RHFS10. To further corroborate this hypothesis, a mass spectrometry-based proteomic analysis on the previously described 72-CFSs of RHFS10 strain treated at increasing temperatures (cfr. 3.4), was performed. Again, the two forms of subtilisin and glucuronoxylanase XynC were identified in the samples retaining the antifungal activity. Interestingly, the two proteins were not detected in CFS from negative control (RHF28), subjected to the same treatment. Although the genome of RHFS10 was in permanent draft stage (SAMN17389611), it allowed us to confirm the presence of the all purified protein genes, which when expressed, could be involved in inhibiting fungal growth.

Table5. The proteins identified on the three peaks are listed with their AC and molecular weight .

Fractions	Mass (Da)[a]	SwissProt AC	significant sequences	score	Description
Peak 1	47.924	XYNC_BACIU	18	1776	Glucuronoxylanase XynC OS=Bacillus subtilis
	39.483	SUBN_BACNA	5	1080	Subtilisin NAT OS=Bacillus subtilis subsp. Natto
	27.42	SUBN_BACNA	5	865	Subtilisin NAT OS=Bacillus subtilis subsp. Natto
	75.961	SACC_BACSU	1	795	Levanase OS=Bacillus subtilis
	38.141	PEL2_BACIU	3	566	Pectin lyase OS=Bacillus subtilis
Peak2	27.365	GUB_BACAM	8	990	Beta-glucanase OS=Bacillus amyloliquefaciens
	39.483	SUBN_BACNA	5	800	Subtilisin NAT OS=Bacillus subtilis subsp. Natto
	27.42	SUBN_BACNA	5	637	Subtilisin NAT OS=Bacillus subtilis subsp. Natto
Peak 3	72.39	AMY_BACSU	1	41	Alpha-amylase OS=Bacillus subtilis

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34.106	MPR_BACSU	1	39	Extracellular metalloprotease OS=Bacillus subtilis

[a] Molecular Mass of the Swiss Prot sequence in the absence of molecule processing

3. Discussion

Fungal pathogens represent one of the most common causes of plant disease and are responsible for losing a third of crops annually [20], causing economic loss and impacting global poverty. To limit the losses in crop yield and to satisfy the increasing demand for food, chemical pesticides have been used recklessly in the last decades with a negative impact on the environment. Recently, novel eco-friendly strategies are being developed to limit plant diseases and stimulate plant growth. In this regard, the employment of PGPR has been taking over. These microorganisms can colonize the rhizosphere improving plant growth directly by either enhancing nutrient uptake or regulating plant hormone production, or indirectly by suppressing a broad spectrum of phytopathogens, including bacteria and fungi, thus regulating the microbial community structure of the rhizosphere.

The focus of our research was to identify promising Bacilli rhizobacteria acting as biofertilizer and biocontrol agents. Bacillus species are a major type of rhizobacteria able to be beneficial to plants and to perform the same role as chemical fertilizers [21] and pesticides [22]. As PGPR, Bacilli spp act both by direct and indirect mechanisms, secreting phytohormones, antioxidants, solubilizing soil P, enhancing nitrogen fixation, or producing cell-wall-degrading enzymes and siderophores that promote plant growth and suppress the pathogens.

Besides, some Bacillus species may also function as plant endophytes [23]. Finally, the ability of the Bacillus spp. of producing endospores, a quiescent cell-forms able to resist harsh environmental conditions and several stresses, makes them more suitable candidates for PGPR-based commercial products since i) the resistance features of the spores can ensure the persistence of the bacteria in the environment for a long-period; ii) spores are more stable than vegetative cells during processing and storage of commercial preparations [24].

To this aim, spore-forming bacteria were isolated from salt-pan rhizosphere (Formentera, Spain), and 22 identified strains were screened for their plant growth-promoting traits and biocontrol activity against the fungus Macrophomina phaseolina, one of the most devastating phytopathogens causing huge economic losses in many crops, such as soy plants [25].

Among these, strain RHFS10, identified as Bacillus vallismortis, showed the best performance for plant growth-promoting applications both as bio-fertilizer and biocontrol agent. The fungal growth inhibition revealed in the cell-free supernatant assay suggested the secretion of antifungal extracellular metabolites not induced by the direct contact with the fungus. This data was in agreement with the stereoscopic observation of co-cultures experiments. Besides, the antagonist activity of RHF10 was not influenced by the bacterial growth stage, suggesting a constitutive production of the antimicrobial molecules.

Stability experiments revealed a thermostability of the antifungal molecules up to 75°C and resistance to various organic solvents. Instead, the sensitivity to protease treatment as well as the association of the antifungal activity to the aqueous phase during the extraction suggested a proteinaceous nature of the metabolites.

Purification experiments have associated the antifungal activity to metabolites with a molecular weight between 20KDa and 50 kDa while LC_MS/MS analysis revealed the presence of proteases and hydrolytic enzymes in the active fractions. In particular a

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glucuronoxylanase of 45kDa and a homologous of the serine protease Subtilisin NAT from B.subtilis subsp. natto that could be directly implicated in the fungal growth inhibition. Both proteins were absent in the inactive peak, confirming their involvement in the observed antifungal activity.

There are, indeed, several functions ascribed to the release of these compounds during the stationary phase of growth. It is well known, that during this very phase of their life cycle, bacteria generally release hydrolytic enzymes mainly involved in the cell wall turnover and nutritional functions, which in many cases show an antimicrobial and/or antibiofilm activity [26]. Moreover, it has been lately reported that subtilisin-like proteases and glucuronoxylanases can digest fungal cell wall structural proteins [27](Yia & Qian, 2008, 33), supporting our preliminary results. Recently, it has been shown that B. subtilis natto can use several fungal materials as a carbon source for growth, pointing out the role of constitutively secreted-protease as a nutrient scavenger as well as a potent tool for fungal biocontrol [28].

A further important result is the higher efficiency of the purified antifungal metabolites than the commercial fungicide PCNB, used as a positive control in antagonism assays. The minimum inhibitory concentration assessed for the bacterial bioactive compounds against M. phaseolina growth (50ug/mL), resulted 10 times lower than the one estimated for the commercial fungicide PCNB (0.5 mg/mL). Interestingly, the bacterial metabolites also appeared more stable over time: they retained their antifungal activity up to two weeks, while PCNB registered an efficiency reduction after 6 days only. Hence, the purified bacterial bioactive metabolites might be employed in a lower concentration reaching a higher long term efficiency, compared to chemical fungicides.

Altogether, these results suggest a strong antifungal effect of the protein compounds produced by RHFS10 strain and a promising prospect for agricultural applications. The bacterial extract could represent a valid sustainable eco-friendly fungicide and have potential as a biocontrol agent in alternative to chemical pesticides.

Future studies will focus on the effect of the M. phaseolina on the expression of antifungal metabolites produced by RHFS10, to verify if the fungus itself may enhance the production of the bioactive compounds already detected in this study or, perhaps, trigger the expression of new metabolites.

4. Materials and methods

4.1 Isolation of bacteria

Samples of the rhizosphere of Juniperus sabina plants were collected from the National Park of Ses Salines d'Eivissa, Formentera (Spain). To isolate rhizospheric bacteria, 1 g of roots samples was washed three times with 2mL sterile distilled water to remove impurities, transferred into 9 ml 1x PBS, and vortexed. The selection of spore-forming strains has been promoted through a heat pre-treatment at 80°C to kill all vegetative cells. 1 ml of the mixture was inoculated into 9 ml of LB (8 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract), serially diluted up to 10-6 and 0.1 ml of each dilution were spread on LB agar plates. Plates were incubated at 30 °C±1 °C for 2 - 3 days. Pure cultures were obtained by serial sub-culturing. Glycerol stocks of the isolates were prepared and stored at −80 °C.

4.2 Growth conditions

Each bacterial isolate was characterized by visual inspection for colony colour and morphology, as colony shape, size, margin and appearance. The ability to grow in facultative anaerobic conditions was determined using the AnaeroGen sachets (Unipath

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Inc., Nepean, Ontario, Canada) placed in a sealed jar with bacteria streaked on LB agar plates and incubated at 37°C for 3 - 4days.

To determine the optimum growth conditions, the bacterial isolates were grown in LB agar at different pH (2.0, 4.0, 6.0, 7.0, 8.0, 10.0, 12.0) and temperature (4, 15, 25, 37, 50, 60°C) ranges. Plates were incubated until the appearance of bacterial colonies.

4.3 Isolates identification by PCR amplification of 16S rRNA

Exponentially growing cells were used to extract chromosomal DNA using the DNeasy PowerSoil kit (Qiagen) according to the manufacturer's instructions. 16S rRNA gene was PCR amplified by using chromosomal DNA as a template and oligonucleotides forward 8F (5′-AGTTTGATCCTGGCTCAG-3′; annealing at position +8 / +28) and reverse 1517R (5′-ACGGCTACCTTGTTACGACT-3′ annealing at position +1497 / +1517). Those two oligonucleotides were designed to amplify a 1500 bp DNA fragment and the reaction was carried out according to Grönemeyer et al. [29] in an Esco SwiftTM MaxPro Thermal Cycler. The 1500 bp DNA amplified fragment was sequenced at the Bio-Fab research sequencing facility and analyzed using Basic Local Alignment Search Tool (BLAST). Phylogenetic analyses were carried out using Seaview 4.4.0 software package (http://pbil.univ-lyon1.fr/software/seaview.html) on 16S ribosomal RNA genes aligned using the Muscle algorithm.

Phylogenetic reconstruction for nucleotide alignment was carried out using the Maximum Likelihood algorithm (PhyLM). The gene sequences of the isolated bacteria were aligned to the ones belonging to different strains of related bacteria species obtained from BlastN analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches.

4.4 In vitro screening for plant growth-promoting (PGP) traits

4.4.1 Phosphate solubilization. The ability to solubilize inorganic phosphate was tested by growing the bacterial isolates on Pikovskaya agar (Oxoid Ltd) dyed with bromophenol blue [30] for 10 days at 30 °C. The formation of more transparent zones around the bacterial colonies was indicative of inorganic phosphate solubilization on Pikovskaya agar.

4.4.2 Siderophore production. To test siderophores production, 3 µl of overnight-grown culture in LB medium was spot-inoculated on iron-free S7 agar minimal medium. After 72 hours of incubation at 28 °C, 10mL of Chrome Azurol S (CAS) agar medium [31] were applied over agar plates containing cultivated microorganisms. Development of yellow-orange halo zone around bacterial spots was observed after 1 hour of incubation.

4.4.3 Biofilm Production. To evaluate the ability to produce biofilm, the isolates were separately grown in glass tubes in LB medium as described by Haney et al., 2018 [32]. Cultures were inoculated by adding 10 µL of an overnight culture of bacteria into 1 mL of sterile media, and the tubes were incubated statically at either 37 °C for 48 hours.

4.4.4 Swarming motility. Bacterial isolates were analyzed for their swarming motility using LB with spot-inoculation on agar 0,7% and incubated at 37°C overnight.

4.5 Evaluation of potential biocontrol features

4.5.1 Screening for hydrolytic enzymatic activity. Twenty-two bacterial isolates were grown separately in 5mL of LB broth a 37°C overnight with shaking at 150 rpm.

3 µl of each fresh bacterial culture were spot-inoculated on different assay plates, for testing hydrolytic enzyme activity. The protease activity was performed on Skimmed Milk Agar (SMA) [33] and the lipase activity on Tributyrene Agar medium [34]. After overnight at 37°C, the formation of a clear halo around the colony was considered as positive production for these enzymes. To detect the amylase activity was used the method described by Alariya et al., 2013 [35] with Starch Agar plates. After the overnight

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incubation at 37°C, the plates were flooded with Iodine solution and the hydrolysis of starch was observed as a colourless zone with a violet background around grown colonies. For the detection of cellulase and xylanase activities, it was used XPM (Xylanase Production Medium) agar plates with 0.5% xylan [36] (Megazyme) and a minimal medium with 0.5% carboxymethylcellulose (CMC) [37] as a sole carbon source. The plates were incubated at 37 °C for 3 days after which hydrolysis zones were visualized by flooding the plates with 0.1% Congo Red for 15–20 min and then de-stained by washing twice with 1 M NaCl. Plates, where CMC and xylan were omitted, were used as non-substrate controls. Transparent hydrolytic zones around the colonies are considered positive. For the Chitinase activity and Biosurfactant production, the bacterial strains were spot-inoculated on colloidal chitin-containing medium plates [38] and blood agar plates (BBL™Trypticase™Soy Agar (TSA II) with 5% Horse Blood) [39], respectively. After incubation at 25±2°C for 2-3 days, the clear zones around or within the colonies are considered positive evidence.

All experiments were performed in triplicate.

4.5.2 Dual culture assay. The isolated strains were examined in vitro for antifungal activity against pathogenic fungus Macrophomina phaseolina (Tassi) Goid (ATCC® 64334™). The in vitro antifungal bioassays were carried out based on the dual culture method as previously described by Khamn et al. (2009) [40]. A fungal plug of 6x6mm was placed on Potato Dextrose Agar (PDA) Petri dishes and each bacterial strain was streaked 2 cm away from the fungus. Plates containing the fungal plugs without bacterial inoculation were used as control plates. All plates were incubated at 28 °C for five days. The percentage of inhibition of the fungal growth was calculated using the following formula:

%=[(Rc-Ri)/Rc] x100

where Rc is the radial growth of the test pathogen in the control plates (mm), and Ri is the radial growth of the test pathogen in the test plates (mm). The experiment was repeated thrice. Bacterial strains that showed an inhibition of the growth of pathogenic fungus were observed by stereoscopic microscope 10x magnification.

4.5.3 Antifungal assay of Cell-Free Supernatants (CFS). Bacteria were grown on LB at 28 ± 2 °C and aliquots of the suspensions, collected at 24-hour intervals for the first 96 hours. Cells were removed by centrifugation (7000xg for 30 min) and supernatants were filtered using 0.22µm-pore-diameter membranes (Corning®) and concentrated 1:10. 100 µl aliquots of sterilized supernatant samples were dispensed in four wells (performed with a sterile cork borer, 6 mm diameter) equidistant from the centre of a PDA plate previously inoculated with an actively growing mycelia disc (6x6 mm diameter) of M. phaseolina as described by Kumar et al. 2012 [41]. As a positive control, fungicidal pentachloronitrobenzene ≥94% (PCNB) (Sigma-Aldrich) dissolved in toluene, was used. Toluene alone was used as a negative control. Plates were prepared in triplicate, incubated at 28°C for 5 days, and examined for zones of inhibition of grown colonies.

4.6 Extraction of secondary metabolites

The strains were grown in 300mL of LB at 28°C± 2 °C and for 72h. The broth cultures were then centrifuged at 9,000xg for 30 min at 4 °C and filtered through a 0.22 µm syringe filter. The culture filtrate was extracted at pH7 and pH2 three times for each, mixed with an equal volume of EtOAc into the separating funnel, and shaken for complete extraction. The secondary compounds contained in the solvent phase were separated from the aqueous phase, dried withNa2SO4, and evaporated under reduced pressure to yield the crude extracts. The crude extracts were dissolved in 1 mL 2% methanol at a final concentration of 5mg/mL, the aqueous phase was concentrated 1:10. All fractions were tested against M.phaseolina on PDA plates and incubated at 28°C ± 2 °C for 5 days.

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4.7 Stability of antifungal metabolites at different enzymes, temperatures and organic solvent conditions

100 µg/mL of enzymes (Trypsin, Proteinase K, Pancreatin and Pepsin) and 10% organic solvents (Acetone, Ethyl alcohol, Chloroform, Toluene and Isopropyl alcohol) (see Figure 4) were added to 100 µL of culture supernatant. Enzyme-treated samples were incubated for 3 h at 37 °C (42 °C in the case of proteinase K) and the solvent-treated samples were incubated for 3 h at 25 °C and subsequently, 100 µL aliquots were tested for antifungal activity as described above. To assess the stability of the bioactive compounds at high temperatures, CSFs were incubated at 65, 75 and 80°C for 1 or 3 hours, and their activity toward M. phaseolina eventually tested.

4.8 Size-fractionated supernatants tested for antifungal activity

RHFS10 strain was grown in 100 ml of LB broth for 72 h at 28 °C. The cultures were centrifuged at 7000× g for 30 min at 4°C and the supernatants filter-sterilized with a 0.22-µm filter (Millipore, Bedford, MA, USA). The supernatants were size-fractionated (10-kDa, 30-kDa and, 50-kDa cutoff spin column; Centricon, Millipore). Fractions were tested for antifungal activity and reported as % of growth inhibition as described above.

4.9 LC-MS/MS analyses

Protein extracts were electrophoretically separated on a 12.5 % polyacrylamide gel, under denaturing conditions. Resulting lines were divided into 10 pieces, and each underwent trypsin in gel digestion procedure. NanoUPLC-hrMS/MS analyses of the resulting peptides mixtures were carried out on a Q-Exactive orbitrap mass spectrometer (Thermo Fisher Scientific), coupled with a nanoUltimate300 UHPLC system (Thermo Fisher Scientific). Peptides separation was performed on a capillary EASY-Spray C18 column (0.075 mm × 100 mm, 1.7 µ m, Thermo Fisher Scientific) using aqueous 0.1% formic acid (A) and CH3CN containing 0.1% formic acid (B) as mobile phases and a linear gradient from 3% to 30% of B in 60 minutes and a 300 nL min−1 flow rate. Mass spectra were acquired over an m/z range from 350 to 1500. To achieve protein identification, MS and MS/MS data underwent Mascot software (Matrix Science) analysis using the non-redundant Data Bank UniProtKB/Swiss-Prot (Release 2020_03). Parameters sets were: trypsin cleavage; carbamidomethylation of cysteine as a fixed modification and methionine oxidation as a variable modification; a maximum of two missed cleavages; false discovery rate (FDR), calculated by searching the decoy database, ≤0.05. A comparison between the proteins found in the different samples allowed discriminating those specifically expressed by the strains showing promising antifungal activity.

4.10 Detection of antifungal metabolites

RHFS10 strain was grown in 2L of LB broth at 28°C for 72h with shaking at 150 rpm. The cells were removed by centrifugation (9000xg, 30 min) and the supernatant fluid filter-sterilized using 0.22µm-pore-diameter membranes. The antifungal activity of the preparation was determined against M.phaseolina using the cell-free supernatants assay described above. The culture filtrate (1800 ml) was precipitated with ammonium sulfate (66%w/v saturation) and stored overnight at 4 °C with shaking. The precipitate was removed by centrifugation (12 000 g, 20 min, 4 °C), resuspended in PBS 1X buffer (0·01 mol l_1, pH6·5; 1/10 of the initial volume) and dialyzed against the same buffer for 48 h at 4 °C with several changes (dialysis tube, porosity 24, cut-off 12 kDa; Union Carbide Corporation, Danbury, CT, USA). The dialyzed precipitate, was lyophilized, and the residue (483mg) was dissolved in 6ml ultrapure Milli-Q water and applied to a Sephadex G-50 fine column (Pharmacia, Uppsala, Sweden; 4·5_40 cm; flow rate 2·5 ml min_1). The column fractions (3 ml each) were collected in homogeneous groups according to the chromatogram obtained by monitoring proteins concentration at 280 nm [42]. Fractions were lyophilized, tested for antifungal activity (1mg/dot) against M.phaseolina, and analyzed by SDS-PAGE. The SDS-PAGE was performed with 20 µg of total proteins,

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fractionated on 12.5% SDS polyacrylamide gels and stained by Brilliant Blue Coomassie. Protein concentration was determined with the Bradford assay (Bradford, 1976) with bovine serum albumin used as standard.

4.11 Minimum Inhibitory Concentrations (MIC)

The MIC determination was performed in 24-well culture plates according to the method described by Agrillo et al. (2019) [43], with some modifications. The wells were prepared in triplicate for each concentration. The retentates (peaks 1, 2, and 3) containing the antifungal compounds were diluted separately at different concentrations (1mg/mL; 0,5mg/mL; 200µg/mL; 100µg/mL; 50µg/mL and 25µg/mL) in a volume of 500 µL of ultrapure Milli-Q water and were inoculated with 500 µL of M.phaseolina plugs (4x4mm) resuspended in 2xPD broth. As a control, 500 µL of M.phaseolina plugs (4x4mm) were resuspended in 2xPD broth diluted with 500µL of ultrapure Milli-Q water. The retentates were compared with the fungicidal PCNB ≥94% (Sigma-Aldrich) at the same different concentrations. The plates were incubated at 28 °C for 5 days and after it was taken as MIC the lowest concentration of antifungal agent at which there was no visible growth of the fungus after incubation. Finally, the percentage of inhibition of the fungal growth was calculated using the formula described above.

4.12 Whole-genome sequencing

The most promising bacterial strain RHFS10, that showed outstanding biocontrol performance, was selected for whole-genome sequencing to obtain future relevant genetic information. DNA extraction was performed using the method described above. Genome sequencing was performed by MicrobesNG (Birmingham, UK) with the genomic DNA library prepared using the Nextera XT library prep kit (Illumina) following the manufacturer's protocol. Libraries were sequenced on the Illumina HiSeq using a 250 bp paired-end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 [44] and de novo genome assembly was carried out with SPAdes (version 3.7) via MicrobesNG.

4.13 Statistical analysis

All the statistical analyses were performed using GraphPad Prism 8 software. Data were expressed as mean ± SEM. Differences among groups were compared by ANOVA or t-test as indicated in the Figure legends. Differences were considered statistically significant at $p < 0.05$.

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Supplementary Materials:

Figure S1: Potential plant growth-promoting traits of selected bacterial isolates. The ability to solubilize inorganic phosphate was assessed by growing the bacterial isolates on the Pikovskaya agar assay [30]. The experiment was performed in triplicate with three independent trials.

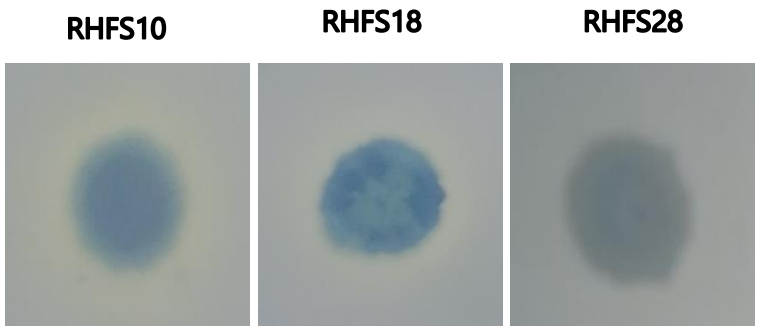

Figure S2: Potential plant growth-promoting traits of selected bacterial isolates. Siderophore production was assessed through O-CAS assay method [31]. The experiment was performed in triplicate with three independent trials.

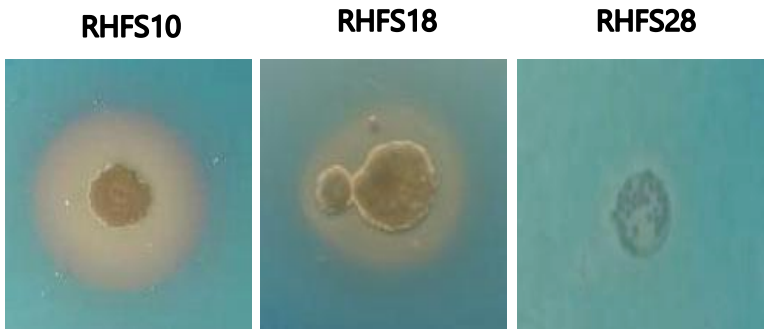

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Figure S3: Hydrolytic activities of selected bacterial isolates [33,34,35,36,37,38,39]. The experiment was performed in triplicate with three independent trials.

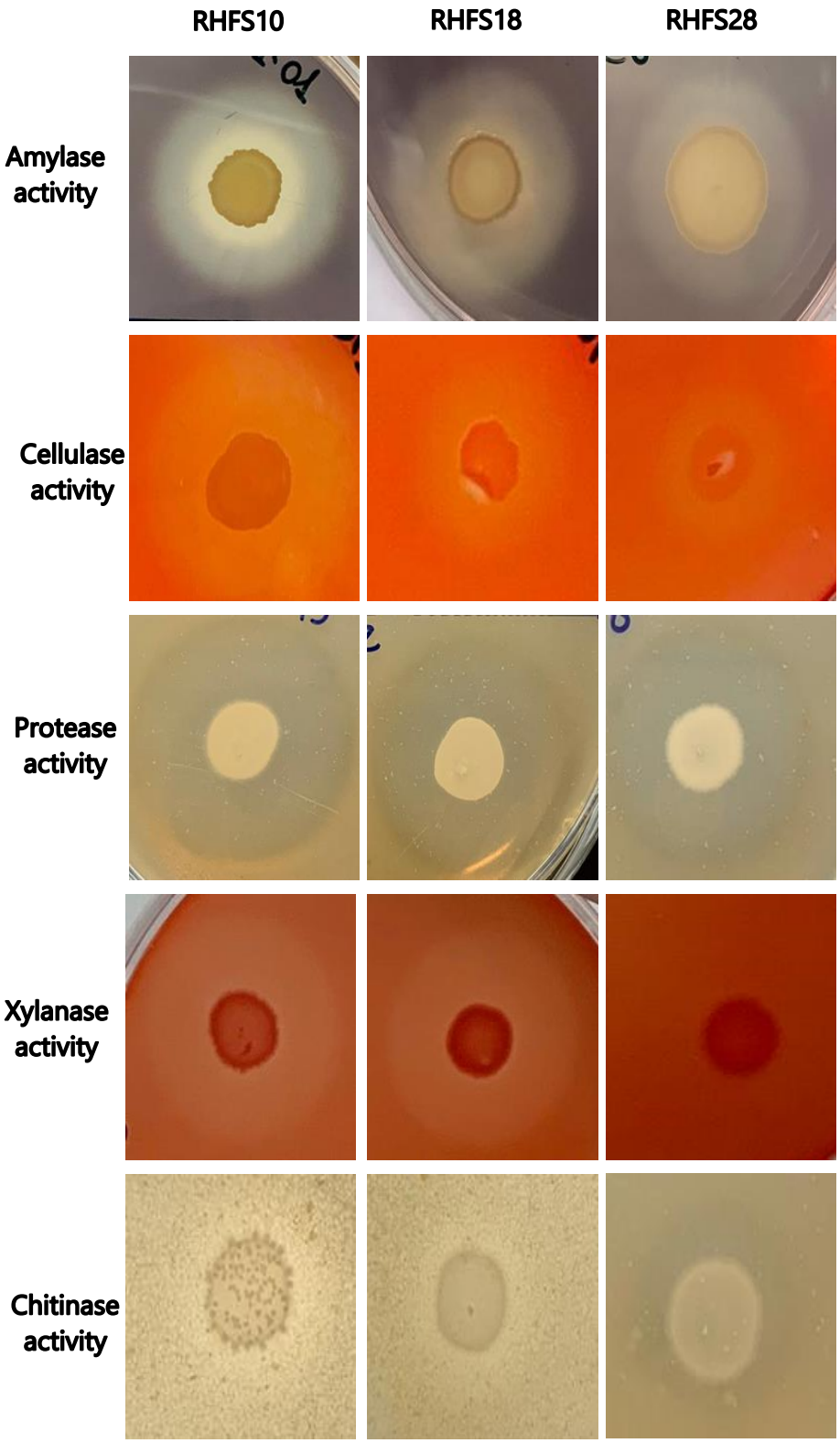

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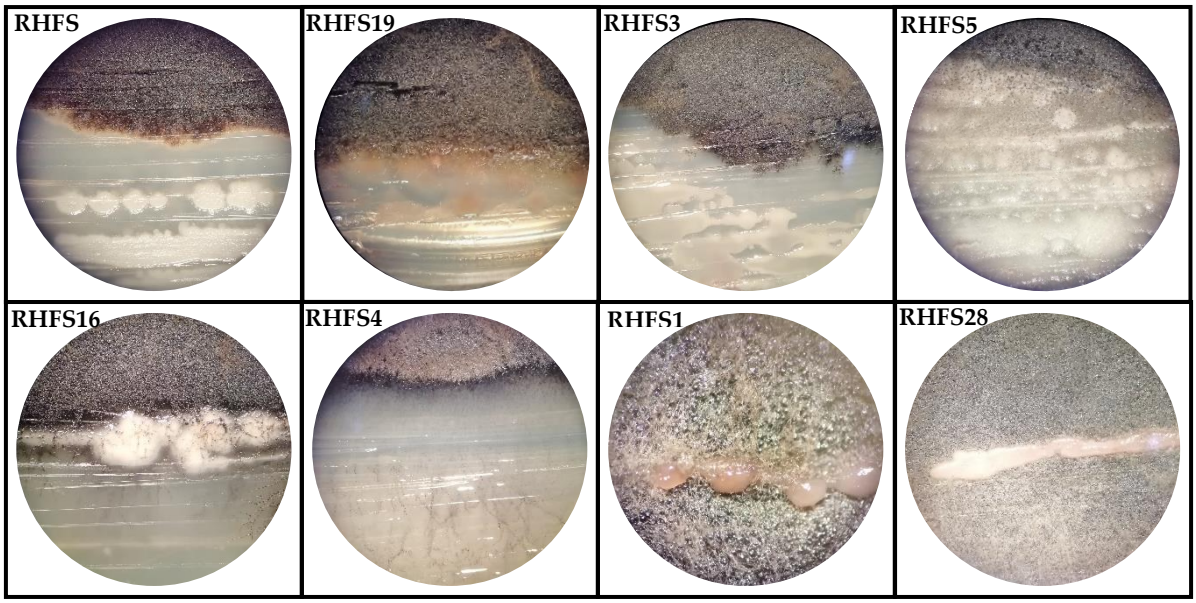
Figure S4: Dual culture assay. Plates with *M.phaseolina* and most significant bacteria observed under a stereoscopic microscope (10x magnification)[40].



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