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RESEARCH ARTICLE



## Biocontrol activity of *Bacillus*, *Paenibacillus* and *Pseudomonas* against Fusarium wilt of chickpea in Morocco

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### ABSTRACT

Fusarium wilt caused by *Fusarium oxysporum* f.sp. *ciceris* is one of the major diseases impacting chickpea productivity. Significant losses are reported by farmers due to the absence of effective wilt management options. Biological control using beneficial microorganisms in agriculture, is one of the promising alternatives and eco-friendly strategies utilised to overcome this disease. The present study investigated the biocontrol effect of 40 bacterial strains isolated from the rhizosphere of healthy chickpea plants collected from major chickpea growing regions in Morocco. Twelve out of 40 strains showed more than 25% *in vitro* inhibition of the pathogen growth. These strains, using the 16S rDNA gene sequencing, were classified into three genera, namely *Bacillus*, *Paenibacillus*, and *Pseudomonas*, represented by different species. Our finding showed that the mode of antagonism was mainly due to the production of diffusible and volatile compounds as well as lytic enzymes. Moreover, a greenhouse experiment of the three selected antagonistic strains showed a significant reduction in the mean of wilt incidence in different chickpea genotypes. Strain B18 reduced the wilt incidence in the susceptible variety from 90% to 18%. Consequently, our antagonistic bacterial strains could be a potential component of integrated management of Fusarium wilt, therefore, increase the yield of chickpea.

### ARTICLE HISTORY

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### KEYWORDS

Antagonism; biocontrol; *Fusarium oxysporum* f.sp. *ciceris*; chickpea

## Introduction



Chickpea (*Cicer arietinum*) is one of the most important food legumes produced in more than 50 countries (Muehlbauer and Sarker 2017). The crop is a good source of protein and plays an important role in improving soil fertility through biological nitrogen fixation in cereal-based cropping systems (Muehlbauer and Sarker 2017). In Morocco, Kabuli chickpea is the second major food legume crop after Faba bean and is mainly produced in Taza-Al Hoceima-Taounate (27%), Gharb-Chrarda-Benihssen (24%) Meknès-Tafilalet (16%), and Fès-Boulmane (12%) regions. The total cultivated area is about 60–200 ha with average productivity of 718 Kg ha<sup>-1</sup> (Houasli et al. 2020).

The production and productivity of chickpea show high yield instability over the years due to biotic (diseases and insect pests) and abiotic production constraints (Houasli et al. 2020). The major diseases are Ascochyta blight and wilt/root rot (Singh et al. 2021). The wilt/root rot disease is prevalent in Ethiopia, India,

Mexico, Morocco, Spain, Tunisia, Turkey, and the United States of America (Jendoubi et al. 2017). The average annual yield losses due to Fusarium wilt /root rot have been estimated between 10 and 90% reaching 100% sometimes. In Morocco, the major pathogens associated with the wilt/root rot complex are *Fusarium oxysporum* f.sp. *ciceris* (59.8%), *Rhizoctonia bataticola* (32.7%) and *R. solani* (7.3%) (Elbouzaoui et al. 2018).

Fusarium wilt can appear at seedling, vegetative and flowering-podding stages of the crop where symptoms can be observed 3 weeks after sowing depending on weather conditions and genotype susceptibility (Jiménez-Díaz et al. 2015). Some races of *F. oxysporum* f.sp. *ciceris* (*Foc*) can cause early and late wilting on different chickpea genotypes (Upadhyaya et al. 1983).

Several strategies are used by chickpea growers to manage Fusarium wilt/root rot complex in different countries that include cultural practices (diversified crop rotation and adjusting sowing dates), growing resistant cultivars, fungicide seed treatment, and

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biological control (Sampaio et al. 2020). In developed countries, fungicide seed treatments are used to control *Fusarium* wilt/root rot complex diseases (Sampaio et al. 2020). In Morocco, farmers are practicing short crop rotation (wheat followed by chickpea) and fungicide seed treatments but their effectiveness in reducing the disease complex is low (Bishaw et al. 2019).

Biocontrol of *Fusarium* wilt/root rot complex diseases is one of the recommended components of integrated disease management in many pulse crops (Pandey et al. 2018). Among the biological control agents (BCA), rhizobacteria are mostly used since they occupy 7–15% of the rhizosphere (Köhl et al. 2019). Moreover, some rhizobacteria act as plant-growth promoting agents that enhance crop growth and development and play roles in reducing soil-borne diseases (Köhl et al. 2019; Ahemad and Kibret 2014; Gupta and Pandey 2019).

The use of BCA in managing *Fusarium* wilt/root rot diseases in chickpea is not widely exploited in Morocco and other developing countries. Therefore, the objectives of this study were to (1) identify potential antagonistic bacterial strains to *Fusarium* wilt; (2) determine the mechanism of actions of antagonistic bacterial strains, and (3) test the effectiveness of selected strains to manage *Fusarium* wilt under glasshouse conditions.

## Materials and methods

### Isolation of antagonistic bacteria strains from soil

Soil samples were randomly collected from rhizospheric parts of healthy chickpea plants (the upper 20–25 cm of the soil) in 11 fields located in the major chickpea growing areas of Morocco, Oulad Said (Latitude 32.985829, longitude -7.778494) located in Casablanca-Settate region, Haj kadour (33.831260, -5.483157) located in Meknes-Fes region, Sidi kacem (34.228233, -5.691842) and Merchouch (33.561319, -6.691883) which are situated in Rabat-Sale-Kenitra region. Bacterial strains were isolated from the composite soil samples using the dilution method ( $10^{-1}$ – $10^{-8}$ ) where soil samples were suspended in physiological distilled water (1%). Each dilution was added to Luria Bertani (LB) medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 1L of distilled water) in Petri dishes (9 cm) and incubated at 27 °C for 24–48 h.

### Pathogen isolation and purification

*Foc* was isolated from infected chickpea plants collected from Merchouch Research Station. The infected stems were cut into small pieces (1 cm), then washed with tap water, sterilised with 20% Chlorox (NaOCl) for 2 minutes, rinsed three times with fresh sterilised distilled

water, and finally dried on sterilised filter papers under a laminar flow hood. The dried stem pieces were plated on Potato Dextrose Agar (PDA) (20 g dextrose, 4 g potato extract, and 15 g agar in 1L of distilled water) and incubated at 25 °C for seven days. The *Fusarium* isolated was identified as *F. oxysporum* using Barnett and Hunter (Barnett and Hunter 1972) key for fungi identification and inoculated on susceptible chickpea genotypes and re-isolated from the wilted plant stem. A single spore of the isolate was prepared according to Leslie's method (Leslie and Summerell 2008) and stored in sterile sand-filled tubes at 4 °C for further experimental uses.

### Screening of antagonist bacteria for their inhibition against *Foc*

Bacterial strains were screened using the dual culture plate technique described by Naing et al. (2015). A five mm agar disk from a fresh culture of *Foc* was placed in the centre of a plate containing PDA, then the bacterial strains were placed at 2.5 cm from the centre of PDA Petri dishes and incubated at 28 °C for 7 days. Petri dishes inoculated only with the pathogen were used as control. The experiment was replicated three times in a completely randomised design.

Percentage of inhibition of radial growth (PIRG) was calculated after measuring the radial growth of the *Foc* colony with and without bacteria, using the following formula (Stracquandano, et al. 2020)

$$PIRG = \frac{R - r}{R} \times 100$$

where,  $r$  is the radius of the fungal colony with bacteria and,  $R$  is the radius of the fungal colony without bacteria (control).

### Molecular identification of antagonistic bacteria

Antagonistic bacterial strains selected based on their ability to inhibit the growth of *Foc in vitro* were identified using sequence analysis of the 16S rRNA gene. DNA of the strains was extracted following the method described by Dong et al. (2013). Twelve antagonistic bacteria were grown on LB medium at 28 °C for 48 h. A hundred mg of fresh pure culture of the antagonistic strains was suspended in 564 µL TE buffer (50 µM Tris-HCl; 50 µM EDTA; 1.5M NaCl, pH 8). Digestion was done by adding 20 mg/mL of lysozyme solution (Sigma-Aldrich, Germany), 10 mg/mL of proteinase K solution (Sigma-Aldrich, Germany), and 30 µL of SDS (15%). The solution was vortexed thoroughly and incubated at 37 °C for 35 minutes. A hundred microliters of NaCl (5M) and 80 µL CTAB/NaCl solution were added and mixed thoroughly

and incubated at 65 °C for 10 minutes. The proteins and the other cell component were removed by adding 800 µL of chloroform/isoamyl alcohol (25:24:1) solution. The aqueous phase, containing the DNA, was transferred twice after centrifuge (10,000 rpm for 5 minutes). The DNA elution/precipitation was performed using 0.7 volumes of isopropanol with 0.1 volumes acetic acid (3M) and centrifuged at 15,000 rpm for 30 minutes. The DNA pellet was washed with ethanol (70%). The dry pellet was resuspended in 50 µL TE buffer. The concentration and the purity of the DNA ( $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  ratios) were detected using the Nanodrop spectrophotometer (Jenway, Genova nano). A brief treatment of the extracted DNA was done by RNAase if the ratios were greater than 2.0.

### **16S rRNA gene amplification, PCR purification, and sequencing**

The small unit 16S rRNA was amplified by PCR using universal primers 27F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGTTACCTTGTACGACTT-3') (Srivastava et al. 2008). The PCR reaction was performed as follows: an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 45 seconds, annealing at 50 °C for 45 seconds, extension at 72 °C for 2 minutes, and final extension at 72 °C for 10 minutes.

The DNA extract and the PCR products were separated on an agarose gel 0.5% (v/w) and 1%(v/w) respectively). Ten µL of the DNA templates with 7 µL loading dye (EZ-Vision, AMRESCO) deposited in the gel for 30–40 minutes at 180 V. The molecular sizes of the amplified fragments were estimated using 1 and 2 kb Hyper Ladder (Bioline).

The PCR products were purified using the ExoSAP-IT purification system (Cleanup Reagent, Invitrogen) following the manufacture's protocol. Sequencing was carried out using Big dye Terminator cycle sequencing Kit V3.1 (Applied Biosystems) for both forward and reverse directions with the same primers as the PCR amplification. The sequence data were collected from an ABI 3730XL DNA capillary sequencer (Applied Biosystems, France) at Genoscreen, France. The obtained nucleotide sequences were blasted using the nucleotide basic local alignment tool (BLASTn) on NCBI website. The antagonistic bacteria raw 16S rRNA sequences were assembled using sequence scanner software 2.0 (ThermoFisher, United States) and EMBOSS Needle tool (<https://www.ebi.ac.uk/>).

### **The phylogenetic analysis of 16S rDNA sequences**

The phylogenetic analysis of the 16S rDNA sequences of the antagonistic bacteria was carried out by comparing

with reference strains with high similarity in sequences and 100% query cover on National Center for Biotechnology Information (NCBI). All the sequences were aligned using the ClustalW programme within MEGA X software (Qin et al. 2019). The phylogenetic tree was constructed based on the aligned sequences adopting the Maximum Likelihood (ML) method (Allison et al. 2017) and the suggested best Model using MEGA X software. The statistical significance of the nodes was assessed by bootstrap resampling analysis of 1000 replicates.

## **Assessment of mechanisms of antagonism**

### **Production of diffusible compounds**

The presence of diffusible compounds produced by antagonistic bacteria was tested by spotting filtered (0.22 µm) supernatant from a 48-h bacterial culture 2.5 cm away from a mycelial disc and incubated for 8 day. The bacteria alone served as control, the PIRG was calculated in the same way as described earlier. The experiment was replicated three times.

### **Production of volatile compounds**

To assess the presence of volatile compounds from the antagonistic bacteria, a plate-within-a-plate system was used (Schmidt et al. 2017). After adjusting the colony-forming unit (CFU) to  $10^8$  bacteria/mL, 50 µL of the bacterial suspension was streaked in a Petri dish containing LB medium. The pathogen was plated on a PDA medium. A Petri dish containing bacteria was used to cover the Petri dish containing the pathogen. The two Petri dishes were sealed with parafilm and incubated at 28 °C for a week. Pathogen cultured on PDA without the antagonistic bacteria was used as control and after one week, PIRG was calculated. The experiment was replicated three times.

### **Production of enzymes**

**Protease production.** The proteolytic activity was determined by placing 10 µL of each antagonistic bacteria in Petri dishes containing Skimmed Milk Agar (SMA) (Yeast extract 3 g, casein peptone 5 g, and agar 15 g in 1 litre of distilled water) supplemented after autoclaving with 250 mL of sterile skimmed milk (Colantuono et al. 2020). After 72 h of incubation at 28 °C, observation was made for the development of a halo around the colonies.

**Chitinase production.** The chitinolytic activity was estimated in a Petri dish containing chitin agar medium (Kapur et al. 2018). Colloidal chitin (10 g),  $(\text{NH}_4)_2\text{SO}_4$  (2 g),  $\text{KH}_2\text{PO}_4$  (0.7 g),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.5 g),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.01 g), and agar (15) gram per liter. Chitin powder

was prepared according to the method of Tan et al. (2020) with slight modifications. Twenty grams of crushed shrimp powder was dissolved in 100 mL HCl (1%) with stirring for one night. Later, HCl was slowly discarded, then protein was removed by adding 3.5% NaOH solution ratio of 10:1, and then heated at 60°C for 2 h. The precipitates were washed with water many times until reaching a pH  $\approx$  7. The formation of clear halos around the colonies was observed. The prepared medium was inoculated with 5  $\mu$ L of each of the antagonistic bacteria, incubated at 28 °C for ten days. The observation was made for the development of a halo around the colonies.

**Glucanase production.** The production of the glucanase enzyme was tested in Petri dishes containing LB medium supplemented with 10 g/L of barley flour (Ben Slama, et al. 2019). The bacterial suspension (10  $\mu$ L) was placed within the Petri dishes and after 72 h of incubation at 28 °C, the formation of a clearing zone around the colonies was observed. The experiment was replicated three times.

**Cellulase production.** Ten microliter of each antagonistic bacteria were cultured on LB medium supplemented with 10 g/L of carboxy-methylcellulose (CMC) medium (Gupta et al. 2012). After 72 h of incubation at 28 °C, Petri dishes were flooded with Congo-red for 10–12 minutes and then washed with 1 M NaCl solution. The observation was made for colonies surrounded by clear halos.

Quantitative comparison among antagonistic bacteria was done by measuring the diameter of both the colony and the clear zone, and then the hydrolysis capacity (HC) was calculated as described by Gupta et al. (2012):

$$HC = \frac{\text{Diameter of clear zone}}{\text{Diameter of colony}}$$

Effect of antagonistic bacteria strains on *Fusarium* wilt diseases on chickpea

Based on their *in vitro* effects in inhibiting *Foc*, three antagonistic bacteria and one fungicide were selected as a seed treatment on two Moroccan varieties; Arifi (FLIP 98-50C), Farehan (FLIP 84-92C), and a breeding line (FLIP-09-C211) using highly *Fusarium* infested soil (3000 CFU/g of soil) collected from Merchouch Research Station. The genotypes have different levels of resistance of the *Fusarium* wilt/rot complex.

Seeds of each chickpea genotype were disinfected with 0.1% Mercuric solution, rinsed three times repeatedly with sterile distilled water for surface sterilisation.

After surface disinfection, seeds were treated with a bacterial inoculum ( $10^8$  CFU/mL) of each isolate using the seed bacterisation method for 20 minutes (Chowdhury et al. 2020). Seeds were also treated with Celest Top (Syngenta) containing Difenconazole (25 g/L) + Fludioxonil (25 g/L) + Thiamethoxam (262,5 g/L) at the rate of 200 g/hl. Untreated seeds were used as control. Treated seeds were planted on infested field soil in cones ( $3 \times 3 \times 4.5$  cm) containing 10 g of infested soil. Greenhouse conditions were maintained at 28 °C, with 80% relative humidity and 14 h/10 h photoperiod (light intensity of 12,000 Lux). The experiment was arranged in a completely randomised block design with three replications and repeated two times. Sixty days after planting, wilt incidence was recorded using the following formula;

$$\text{Wilt incidence (\%)} = \frac{\text{Number of wilted plants}}{\text{Total number of plants}} \times 100$$

### Statistical analysis

Analysis of variance (ANOVA) was done for percent inhibition and wilt incidence using GenStat software Genstat V 20. Percent *Fusarium* wilt incidence values were converted into Bliss angular values ( $\arcsin \sqrt{\% \text{ wilt} + 1}$ ) to normalise residuals for data analysis. The means were separated using the LSD test.

## Results

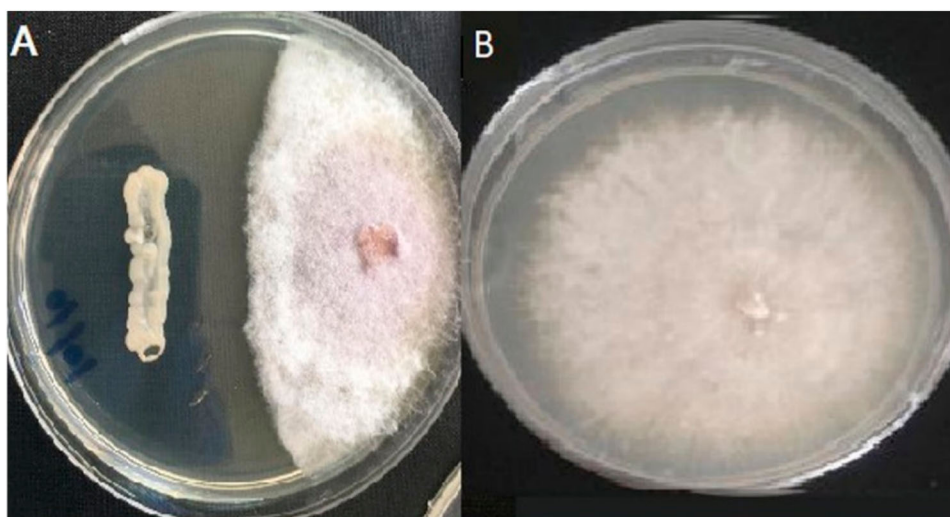
### Screening of bacterial strains

Bacterial isolates showed different levels of antagonistic activities against *Fusarium oxysporum* f.sp *ciceris* (*Foc*) (Figure 1). Highly significant differences ( $p \leq .05$ ) were observed among bacterial strains. The mean percentage inhibition of the strains ranged from 1 to 75% of which 12 showed more than 25%. The highest inhibitions were observed from StrainCR18, StrainCR17, and StrainCR3 with mean inhibition percentages of 75%, 58%, and 54% respectively (Figure 2).

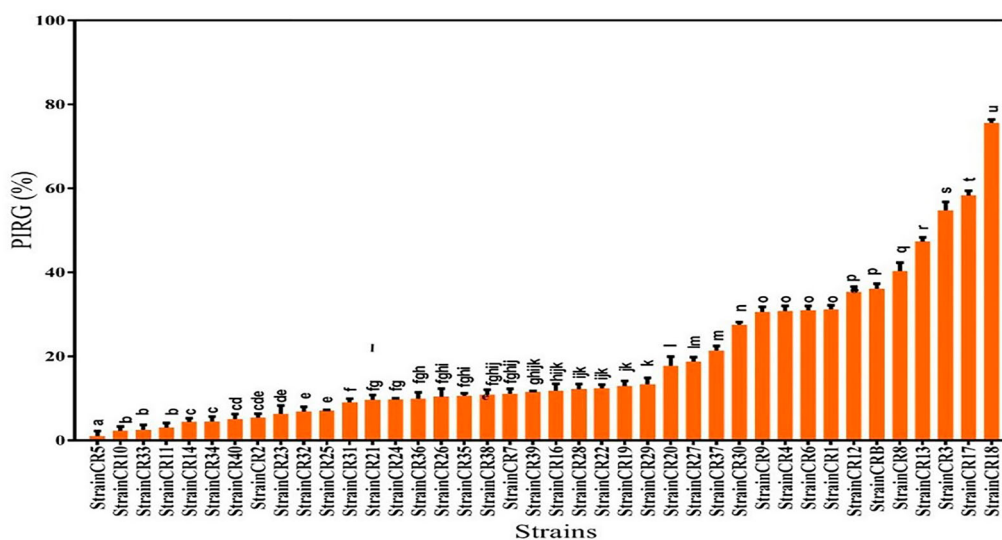
### Identification of bacterial strains

Based on *in-vitro* tests, 12 antagonistic strains were selected and identified using 16S rRNA gene (Figure 3) and were clustered into three genera (Figure 4). Seven strains (StrainCRB, StrainCR12, StrainCR18, StrainCR6, StrainCR30, StrainCR3, and StrainCR1) were grouped in the genus *Bacillus*. Two strains (StrainCRB and StrainCR12) were affiliated to *B. subtilis*, one strain StrainCR18 to *B. majovensis* and four strains (StrainCR6, StrainCR30, StrainCR3 and StrainCR1) to *B. amyloliquefaciens*.

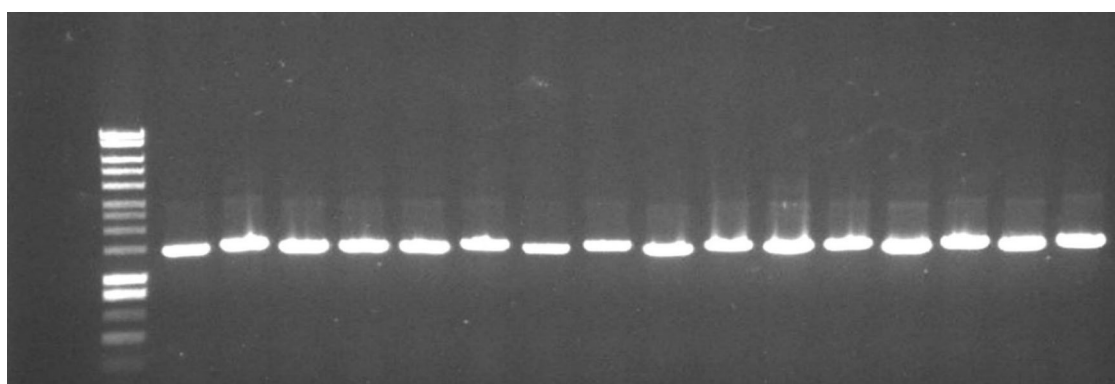




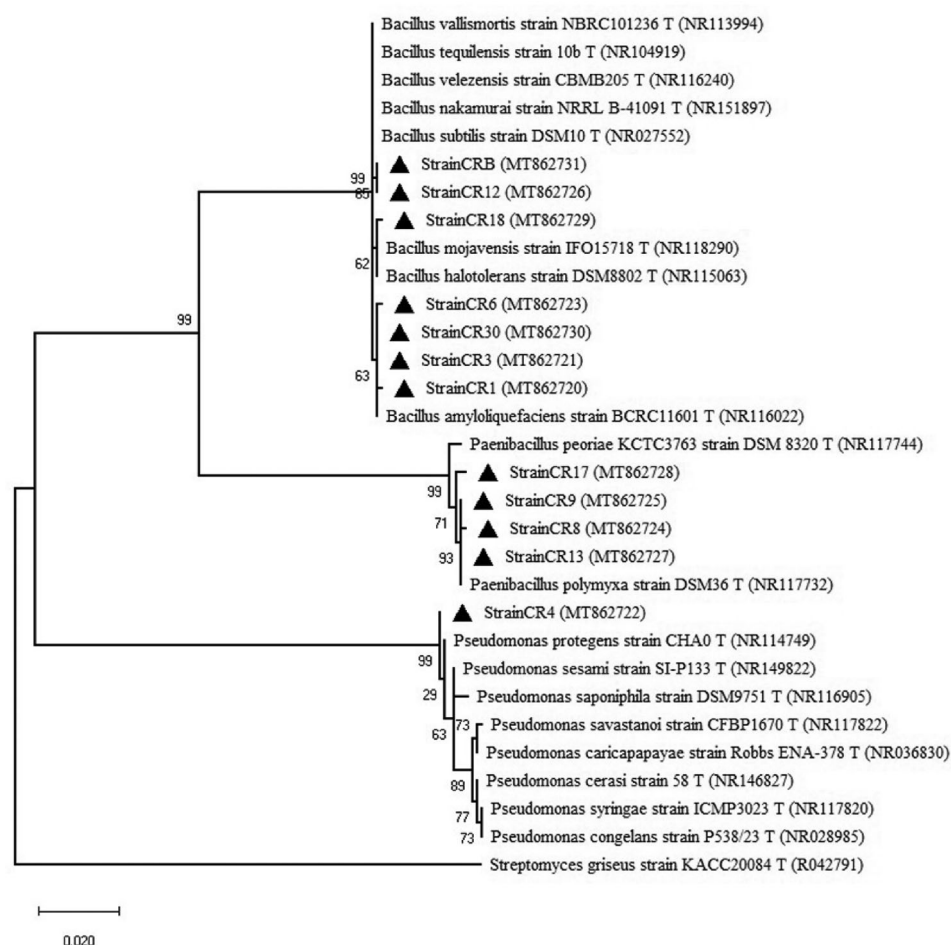
**Figure 1.** *In vitro* dual cultures on P DA medium at 28 °C for 7 days. (a) antagonistic effect of bacterial strain against *Foc*. (b) control.



**Figure 2.** Mean percentage of *in vitro* inhibition of bacterial strains on *Foc*. Treatments with the same letters do not differ significantly ( $p \leq .05$ ) according to protected Fisher's LSD test. The vertical bars represent standard deviation with three replicates.



**Figure 3.** PCR products of 12 antagonistic bacteria captured by the imaging system (Enduro, Labnet). Line 1: Hyperactive ladder 1 kb (Bioline). Lines 2-12: StrainCR1; StrainCR3; StrainCR4; StrainCR6; StrainCR8; StrainCR9; StrainCR12; StrainCR13; StrainCR17; StrainCR18; StrainCR30; StrainCRB. Bands are positioned in 1500 bp size of the DNA ladder.



**Figure 4.** Phylogenetic tree constructed based on partial 16S rRNA gene sequences of the antagonistic bacteria and the closest type strains sequences in the NCBI database. The tree was generated using the MEGA-X Kimura 3-parameter (T92) distance model and Maximum Likelihood (ML) method with 1000 bootstrap analysis and bootstrap value (B.V  $\geq$  50%). The strains were out-grouped by *Streptomyces griseus* strain KACC20084. Bars: 2% nucleotide substitutions. The black triangle represents the sequences of the antagonistic bacteria in this study.

StrainCR17, StrainCR9, StrainCR8, and StrainCR13 were affiliated to the genus *Paenibacillus*, while StrainCR4 belonged to the genus *Pseudomonas* in the species *P. protegens* (Figure 5).

All bacterial 16S rRNA gene sequences were submitted and registered on NCBI (National Center for Biotechnology Information) under the following accession numbers: StrainCR1 (MT862720); StrainCR3 (MT862721); StrainCR4 (MT862722); StrainCR6 (MT862723); StrainCR8 (MT862724); StrainCR9 (MT862725); StrainCR12 (MT862726); StrainCR13 (MT862727); StrainCR17 (MT862728); StrainCR18 (MT862729); StrainCR30 (MT862730); StrainCRB (MT862731).

#### Production of diffusible and volatile compounds

Antagonistic strains varied in their ability to produce diffusible and volatile compounds. All strains produced

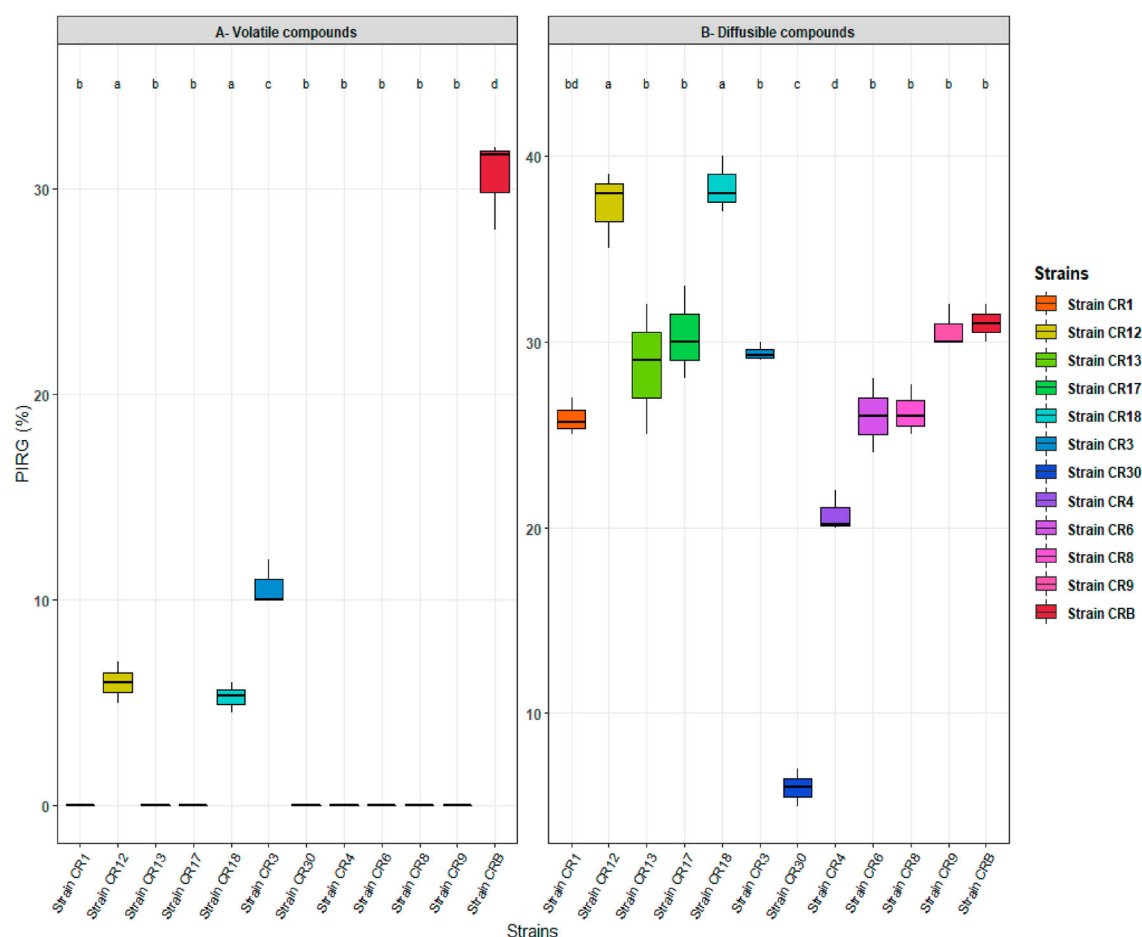
diffusible compounds where the mean percentage inhibition of the pathogen varied from 6% for StrainCR30 to 38.33% for StrainCR18 (Figure 5).

Regarding volatile compounds, among the 12 tested strains, only four were able to inhibit the growth of *Foc* through volatile substances. The highest inhibition was obtained from StrainCRB with the high percentage of inhibition exceeding 30% (Figure 5).

#### Production and inhibition effects of lytic enzymes on in vitro growth of *Foc*

Four lytic enzymes namely protease, glucanase, cellulase, and chitinase were produced by different isolates tested (Figures 6 and 7). Among the enzymes produced the major ones were protease and glucanase.

All strains produced protease with maximum production from StrainCR18. While StrainCR17, StrainCR9,



**Figure 5.** Box plot of percentage inhibition of radial growth (PIRG) by bacterial strains using volatile (A) and diffusible (B) compounds. Treatments with the same letters do not differ significantly ( $p \leq .05$ ) according to protected Fisher's LSD test. The vertical bars represent standard deviation with three replicates.

StrainCR18, StrainCR8, StrainCR13, and StrainCR30 were able to produce cellulase with the highest production recorded from StrainCR17. Chitinase was produced by six strains where the highest production was observed from StrainCR3. For glucanase production, out of the 12 strains 11 were positive with StrainCR17 having the highest hydrolysis activity (Figure 7).

In order to understand the relationship between PIRG and bacterial strains activities, a correlation test was performed, results showed that the PIRG is correlated positively with all activities, the highest correlation was observed with gulanase, chitinase and diffusibles compounds (Figure 8).

### Effects of antagonistic bacteria strains on *Fusarium* wilt of chickpea

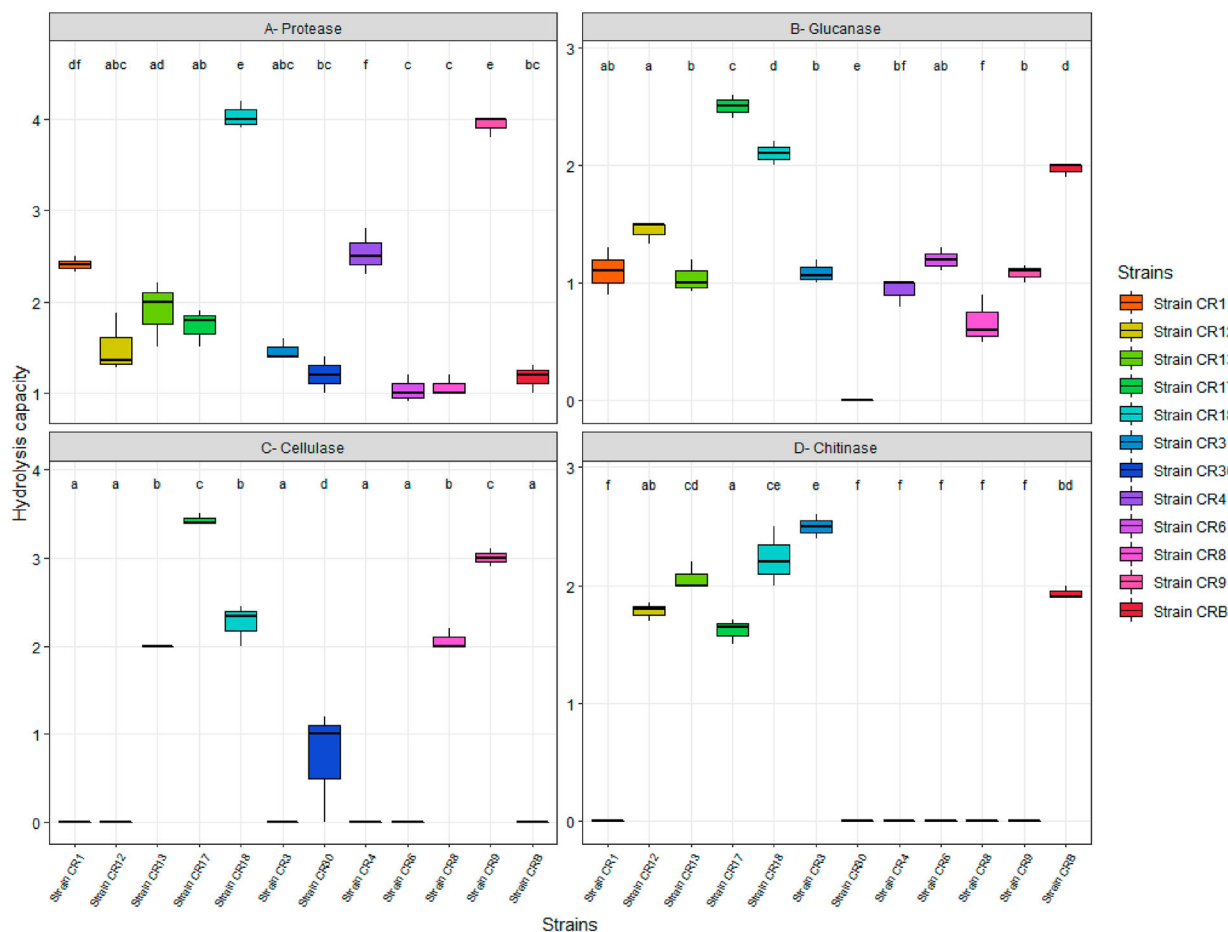
*Fusarium* wilt was developed in all chickpea genotypes where the highest infection percentage (90%) in this experiment was obtained with susceptible variety sown in sick soil without any treatment (Figure 9). All

treatments reduced the mean percentage of *Fusarium* wilt incidence in the three chickpea genotypes. The interaction seed treatment and genotypes were highly significant ( $p < .05$ ). StrainCR18 was most effective, it reduced *Fusarium* wilt incidence in the three genotypes (Figure 10).

### Discussion

The negative impacts of the soil-borne disease complex remain an important biotic factor that requires suitable solutions. For that purpose, bacteria were isolated then confronted using dual culture, this technic is usually used to screen biocontrol agents against phytopathogens (Siameto 2011). The antagonistic isolates selected and tested in this study were grouped into three genera (*Bacillus*, *Paenibacillus*, and *Pseudomonas*) (Figure 4). This diversity could be explained by the fact that the isolation was made from different regions and the screening were based on the antagonistic activity.





**Figure 6.** Box plot of hydrolysis capacity of different antagonist bacteria after culture for enzymes production. (A) Protease, (B) Glucanase, (C) Cellulase, and (D) Chitinase. Treatments with the same letters do not differ significantly ( $p < .05$ ) according to protected Fisher's LSD test. The vertical bars represent standard deviation with three replicates

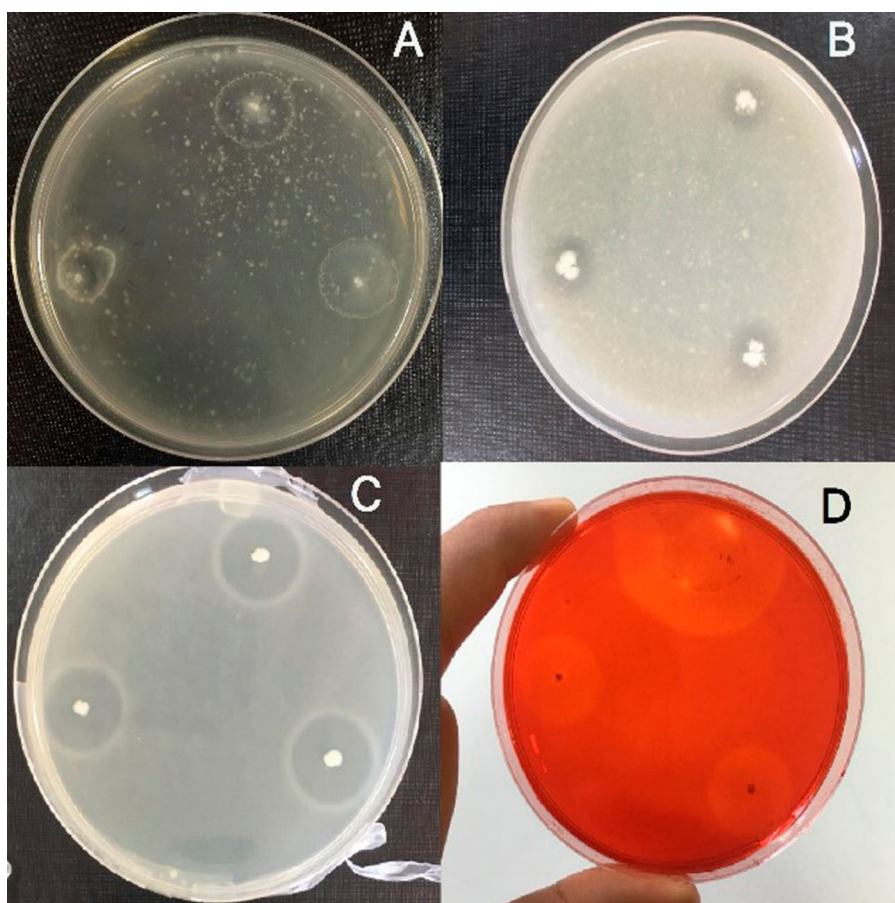
The three genera are broadly known for their potential antagonistic activities and their uses as BCA in managing soil-borne crop diseases (Grover et al. 2021; Ruii 2020). Our findings are in line with previous studies which showed that the application of *B. subtilis*, *P. fluorescens*, and strains from the genus *Paenibacillus* control *Fusarium* wilt of chickpea (Anusha et al. 2019; Sahane et al. 2021; Zaim et al. 2013).

The mode of action and/or the type of metabolite produced by bacterial strains may explain this disparity among the antagonistic strains. Our results showed that the percentage of inhibition with diffusible compounds were ranging from 6% to 38% (Figure 4). Similar results were reported by Prashar et al. (2013), where the diffusible compounds reduced the growth of *F. oxysporum* by up to 48%. The inhibition was showed not only with diffusible compound but also with volatile compounds and reached 30% with StrainCRB. Our results collaborate with the results of Souad et al. (2013) which revealed that some antagonistic rhizobacteria tested were able to inhibit the

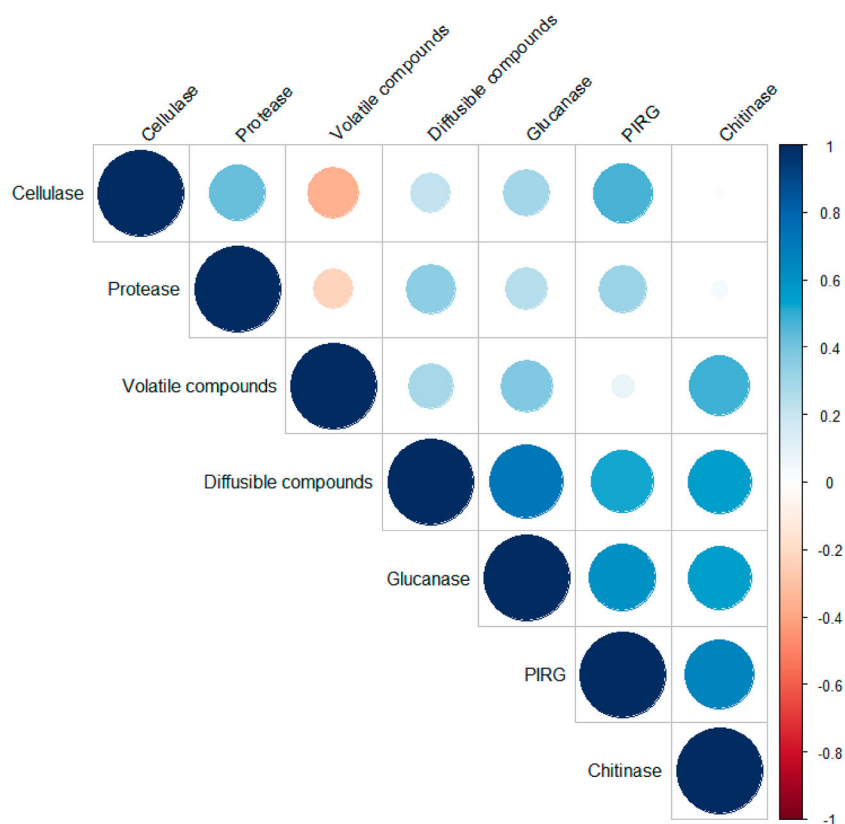
growth of *Foc* by volatile metabolites up 40%. Several studies showed the ability of bacteria belonging to *Bacillus* and *Pseudomonas* spp. To inhibit the growth of *F. oxysporum* by producing volatile compounds (Tilocca et al. 2020; Kong et al. 2020).

Bacteria belonging to *Bacillus* spp. can produce a large spectrum of antifungal compounds classified into different families, these molecules have been reported efficient against pathogenic microorganisms including *Fusarium oxysporum* (Xie et al. 2018).

Enzymes are among the substances secreted by antagonistic bacteria, which degrade a complex of organic molecules constituting the membrane of the fungi. *Fusarium* cell wall contains  $\alpha$ -1,3-glucan and chitin where some enzymes produced by antagonistic bacteria can degrade and inhibit the growth of the pathogen (Schoffemeer et al. 1999). In our study, antagonistic bacterial strains with higher inhibitions produced glucanase and chitinase enzymes. These enzymes are capable of breaking down glycosidic bonds in chitin (Jadhav et al. 2017). Several attempts have been



**Figure 7.** *In-vitro* lytic enzyme activity. (A) Chitinase, (B) Glucanase, (C) Protease, and (D) Cellulase.



**Figure 8.** Correlogram showing correlation between percentage of inhibition and antagonistic activities.

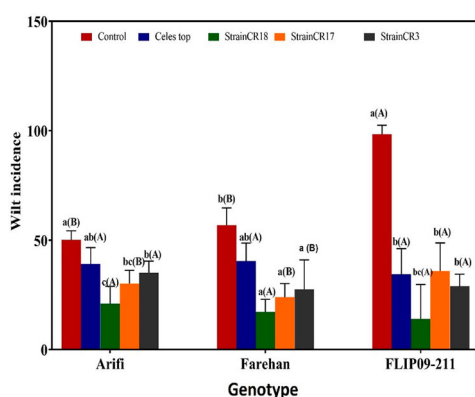


**Figure 9.** Effects of bacterial strains and fungicide seed treatments on *Fusarium* wilt on susceptible chickpea genotype (FLIP09-211) after 60 days under greenhouse conditions. (B1) StrainCR18, (B17) StrainCR17, (B3) Strain CR3, (F) Celest Top fungicide, (C) control.

made to use Chitinolytic bacteria to control some diseases (Swiontek et al. 2014; Veliz et al. 2017). In addition to their ability to degrade chitin and  $\beta$ -1,3-glucan of fungal cell walls, chitinolytic bacteria can also stimulate

various defense responses in plants (Ali et al. 2020; Kumar et al. 2018).

In order to confirm the anti-fungal activity, three antagonistic bacteria were selected for *in vivo* test (Figure 10), all analysed bacteria decreased the wilt incidence, this result is in accordance with the results of Landa et al. (2004) which showed that Soil treatment with *Pseudomonas fluorescens*, *Bacillus megaterium* and *Paenibacillus macerates* inhibited *Fusarium* wilt in chickpeas (*Cicer arietinum*), ours results agree also with those of other authors which showed that chickpea plants could be protected against *Fusarium oxysporum* by inoculating the soil with antagonistic bacteria (Kumari and Khanna 2014; Jamali et al. 2004; Subhani et al. 2013; Zaim et al. 2013). The *in-planta* effect of these bacteria could be explained by a directed interaction against the pathogen (parasitism, competition for nutrients, and antibiosis) or by an indirect action through induced resistance of the host plant (Srivastava et al. 2021). Our results showed the importance of an environment-friendly method for suppressing diseases which is the use of rhizobacteria. However, the mode of action of the antagonistic in reducing the disease incidence was



**Figure 10.** Effects of bacterial strains and fungicide seed treatments on mean percentage of *Fusarium* wilt incidence on chickpea. Vertical bars represent the standard deviation of the mean. Lowercase letters indicate significance in the same genotype, capital letters in parentheses indicate significance in each treatment ( $p < .05$ )



not included in this study and needs further investigation.

## Conclusions

In this study, we were able to identify potential antagonistic bacterial strains that can be used to manage *Fusarium* wilt of chickpea in Morocco. The majority of the potential strains are belonging to the genus *Bacillus* and *Penibacillus*. The potential antagonist bacterial isolates employed varying levels of diffusible and volatile compounds and different enzymes. Three potential antagonist strains (StrainCR18, StrainCR17, and StrainCR3) were very effective in reducing *Fusarium* wilt incidence on chickpea genotypes with varying levels of host resistance to the disease. These potential antagonist bacterial strains should be further tested under field conditions for future uses as components of integrated disease management. Moreover, the mechanism of disease control of the potential antagonistic strains *in-planta* will be investigated in the future.

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