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RESEARCH

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Acinetobacter calcoaceticus SJ19 and *Bacillus safensis* SJ4, two Algerian rhizobacteria protecting tomato plants against *Botrytis cinerea* and promoting their growth

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Abstract

Background: *Botrytis cinerea*, the causal agent of grey mould, is a polyphagous fungus that infects a wide range of plants, including tomato. In many countries, including Algeria, the management of grey mould is a challenging problem, even with chemical control. This necessitates the search for other strategies. The objective of this study was to evaluate the biocontrol potential of two rhizospheric bacterial isolates for the protection of tomato against *B. cinerea*.

Results: On tomato plants, the two bacterial isolates consistently provided high levels of protection (on average 72.1% for SJ19 and 52.3% for SJ4). Their applications as a 1:1 mixture reinforced their effect (87.8% protection), indicating their compatibility and a potential use as a consortium. In vitro, the isolates significantly inhibited the mycelial growth of *B. cinerea* strains, both through direct confrontation in dual-culture assays (12–69% inhibition) and through the production of volatile compounds (36–46% inhibition). The two isolates, applied as seed treatment and as drench on seedlings, also showed strong growth-promoting effects on tomatoes. They substantially increased the length and fresh weight of shoots and roots, as well as stem diameter, leaf number and chlorophyll content, compared to untreated plants.

Conclusion: The two bacteria tested in this study showed a high potential for use as biostimulants and as biofungicides against tomato grey mould.

Keywords: Biocontrol, PGPR, *Botrytis cinerea*, *Acinetobacter calcoaceticus*, *Bacillus safensis*, Tomato, Algeria

Background

Tomato grey mould caused by *Botrytis cinerea* Pers (anamorph of *Botryotinia fuckeliana* (de Bary) Whetzel) is becoming one of the most serious concerns in Algeria's unheated greenhouses (Aissat et al. 2008). This necrotrophic and polyphagous fungus can attack the tomato's

aerial parts, causing symptoms such as concentric spots on the leaves, lesions and stem cankers. Fruits, whether pre- or post-harvest, have been known to be affected by this disease. The most well-known symptoms on tomato fruits are “ghost spots” (Panno et al. 2021). Algerian farmers in the coastal districts, such as Bejaia and Jijel, are accustomed to installing their greenhouses close to the sea, which results in particularly humid conditions. Under these conditions, several fungal diseases like grey mould become a challenging problem to manage (Aissat et al. 2008).

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In fact, to manage this disease, which is responsible for significant annual losses around the world, farmers often rely on chemical fungicides, which are viewed as the most effective tool to control this disease (Panno et al. 2021). A common practice was to spray the aerial parts of plants as a preventative strategy before the disease was observed (Liu et al. 2021). However, this type of control has been associated with a number of issues all over the world. It has been demonstrated that *B. cinerea*, due to its genetic variability, can develop resistance to a variety of commonly used chemical fungicides (Liu et al. 2021). Undesirable side effects of pesticides have increasingly generated concerns about possible public health and environmental issues. All of these limitations have prompted researchers and agronomists to seek out alternate strategies of disease and pest management (Walia et al. 2021). Biological control has increasingly been used in various countries as a key tool for these new control strategies. It entails the use of either living (such as microorganisms) or natural products, such as plant extracts and essential oils derived from natural sources (Panno et al. 2021). Currently, however, no microbial bio-fungicide is authorized for agricultural use in Algeria (Anonymous 2017). In this context, this study was initiated with the objective of evaluating biocontrol potential of two indigenous rhizobacteria, applied singly or in combination, for the protection of tomato against *B. cinerea*. Their ability to promote plant growth was also assessed.

Methods

Bacterial isolates

The two bacterial isolates used in this study (SJ4 and SJ19) were isolated in 2018 from the rhizospheric soil of healthy tomato plants cultivated in unheated greenhouses near Jijel, Algeria. Isolation, purification and storage were performed according to Akter et al. (2015). Their identification was carried out on the basis of their molecular characterization. For this, their DNA was extracted and the amplification of the 16S rDNA was carried out using the universal primers fd1 and S17 as described by Bouaoud et al. (2018). The PCR results were sent for sequencing. The NCBI database (<https://www.ncbi.nlm.nih.gov>) and the EzBioCloud platform (<https://www.ezbiocloud.net>) were used to compare the sequences and determine the species. Based on this analysis, the bacterial isolates SJ4 and SJ19 were assigned, respectively, to the species *Bacillus safensis* (GenBank accession number: OK562384) with 100% of similitude and to *Acinetobacter calcoaceticus* (GenBank accession number: OK562383) with 99% of similitude.

Botrytis cinerea strains

Five strains of *B. cinerea* were used in this study. Four strains (BCJ1, BCJ2, BCJ3 and BCJ4) were isolated in the Jijel District from tomato plants cultivated in unheated greenhouses and exhibiting grey mould symptoms. Strain BC21 was provided by the Mycology Laboratory of INRAE Avignon, France. All the strains were stored at -20°C . For revivification, culture and inoculum production, PDA medium (Difco Laboratory Detroit, USA) was used according to Bouaoud et al. (2018).

Tomato plants

Tomato plants (variety Clodano; Syngenta Seeds, Switzerland) were grown in individual pots in a heated greenhouse and used 6 weeks after sowing, for *in planta* biocontrol assays.

In vitro antagonistic effect of the bacteria against *Botrytis cinerea*

Direct confrontation

To evaluate the direct *in vitro* antagonistic effect against five strains of *B. cinerea*, the bacterial isolates were placed in contact with the fungal pathogen on the surface of PDA medium (dual culture assay) as described by Xu and Kim (2014). Petri dishes containing only the fungus without bacteria were prepared in parallel with the other plates. After 72 h of incubation at 22°C , the percentage of mycelial growth inhibition was calculated according to Whipps (1987). Three plates were used per bacterial isolate, and the experiment was repeated three times independently.

Indirect confrontation

The two bacteria were assessed for their ability to produce volatile antifungal compounds according to the protocol described by Barakat et al. (2014). Strain BC21 of *B. cinerea* was used in these tests. To determine the percentage of mycelial growth inhibition, control plates without the bacteria were prepared and incubated together with the other plates at 22°C for 72 h. Three plates were used per bacterium, and three independent repetitions of the whole experiment were carried out.

In planta biocontrol assays

To evaluate the *in planta* antagonistic activity of the two bacterial isolates against *B. cinerea*, 6-week old tomato plants were used. Two leaves were removed from each plant, and the wounds were inoculated using 10- μl aliquots of a spore suspension of *B. cinerea* (strain BC21) at 10^6 spores/ml. The wounds were then immediately treated either with a bacterial suspension (10 μl

per wound; 10^9 CFU /ml) or left untreated as control plants. The two bacterial isolates were applied either singly, or in combination using a 1:1 (V/V) mixture of the cell suspensions. To evaluate a possible effect of the time of application of the bacteria on their protective efficacy, additional modalities were used consisting of plants with an application of the bacteria on the wounds either 1 h before or after the inoculation with *B. cinerea*. Thus, in total, the biocontrol assay entailed 10 modalities of treatments, and 5 replicated tomato plants were used per modality. The whole assay was carried out three times independently.

Following inoculation, all the plants were incubated in a growth chamber (14-h photoperiod; 22 °C; 90% RH) for 7 days (Bouaoud et al. 2018). The development of disease from each inoculated wound was monitored by measuring the length of stem lesions between the 3rd and the 7th day after inoculation. Then, an area under the disease progress curve (AUDPC) was computed for each wound. The percentage of protection conferred by each bacterial isolate was assessed as follows: Protection (%) = $100 \times (\text{average AUDPC}_{\text{control}} - \text{average AUDPC}_{\text{bacterial treatment}}) / \text{average AUDPC}_{\text{control}}$ (Decognet et al. 2009).

Pathogenicity and induction of hypersensitive response (HR)

In conjunction with the biocontrol assays described above, batches of tomato plants were used to evaluate a possible deleterious effect of the bacterial isolates. For this, leaves were removed from the plants as described above, but the wounds were not inoculated with *B. cinerea*. Instead, 10- μ l aliquots of the bacterial suspensions were applied to the wounds and the plants were incubated in a growth chamber as described above. After 7 days of incubation, the wounds were examined for possible symptoms. Five plants were used for each bacterial isolate, and the whole experiment was carried out three times independently.

Another test was used to evaluate the ability of the two bacteria to induce a hypersensitivity response. Tobacco (*Nicotiana tabacum*) leaves were infiltrated with suspensions of the two bacterial isolates (10^9 CFU/ml) according to Morris et al. (2010). Control plants were infiltrated with a suspension of *Pseudomonas syringae* strain CC94 as a positive control, while plants infiltrated with sterile distilled water were used as a negative control. The plants were then incubated for 48 h in a growth chamber at 22 °C with a 14-h photoperiod, and the plants were examined for possible necroses around the inoculation point, as a sign of hypersensitive response.

Tomato growth promotion

To assess the potential of the two bacteria to enhance tomato growth, tomato seeds (variety Clodano; Syngenta Seeds, Switzerland) were surface-sterilized by tipping its 3 min in 70% ethanol and 4 min in 0.9% (v/v) sodium hypochlorite solution. The seeds were then washed three times using sterile distilled water (Boukaya et al. 2018).

Batches of 25 seeds were then soaked for 2 h at room temperature in a suspension (10^9 cells/ml) of either SJ4 or SJ19. Control batches were soaked in sterile water. The seeds were then sown in sterile potting soil and placed in a heated glasshouse. Seven days after sowing, seed germination was assessed for each seed batch, and the seedlings were carefully uprooted and washed. Seedling growth was assessed, and a seed vigor index was computed according to Syed-Ab-Rahman et al. (2018) as the mean root length \times percentage of seed germination. This assessment was carried out on 20 seedlings for each modality of seed treatment. The seven-day old seedlings were then transplanted to individual (10 \times 15 cm) pots containing a sterile potting soil and grown in the glasshouse. Five seedlings were transplanted for each modality of seed treatment. Two days after transplanting, each seedling received a drench treatment with 5 ml of a similar bacterial suspension as the seed it had germinated from (drench with sterile distilled water for the control seedlings) (Mohamed et al. 2020). Four weeks after this treatment, the following morphometric parameters were measured on the plants: root and shoot lengths (cm), stem diameter above the second leaf (mm), number of leaves, and fresh weight of roots and shoots (g). The chlorophyll content of the leaves was measured with the help of a portable chlorophyll meter (Konica Minolta SPAD 502). The whole experiment was carried out three times independently.

Statistical analyses

Analysis of variance (ANOVA) with post hoc test (Newman–Keuls multiple range test) was used to test the growth-promoting *in vitro* and *in planta* effects of the two bacterial isolates. However, a Mann–Whitney test was used to check the effect of the two bacterial strains on seed germination. Statistica (StatSoft Inc., Tulsa, USA) was used to conduct all the statistical analyses.

Results

In vitro inhibition of *Botrytis cinerea* by the bacterial isolates

Direct confrontation

In dual culture tests, both bacterial isolates significantly inhibited the mycelial growth of the five strains of *B. cinerea* (Table 1 and Fig. 1). With average inhibition rates

Table 1 In vitro effect of two bacterial isolates against various *Botrytis cinerea* strains

| Isolates | Bacterial species | GenBank accession number | Mycelial Growth Inhibition (%) ¹ | | | | | Volatile compounds |
|----------|------------------------------------|--------------------------|---|----------------|----------------|----------------|----------------|--------------------|
| | | | Direct confrontation | | | | | |
| | | | BC21 | BCJ1 | BCJ2 | BCJ3 | BCJ4 | |
| SJ4 | <i>Bacillus safensis</i> | OK562384 | 69.02 ± 5.35 Δ | 59.52 ± 1.59 Δ | 43.65 ± 6.88 Δ | 57.94 ± 1.06 Δ | 66.67 ± 1.59 Δ | 46.22 ± 2.92 Δ |
| SJ19 | <i>Acinetobacter calcoaceticus</i> | OK562383 | 38.67 ± 0.5 Δ | 19.84 ± 2.12 Δ | 23.02 ± 1.06 Δ | 22.22 ± 1.06 Δ | 11.90 ± 2.51 Δ | 36.02 ± 1 Δ |

Values represent the mean from three independently repeated experiments (each with three elementary replicates) ± SE

¹ Within a column, Δ symbols indicate a significant difference between the values of each modality and the control as indicated by Newman–Keuls multiple range tests applied to the diameter values ($P < 0.05$)

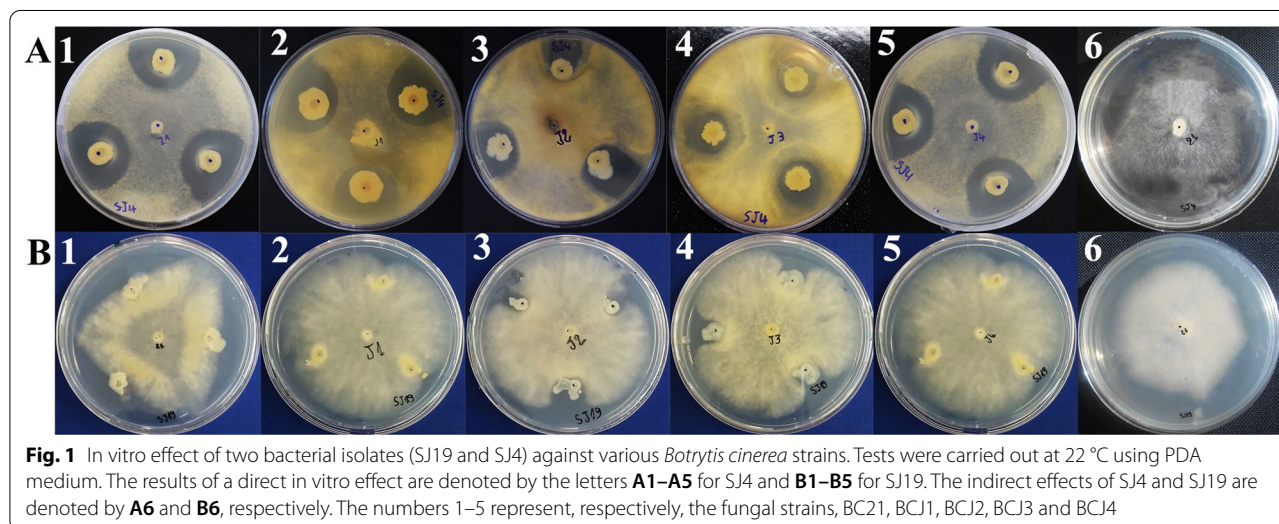


Fig. 1 In vitro effect of two bacterial isolates (SJ19 and SJ4) against various *Botrytis cinerea* strains. Tests were carried out at 22 °C using PDA medium. The results of a direct in vitro effect are denoted by the letters **A1–A5** for SJ4 and **B1–B5** for SJ19. The indirect effects of SJ4 and SJ19 are denoted by **A6** and **B6**, respectively. The numbers 1–5 represent, respectively, the fungal strains, BC21, BCJ1, BCJ2, BCJ3 and BCJ4

of 43.7–69.0%, isolate SJ4 showed a high level of inhibition against the strains of *B. cinerea* than isolate SJ19 (11.9–38.6% inhibition).

Indirect confrontation

Both bacterial isolates significantly inhibited the mycelial growth of *B. cinerea* (strain BC21) through the production of volatile antifungal compounds (Newman–Keuls multiple range test; $P < 0.05$ for SJ4 and SJ19) (Table 1 and Fig. 1). Isolate SJ4 provided significantly higher inhibition than SJ19.

Protective effect of the bacteria on tomato plants

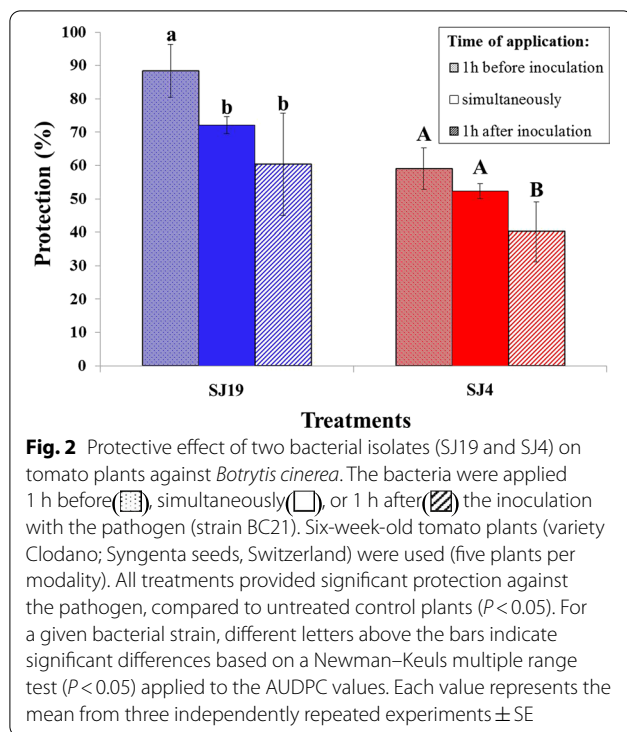
On tomato plants, treating the wounds with the bacterial isolates immediately after inoculation resulted in a significant protective effect against *B. cinerea* (Fig. 2). With a protection index of 72.1%, isolate SJ19 was significantly more efficient than SJ4 (52.3%) (Newman–Keuls multiple range test; $P < 0.05$). The 1:1 combination of the two bacteria had the greatest protective effect (87.8%), suggesting that these antagonists can work together to protect tomato plants against *B. cinerea*.

Applying the bacterial isolates on the wounds, one hour before or after inoculation with *B. cinerea*, also resulted in a significant protection (Fig. 2). The highest level of protection was observed, for both bacterial isolates, when the treatment was applied one hour before inoculation (Fig. 2).

Plant growth-promoting effects of the bacterial isolates

Soaking the tomato seeds in suspensions of the bacteria did not substantially alter their germination rate (Mann–Whitney test, $P = 0.3$ for SJ4 and $P = 0.072$ for SJ19), but the seed vigor index was increased by 52 and 23%, respectively, by isolates SJ19 (Newman–Keuls multiple range test, $P < 0.0001$) and SJ4 (Newman–Keuls multiple range test, $P = 0.006$) (Fig. 3).

One month after the drench treatment of seedlings obtained from treated seeds, the plant growth indicators also showed a significant effect of the bacteria, in comparison with untreated controls. Treatments with isolate SJ19 significantly enhanced the lengths of roots and shoots by 99 and 43%, respectively, than the untreated controls (Newman–Keuls multiple range test, $P < 0.05$),



while isolate SJ4 increased these two parameters by 88 and 45%, respectively (Newman–Keuls multiple range test, $P < 0.05$) (Fig. 4).

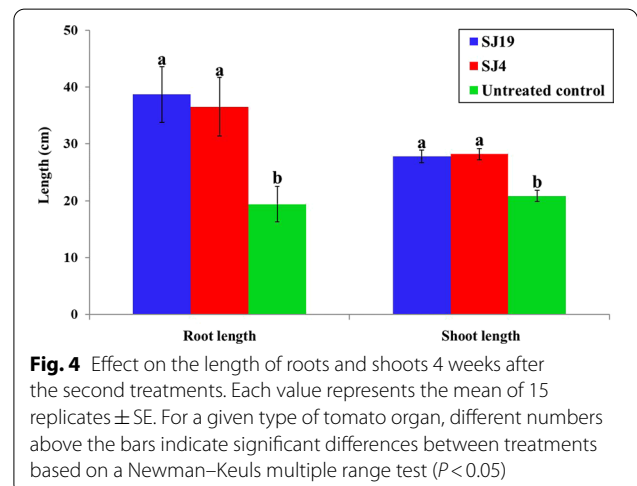
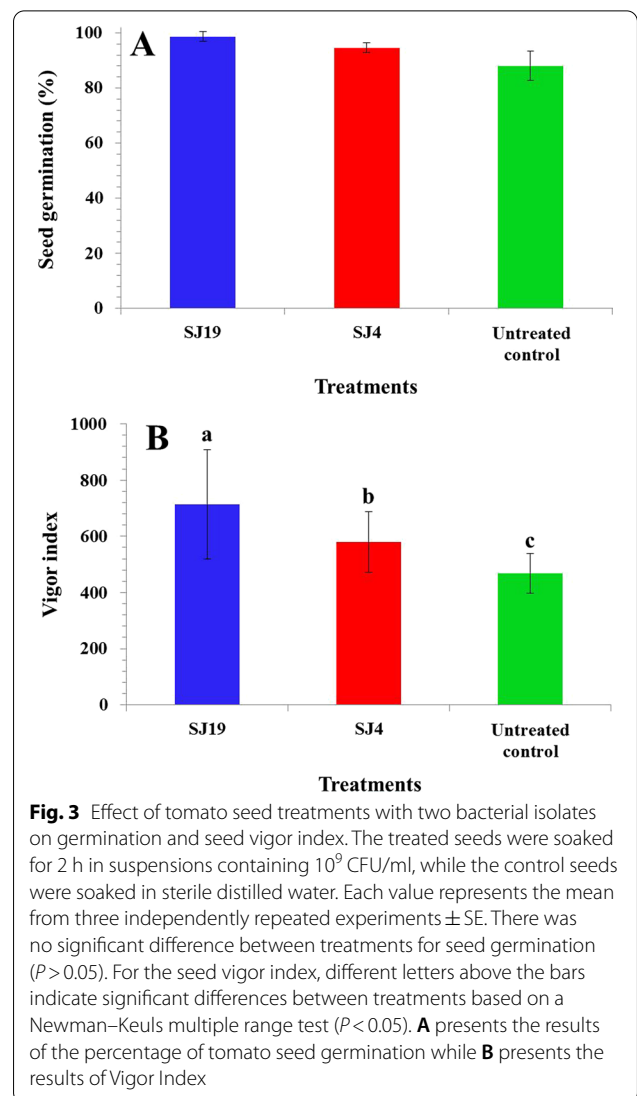
In addition, treatments with the bacteria significantly enhanced the fresh weight of shoots (Newman–Keuls multiple range test, $P < 0.05$; Fig. 5). In comparison with the control plants, the fresh weight of shoots was increased by 102 and 83% and the roots by 69 and 58%, respectively, by SJ19 and SJ4. The treatment of tomato seedlings with the bacteria also had a positive effect on stem diameter, number of leaves and chlorophyll content (Table 2).

Pathogenicity and induction of hypersensitive response (HR)

Following the application of the bacterial isolates on wounds of tomato plants, no disease symptoms were observed. Similarly, no necrosis or other symptoms were observed following their infiltration in leaves of tobacco plants.

Discussion

The present study provided evidence for the plant growth-promoting effect and for the biocontrol potential of two bacteria isolated from rhizospheric soil in Northern Algeria, which were identified as *Acinetobacter calcoaceticus* (isolate SJ19) and *Bacillus safensis* (isolate SJ4). The combination of *in vitro* and *in planta* experiments



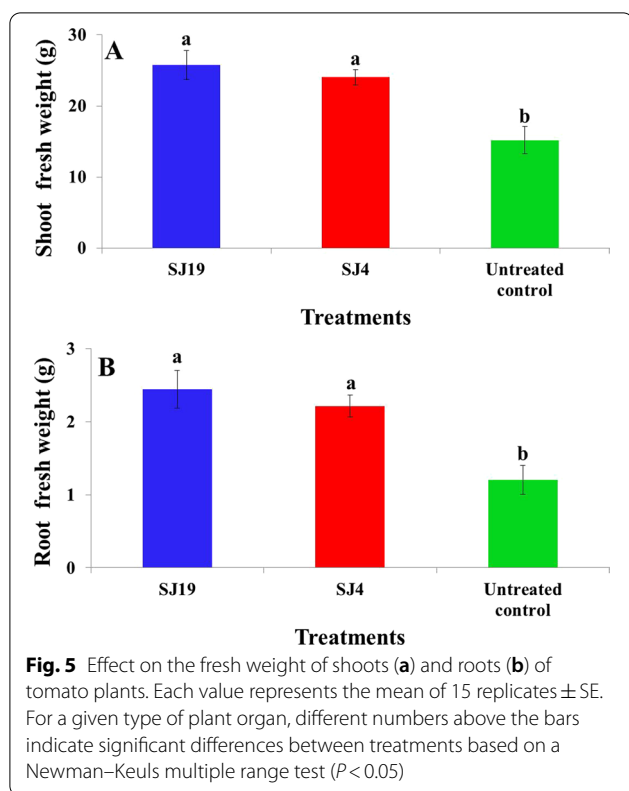


Table 2 Effect on stem diameter, chlorophyll content and number of leaves of tomato plants

| Treatments | Stem diameter (mm) | Chlorophyll content (SPAD) | Number of leaves |
|------------|--------------------|----------------------------|------------------|
| Control | 6.8 \pm 0.24b | 38.09 \pm 2.46b | 5.6 \pm 0.48b |
| SJ4 | 7.6 \pm 0.56b | 43.71 \pm 1.27a | 7.2 \pm 0.32a |
| SJ19 | 8.3 \pm 0.24a | 44.97 \pm 1.60a | 7.2 \pm 0.32a |

Values represent averages of 15 replicates \pm SE. Within a column, the same letters next to each value indicate that there is no significant difference as indicated by Newman–Keuls multiple range test ($P < 0.05$)

carried out in the study also allowed to propose hypotheses on the possible mode of action implicated in the protection of tomato against one of its key pathogens, *B. cinerea*. In vitro assays showed that the mycelial growth of *B. cinerea* was significantly inhibited by the two isolates SJ4 and SJ19 with varying degrees of effectiveness. In accordance with the present results, several studies have reported the in vitro antagonistic effect of *Bacillus* (You et al. 2021) and *Acinetobacter* (Faria et al. 2021) species against a broad range of phytopathogenic fungi. Furthermore, species belonging to these two genera are known for producing diffusible and volatile antifungal compounds (You et al. 2021). Indeed, in the present study, SJ4

and SJ19 reduced the development of disease on tomato plants. Despite this, isolate SJ19 was not very effective against the pathogen in Petri plates. In line with these results, the efficacy of isolate SJ19 on plants suggested that it may suppress the disease by inducing plant resistance. This mechanism of action was shown by Trotel-Aziz et al. (2008) who revealed the capacity of two strains of *Acinetobacter lwoffii* that induced the plant defences against *B. cinerea* on grapevines. *Bacillus* species have also been found to stimulate plant defences against a wide range of diseases. Alamri et al. (2019) showed that *B. subtilis* HQ656002 was able to induce lettuce defence mechanisms against root rot caused by *Exserohilum rostratum* and *Fusarium oxysporum*. GTPase-activating protein, which played an important role in plant defence against pathogenic fungi, was identified, and the defensin genes were expressed in the presence of this bacterium. In fact, the influence of rhizospheric bacteria was demonstrated against a broad spectrum of tomato pathogens including fungi and bacteria (Zheng et al. 2019).

The present study provided results which could support the hypothesis that the tested isolates can induce defence mechanisms on tomato plants. The application of these isolates, one hour prior to the pathogen inoculation, improved their ability to protect tomato plants against *B. cinerea*, suggesting that these isolates can induce a defence reaction on tomato plants against the pathogen, and this mechanism of action was unable to express itself in vitro (at least for SJ19). According to Pascholati and Leite (1995), the time interval between the antagonist application and inoculation of the pathogen plays an important role in the protection induced by a biocontrol agent. For instance, in comparison with the curative treatment (24 h after pathogen inoculation), the preventive treatment (24 h before pathogen inoculation) with *Bacillus amyloliquefaciens* strain QST713 better protected bean plants against four mutants (among six mutants tested) of *B. cinerea* (Samaras et al. 2021).

In the present study, a synergistic effect of SJ4 and SJ19 was recorded. The mixture of the two antagonists enhanced the protective effect against *B. cinerea* on tomato plants. The results are consistent with those of other research showing that combining multiple PGPRs can improve their biocontrol potential (Sharma et al. 2018). In general, PGPR mixtures employed as biocontrol agents have the advantage of being able to combine their multiple traits, especially those that are difficult to find in a single bacterium. This distinguishing characteristic plays a critical role in enhancing the effectiveness and reliability of disease control (Wang et al. 2021).

SJ19 and SJ4 isolates significantly promoted the growth of tomato plants by increasing seed vigor index, number of leaves, stem diameter, contents of chlorophyll, length

of roots and shoots and plants fresh weight. Other studies have found similar results (Alamri et al. 2019). Indeed, the tested *Acinetobacter* and *Bacillus* species are known as power PGPRs enhancing plant growth including tomatoes (Mohamed et al. 2020). The PGP effect depended on the inoculation method. Xue et al. (2009) showed that the inoculation of *Acinetobacter* sp. strain Xa6 by drenching method promoted tomato growth better than the damping method. Besides, the ability of *Acinetobacter* and *Bacillus* species and the other PGPRs to promote plants growth is often assured by the synthesis of certain secondary metabolites.

Conclusions

In conclusion, the present study highlights the capacity of two rhizospheric bacteria (SJ4 and SJ19) to protect tomato plants against *B. cinerea*. *B. safensis* strain SJ4 and *Acinetobacter calcoaceticus* strain SJ19 also promoted the growth of tomato plants. These encouraging findings could be the first step towards using these two bacteria in the field as bio-fungicidal agents to control grey mould and biofertilizers to promote plants growth. Nevertheless, additional research appears to be required to understand the behaviour of these bacteria in the environment.

Abbreviations

AUDPC: Area under the disease progress curve; CFU: Colony-forming unit; HR: Hypersensitive response; NCBI: National Center for Biotechnology Information; PDA: Potato dextrose agar; PGPR: Plant growth-promoting rhizobacteria; RH: Relative humidity.

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Authors' contributions

AF involved in the sampling and isolation of bacteria and fungi, and the writing of the manuscript. This research is part of his doctoral thesis project. YB contributed to the in vitro tests and the identification of *B. cinerea* strains. CC involved in the molecular characterization of bacteria. MD prepared culture media and conserved the bacteria and the fungal strains. MT is a plant pathologist; he was involved in the detection of grey mould on tomato plants and carried out pathogenicity tests of *B. cinerea* on tomato plants. KA revised the article. He is the co-supervisor of AF PhD. PN supervised the study of AF PhD and revised the article. All authors read and approved the final manuscript.

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Availability of data and materials

We confirm the availability of all the data included in this study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The corresponding author declares that there are no competing interests on behalf of all authors.

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