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In vitro and *in vivo* antifungal activity of different bacterial isolates against *Botrytis* gray mold of tomato

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Abstract

Gray mold rot, caused by *Botrytis cinerea*, is considered as one of the most harmful fungal diseases affecting postharvest tomato fruits. In the current work, we explore the biocontrol potential of 174 bacterial strains isolated from tomato rhizosphere, argan rhizosphere and a vermicompost to control this fungal pathogen. The *in vitro* dual culture study of isolated bacterial strains and the fungus-causing pathogen revealed that 31 isolates exhibited a substantial antifungal activity against *B. cinerea* mycelial growth. The inhibition rates ranged from 55.36 to 85.07%. Twelve bacterial strains which showed the highest antifungal properties (more than 79.6%) were subsequently investigated for their ability to produce compounds with antifungal activity, including diffusible substances, volatile compounds and hydrocyanic acid. These strains were then evaluated *in vivo* for their ability to reduce fruit decay caused by the fungus. Indeed, isolates VC-B1 (from vermicompost), RS-TB3, and RS-T6 (from the rhizosphere of tomato root), and RH-TB11 (from the tomato rhizoplane) released the most active diffusible substances, which totally inhibited the mycelial growth of *B. cinerea*. In addition, bacterial strains RS-TB1 and RS-T6 isolated both from the tomato rhizospheric soil were among the isolates that produced the most effective volatile compounds. They reduced the mycelial growth of the fungal pathogen by 75.36% ± 7.24% and 72.94 ± 4.129% respectively. Moreover, RS-TB3, RS-TB4, RH-TB1, and VC-B1 exhibited the highest production of hydrocyanic acid, followed by RS-TB1, VC-3, and VC-13. *In vivo* bioassays showed disease reduction ranging from 37.92% to 93.14%, with VC-1, VC-5, and RS-T4 showing the highest efficacy. This study identifies several bacterial strains with high potential for biocontrol of *B. cinerea* in postharvest tomatoes, presenting a promising alternative to chemical fungicides for managing gray mold rot.

Keywords: biological control; fungal disease; gray mold rot; postharvest; tomato

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Introduction

Tomato (*Lycopersicon esculentum*) is one of the world's most popular fruit crops. It is grown for both fresh produce markets and the processed food industry (Fagundes *et al*., 2013). Tomato crop has an important nutritional value due to its high content of vitamins (A, C) and minerals (Bu *et al*., 2021). In Morocco, tomato fruit is regarded as the main fruit crop exported, contributing significantly to the growth of the country's economy, the annual production of tomatoes exceeds 1.3 million tons (Faostat, 2023). Sixty per cent of the national tomato production is produced in the Souss-Massa region, mainly under greenhouse conditions (Ait Hou *et al*., 2015; Qessaoui *et al*., 2021). However, tomato fruit is attacked by a wide range of pathogenic microorganisms and numerous phytosanitary issues have arisen as a result of its intensive cultivation. In addition to phytopathogenic bacteria, viruses and deleterious microorganisms (Amkraz *et al*., 2010; Li *et al*., 2019), fungal attacks are important factors that reduce the fruit quality and performance of the crop. One of the most harmful fungal pathogens of tomatoes is gray mold, which is caused by *Botrytis cinerea*. Gray mold is considered as the second-most significant disease in agriculture in the world, with losses exceeding $10 billion (Dean *et al*., 2012). This necrotrophic fungus causes significant financial losses in tomatoes grown in both open fields and greenhouses (Ni and Punja, 2019). These losses are mainly occurred during fruit picking, handling and transportation to the markets.

Botrytis cinerea attacks various parts of a host plant including leaves, stems, flowers and fruits (Borges *et al*., 2014) either directly or through wounds induced by cultivation practices (Peng *et al*., 1996). The decay is more prevalent after the harvest and during the rainy season. Measures to control gray mold are essentially based on repeated applications of synthetic chemical fungicides. Depending on the severity, anywhere from one or two treatments to more than twenty may be necessary per season. However, due to severe regulations, the emergence of disease resistance and growing public concern over chemical residues in fruit, the widespread use of fungicides is progressively restricted (Nigro *et al*., 2006). Moreover, the long-term use of chemicals has been shown to affect non-target microorganisms and result in soil contamination and environmental toxic effects (Martins *et al*., 2019).

Several investigations have demonstrated that *B. cinerea* is extremely resistant towards the most used anti-Botrytis fungicides. Indeed, according to Elad *et al*. (1992) *B. cinerea* rapidly acquired resistance to a variety of fungicides including benzimidazoles, dicarboximides and inhibitors of sterol biosynthesis. A significant level of *B. cinerea* resistance to Fenhexamid, the main fungicide used in Moroccan tomatoes cultivated under greenhouse conditions was recently reported by Halime *et al.* (2019). Thus, it is critical to develop a strategy that is both safer and more environmentally friendly for managing gray mold on tomato crops before and after the harvest.

Wang *et al.* (2021) reported that various microorganisms, including many bacteria such as *Pseudomonas chlororaphis* ZL3, have been studied extensively as a potential substitute for preventing postharvest gray mold on fruits and vegetables. Lian *et al.* (2017) reported the effectiveness of *Streptomyces pratensis* LMM15 as a biocontrol agent to suppress *B. cinerea* in tomato. *Pseudomonas* strain QBA5 has been reported to significantly inhibit the mycelial growth, spores' germination and germ tube elongation of *B. cinerea* (Gao *et al*., 2018).

Microbial biocontrol agents have been used to suppress post-harvest diseases because of their safety and compatibility with the environment (Jiang *et al*., 2015). Importantly, microbial biocontrol agents involve various mechanisms of action including induction of systemic resistance, competition for nutrients and space, direct hyphal parasitism and antibiosis production (Veloso and Díaz, 2012; Spadaro and Droby, 2016; Arrarte *et al*., 2017; Martins *et al*., 2019). A combination of the mechanisms mentioned above may be responsible for the biological control of pathogens (Jiang *et al*., 2015). Sadfi-Zouaoui *et al.* (2008) reported that in Europe and the United States, several microorganism species are receiving commercial approval for treatment against fungal plant diseases. These include fungi (*Glicoladium virens* G.21, *Glicoladium catenulatum* J1446, *Trichoderma*

harzianum (Prestop, KRLAG2, Trichodex, RootShield), *Candida oleophila* 182), Gram-negative bacteria (*Pseudomonas fluorescens* EG1053, *P. syringa* ESC-10 and ESC-11, *Burkolderia cepacia*) and Gram-positive bacteria such as *Bacillus subtilis* Quadra 136.

The objective of this work is two-fold; (i) to screen isolated bacteria for their potential effect on *Botrytis cinerea,* the causal agent of tomato gray mold and (ii) to assess, *in vivo* performance of selected antagonistic bacteria on tomato fruits.

Materials and Methods

Botrytis cinerea isolation and conidial suspension preparation

A virulent *B. cinerea* isolate was obtained from infected tomato fruits with typical gray mold symptoms. The isolated fungus was subcultured on potato dextrose agar (PDA) and incubated in darkness at 25 °C for 5 days. The conidial suspension was prepared by scratching a 5-day-old fungal colony flooded with 9 ml of sterile distilled water (SDW) supplemented with 0.05% of Tween 20. A fine scalpel was used to assist the dispersal of conidia. To remove medium debris and hyphal fragments, the resulting liquid was filtered through four layers of sterile cheesecloths. The spore concentration was adjusted to 10^5 spores/ml under an optical microscope using a haemocytometer.

Isolation of potential antagonistic bacteria

Isolation of bacteria from tomato rhizosphere (rhizospheric soil, rhizoplane and endorhizosphere)

Samples of rhizospheric soil with tomato roots were thoroughly collected from a tomato field in the Souss-Massa Valley, Agadir, Morocco and stored at 4 °C before analysis. The bacterial populations of the rhizospheric soil (RS), the rhizoplane (RH) and the endorhizosphere (EN) were isolated as described by Amkraz *et al.* (2010).

To isolate bacteria from tomato rhizospheric soil (RS-T), 100 ml of sterile physiological water (0.9% NaCl) was mixed with 10 g of rhizospheric soil obtained by shaking tomato roots. The resulting suspension was shacked for 40 min using a magnetic stirrer.

Bacteria from the tomato rhizoplane (RH-T) were isolated as follows: rhisopheric soil was removed and 1 g of fresh root fragments was placed in a 100 ml bottle containing 10 ml 0.9% NaCl and agitated for 2 h using a magnetic stirrer.

To isolate bacteria from the tomato endorhizosphere (EN-T), fragments of tomato roots that had been cleaned of rhizospheric soil were subsequently surface-disinfected using a 2.5% sodium hypochlorite solution for 3 min, followed by three washes with sterile distilled water (Mew and Rosales, 1986). Afterwards, 10 ml of sterile physiological water was added to a sterile mortar, and one gram of these fragments was ground to fine powder.

Serial ten-fold dilutions were then prepared from each extract (RS-T, RH-T and EN-T) and 0.1 ml of the resulting dilutions were distributed in three replicates on Petri dishes containing tryptic soy agar medium (TSA) and they were then incubated at 28 °C for 48 hours (Amkraz *et al.*, 2010). Colonies with various morphologies were selected and re-streaked on TSA until pure cultures were obtained.

Isolation of bacteria from the rhizospheric soil of the argan tree and from a vermicompost

The isolation of bacteria was conducted as follows; 10 g of sieved rhizospheric soil of the argan tree (RS-A) or a vermicompost (VC) collected from several areas in the Souss-Massa Valley was deposited in a bottle containing 90 ml of sterile physiological water. The resulting suspension was shacked for 40 min using a magnetic stirrer. A series of decimal dilutions was then performed (Kamali *et al.*, 2019). Isolation and enumeration of bacteria were carried out as previously described procedure.

For isolation and enumeration of spore-forming bacteria, the dilutions were heated in an 80 °C water bath for 10 minutes, and then 0.2 ml of each dilution was spread on TSA agar culture medium. Three replicates were performed for the first three dilutions, and the plates were incubated at 28 °C for 72 h (Kamali *et al.*, 2019). A total of 174 colonies were collected and coded, then stored in TSB medium supplemented with 40% glycerol at – 20 °C until further use.

Fruit preparation

Red tomatoes (*Lycopersicon esculentum*) used in this study were harvested from a commercial orchard in the Souss-Massa Valley, with no prior postharvest treatment. The selected fruits were homogeneous in size and color, free from decay and injuries. All fruits were surface-disinfected by soaking them into 0.1% sodium hypochlorite solution for 10 min, then washed thoroughly three times with sterile distilled water and air dried for 1 hour at room temperature under laminar flow cabinet (Tahiri Alaoui *et al.*, 2017). After drying, two artificial wounds (3 mm in diameter and 3 mm in depth) were performed on each fruit's equatorial sides using a sterile needle. Wounded fruits were placed in a laminar flow cabinet till the wounds were dry prior to being used in the various *in vivo* experiments (Sadfi-Zouaoui *et al.*, 2008).

Screening of antagonist bacteria

The purified bacterial isolates were tested against *Botrytis cinerea* using dual culture technique by direct confrontation in Petri dishes as described by Ezrari *et al.* (2021). Along the edge of the Petri dish, each bacterial isolate was streaked at four equally spaced intervals. After being incubated at 25 °C for 24 hours, a 7 mm diameter mycelial plug taken from the margin of a 5-day-old *B. cinerea* colony was inserted onto the center of the agar plate containing PDA medium between various streaks of each bacterium. Plates inoculated with the pathogen alone were served as controls. Afterwards, the plates were then incubated at 25 °C for 7 days. The inhibition rate of mycelial growth (IR) of *B. cinerea* was calculated using the following formula: IR (%) = [(DC – DT) /DC] × 100 where DC is the diameter of the colony in control treatments while DT corresponds to the diameter of the colony in confrontation treatments. For each treatment, three replicates were performed and only isolates showing a mycelium growth inhibition rate higher than 79.6% in the screening test were retained for further analysis.

Indirect antagonist activity of selected bacteria

Production of diffusible antifungal compounds

Each bacterial isolate was seeded in spots form on a 0.2 µm nylon membrane disc filters, placed in the center of a Petri dish containing PDA medium. Three replicates were prepared for each isolate. Petri dishes containing uninoculated membranes served as controls. These plates were first incubated at 28 °C for 72 h. Afterwards, the membranes containing the bacterial cultures were removed and a 6 mm diameter disc of *B. cinerea* was placed in the center of each Petri dish (Etesami *et al.*, 2019). Inhibition rates were measured as previously mentioned after 7 days of incubation at 25 °C. Three replicates were performed for each treatment and the experiment was repeated twice over time.

Volatile organic compounds (VOCs) bioassay

The two-sealed-base plates method was used to study the antifungal activity of the volatile organic compounds released by selected bacterial isolates (Lahlali *et al.*, 2020). Three parallel streaks of each bacterial isolate were first subcultured on TSA medium for 24 hours. Afterwards, the bottom of a second Petri plate containing a 6 mm-mycelial plug from the edge of a *B. cinerea* fungal colony that had been growing actively for seven days on PDA medium replaced the lid of each Petri plate. Then, parafilm was used to seal tightly both bottom plates that were incubated at 25 °C for 7 days. The plates prepared in the same way but without bacterial isolates were utilized as a control. The mycelial growth inhibition rate (IR) as previously mentioned,

was used to calculate the antifungal activity of VOCS. The experiment was repeated twice over time with three replicates for each treatment.

Production of hydrocyanic acid (HCN)

The ability of the isolates to produce hydrocyanic acid (HCN) was evaluated according to the protocol developed by Ezrari *et al.* (2021). Each bacterial isolate was streaked on Petri dishes containing LPGA medium enriched with 4.4 g/l glycine. Sterile Whatman filter paper No. 1 discs were soaked in alkaline picrate solution and then placed on the inside of the lids of these dishes. Then the plates were sealed with the parafilm to prevent the leakage of HCN, inverted, and then incubated at 28 ◦C for 4 days. Plates prepared in the same way, but without the bacterial inoculum were used as a control. HCN production was evaluated visually by observing the color of Whatman filter paper discs and confirmed by the turning of color from yellow to orange, red or reddish brown (Lahlali *et al.*, 2020; Ezrari *et al.*, 2021).

In vivo effects of antagonists on B. cinerea

Disinfected and wounded tomato fruits were treated with 20 µl of fresh culture of bacterial antagonists grown on TSA for 48 h and adjusted to 10^8 colony-forming units (CFU)/ml with sterile distilled water (SDW). Four hours later, the treated fruits were inoculated with 20 µl of conidial suspension of *B. cinerea* containing 10^5 spores/ml. Fruits inoculated only with 20 µl of SDW served as a control. The fruits were then stored at 20 °C and 95% RH for 5 days. The experiment was repeated twice over time with three replicates (of 9 fruits, 18 wounds) for each treatment. Disease reduction of gray mold was measured on the tomato fruits and calculated as follows:

The disease reduction (%) = $[(A - B)/A] \times 100$, where A is the lesion diameter recorded in tomato fruits inoculated with the pathogen alone and B is the lesion diameter in infected tomato fruits treated with the bacterial isolates (Sadfi-Zouaoui *et al.*, 2008; Berrada *et al.*, 2012).

Statistical analysis

All data were subjected to statistical analysis of variance (ANOVA) with one factor of variation, using STATISTICA software, version 6 (Stat-Soft, 2001) France. Percentage values were subjected to arcsine square root transformation before analysis of variance. Means were separated by Fisher's Least Significant Difference (LSD) test at the level P = 0.05.

Results

Screening for potential antagonist bacteria against B. cinerea

A total of 174 morphologically different bacteria were isolated and purified from tomato rhizosphere soil, rhizoplane, endorhizosphere, vermicopost and argan rhizosphere soil. These bacterial isolates were evaluated for their antagonistic effect against *B. cinerea* using the direct confrontation method. The inhibition zone produced by each bacterial antagonist was assessed after 7 days of the co-incubation period. The results showed that 31 isolates (Table 1) display significant antifungal activity against *B. cinerea*.

Table 1. List of effective antagonistic isolates according to their origin

Samples	Tomato rhizosphere			Argan rhizosphere	Vermicompost
	RS-T	RH-T	EN-T	RS-A	VC
Number of antagonist bacteria	15	5	1	1	9
Total	31				

RS-T: Tomato rhizospheric soil; RH-T: Tomato rhizoplane; EN-T: Tomato endorhizosphere; RS-A: Rhizospheric soil of the argan tree; VC: Vermicompost

These isolates are dominated by bacteria originated from the tomato rhizosphere (21 out of 31 isolates). Thirteen isolates out of 21 consisted of spore-forming bacteria obtained after the heat treatment of previously prepared dilutions (data not shown). The mycelium growth inhibition rates (IR%) ranged between 55.36 and 85.07% (Figure 1). The most effective bacterial isolates (VC-1, VC-13, RS-TB3, RS-T4, RH-TB12, RS-TB1, RS-TB4, VC-5, VC-B1, RH-TB1, RH-TB11, RS-T6 and RS-TB8) showing the highest percentages of inhibition of *B. cinerea* mycelial growth (IR % ≥ 79.60%) were selected for further tests.

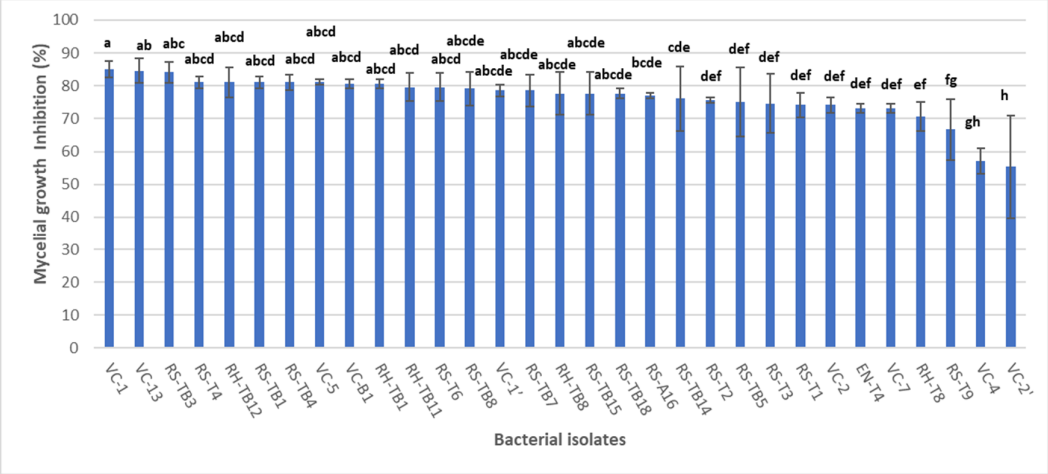

Figure 1. The *in vitro* inhibition rates (%) of mycelial growth of *Botrytis cinerea* obtained with the 31 selected bacterial isolates

Histograms represent the mean value of mycelial growth inhibition rate (%). Error bars indicate standard errors. Values with the same letters are not significantly different according to Fisher's Protected Least Significant Difference (LSD) test at the level P = 0.05

Mode of action of selected bacterial isolates against B. cinerea

Production of diffusible antifungal compounds

The test for the production of diffusible compounds revealed that all selected isolates produced diffusible substances, having an inhibitory effect towards *B. cinerea* (Figure 2). The isolates VC-B1, RS-TB3, RH-TB11 and RS-T6 released the most active diffusible substances; they totally inhibited the mycelial growth of *B. cinerea*, followed by VC-1, VC-13, VC-5, RS-TB1, RH-TB1, RS-TB4 and RH-TB12 isolates that inhibited the mycelial growth of the tested fungus by more than 80%. While, the antagonistic effect of the diffusible compounds generated by isolate RS-T4 was the lowest with an inhibition rate of mycelial growth of only 23.03% ± 4.57%, compared to the control.

Production of volatiles antifungal compounds

The indirect confrontation test revealed that all the tested isolates produce volatile compounds with inhibitory activity against *B. cinerea* (Figure 3). Indeed, the inhibitory effect of these substances against *B. cinerea* varied significantly (P-value< 0.05) between the selected bacterial isolates. Isolates RS-TB1 and RS-T6 isolated from the tomato rhizospheric soil were among the isolates that produced the most effective volatile compounds against *B. cinerea*. They reduced the mycelial growth of *B. cinerea* by 75.36% ± 7.24% and 72.94 ± 4.129% respectively, compared to the control. Whereas, the lowest mycelial growth inhibition rates of *B. cinerea* 34.30% ± 2.21% and 30.43% ± 4.35% were obtained with RS-TB3 and RS-TB4 isolates, respectively.

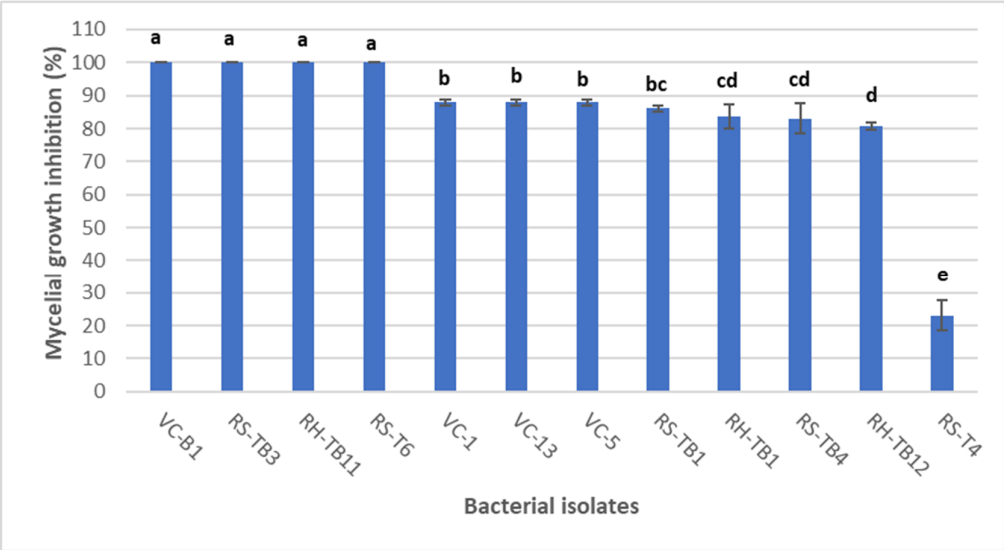

Figure 2. Effect of diffusible antifungal compounds on the *in vitro* mycelial growth of *Botrytis cinerea* after 7 days of incubation at 25 °C

Histograms represent the mean value of mycelial growth inhibition rate (%). Error bars indicate standard errors. Values with the same letters are not significantly different according to Fisher's Protected Least Significant Difference (LSD) test at the level P = 0.05

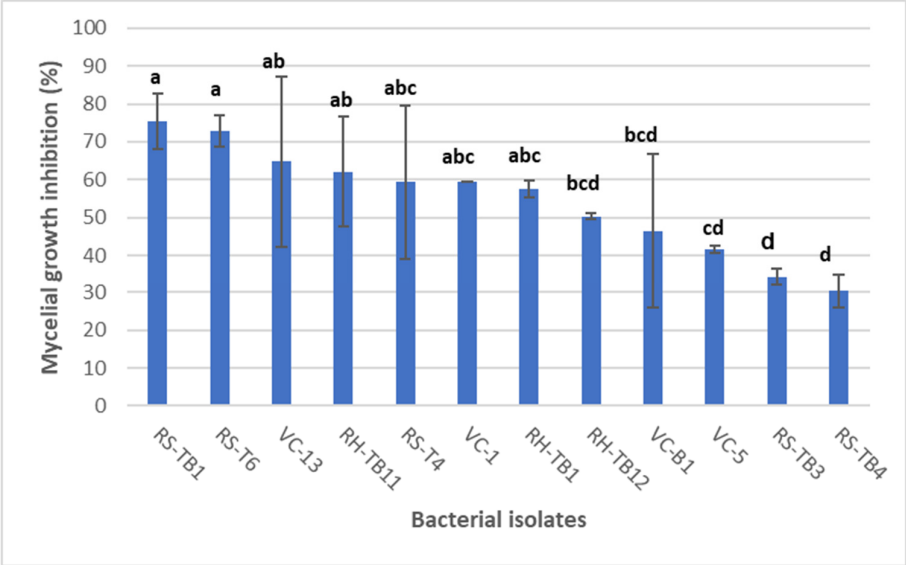

Figure 3. Effect of volatile antifungal compounds on the *in vitro* mycelial growth of *Botrytis cinerea* after 7 days of incubation at 25 °C

Histograms represent the mean value of mycelial growth inhibition rate (%). Error bars indicate standard errors. Values with the same letters are not significantly different according to Fisher's Protected Least Significant Difference (LSD) test at the level P = 0.05

Production of hydrogen cyanide

The production of hydrogen cyanide (HCN) was determined by the staining turn of alkali picrate-soaked Whatman paper from yellow to orange, was produced by isolates RS-TB1, RS-TB3, RS-TB4, RS-T4, RH-TB1, RH-TB12, VC-B1, VC-5, and VC-13 at variable intensities (Table 2). Isolates RS-TB3, RS-TB4, RH-TB1 and VC-B1 produced the highest amounts of HCN based on staining intensity. Whereas, the lowest amounts were produced by isolates RS-T4 and RH-TB12. For the RS-T6, RH-TB11and VC-1 isolates the yellowish staining of Whatman paper remained unchanged, indicating that these isolates do not act through HCN production.

Table 2. Ability of selected bacterial isolates to produce Hydrogen cyanide (HCN)

Substrates	Bacterial isolates	HCN production
Tomato rhizospheric soil (RS-T)	RS-TB1	++
	RS-TB3	+++
	RS-TB4	+++
	RS-T4	+
	RS-T6	-
Tomato rhizoplane (RH-T)	RH-TB1	+++
	RH-TB12	+
	RH-TB11	-
Vermicompost (VC)	VC-1	-
	VC-B1	+++
	VC-5	++
	VC-13	++

- : no HCN production; +: low production; ++: moderate production; +++: high production

Evaluation of biocontrol activities of selected bacterial isolates against B. cinerea

The most relevant isolates showing *in vitro* mycelium growth inhibition greater than 79.6% were tested *in vivo* on tomato fruits using the fruit acupuncture method. Indeed, the injection of 10^8 ufc/ml into the wounds, followed by the injection of the pathogen spore solution after 4 hours, resulted in variable percentages of disease reduction ranging from 37.92 to 93.14% (Figure 4).

The most effective strains, VC-1 and VC-5, were isolated from the vermicompost sample. These strains showed disease reduction rates of 93.14% and 89.42%, respectively, followed by isolates from tomato rhizospheric soil then from the rhizoplane. Infection of *B. cinerea* lead to only a slight hydrophanous lesion around the inoculation sites on tomato fruits that were previously treated with isolates VC-1, VC-5 and RS-T4. While *B. cinerea* infection led to large necrotic lesions with a massive layer of gray mold mycelia on the tomato fruits treated with isolates RH-TB1, RH-TB12 originated from the tomato rhizoplane as well as on the tomatoes in the negative control. Inoculation of wounded tomato fruits with various selected bacterial antagonists alone did not show any disease development.

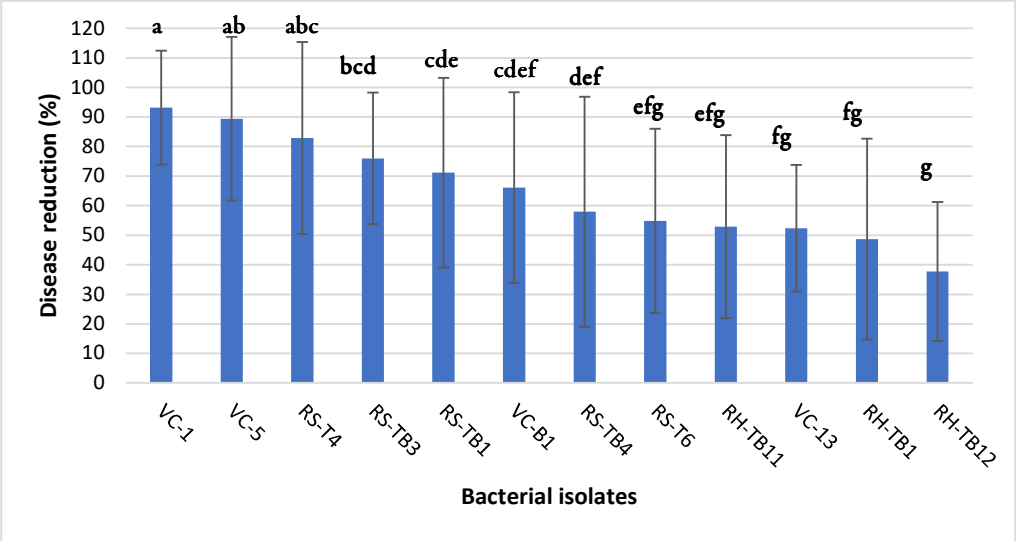

Figure 4. Disease reduction (%) observed on tomato fruits treated with antagonistic bacterial isolates and inoculated with *Botrytis cinerea* (10^5 conidies/ml) after incubation at 20 °C for 5 days

Histograms represent the mean value of disease reduction (%). Error bars indicate standard errors. Values with the same letters are not significantly different according to Fisher's Protected Least Significant Difference (LSD) test at the level P = 0.05

Discussion

Gray mold disease caused by the fungus *Botrytis cinerea* is one of the most devastating post-harvest diseases threatening tomato, especially in regions where it is grown intensively under greenhouse conditions (Berrada *et al.*, 2012). Generally, postharvest diseases affecting fruits or vegetables are extremely prevalent during storage and shelf life; they result in significant losses and eventually have a considerable negative impact on the economic value of the crops (Zhang *et al.*, 2020). Management of this disease often depends on the use of chemical fungicides. However, due to the growing public concern about chemical residues in fruits and the severe regulation, the use of fungicides is becoming progressively restricted (Tahiri Alaoui *et al.*, 2017). Moreover, the emergence of resistance to different fungicides has been detected in Moroccan tomato greenhouses (Hmouni *et al.*, 2003). Given these constraints, the development of environmentally friendly control methods is necessary in a modern and sustainable agricultural system. Among these methods, the use of biological control agents has been developed.

In this context, the present work aims to study the potential of bacterial agents for the control of the gray mold of tomato. Isolation of antagonists was carried out from five different substrates, namely tomato rhizosphere soil (RS-T), rhizoplane (RH-T), endorhizosphere (EN-T), argane rhizosphere soil (RS-A) and vermicompost (VC). The selection of these substrates was made on the basis of several studies that reported that they included bacteria and fungi, having inhibitory properties towards *B. cinerea* (Cloutier *et al.*, 2020; Köhl *et al.*, 2020).

In the current work, 174 bacterial strains were isolated and purified from the above-mentioned substrates. These isolates were evaluated for their inhibitory effect against *B. cinerea* using the *in vitro* direct confrontation assays. These assays are frequently used as an initial method to narrow down a collection of potential antagonists (Kefi *et al.*, 2015).

The direct confrontation assay between the bacterial isolates and *B. cinerea* showed that the tested isolates reduced the mycelial growth of *B. cinerea* to varying degrees. The difference between the percentages of

inhibition of the mycelial growth of *B. cinerea* by the bacterial isolates suggests that the mode of action and/or the type of metabolites released vary from one isolate to another, but also that these isolates are taxonomically different (Haidar *et al.*, 2016).

The 31 isolates that were antagonistic to *B. cinerea* have severely inhibited its mycelial growth, the inhibition rates (IR%) ranged between 55.36 to 85.07%. These isolates have created an area of inhibition, preventing physical contact between the bacteria and *B. cinerea*. This could imply that these isolates release various antifungal compounds including antibiotics such as 2,4-diacétophluoroglucinol, volatile chemicals such as cyanure of hydrogen or enzymes such as chitinase (Köhl *et al.*, 2019).

The majority of the 31 isolates were obtained from tomato rhizospheric soil (RS-T). This suggests that tomato rhizospheric soil isolates are more effective against *B. cinerea* compared to isolates from remaining substrates. These results are in accordance with those of Wang *et al.* (2018) who demonstrated that isolates from tomato rhizospheric soil had the strongest inhibitory effect on *B. cinerea*'s growth. The most active bacteria from the tomato rhizosphere were dominated with sporogenic bacteria. These results are in line with those of Wang *et al.* (2018) who reported that the species of the genus *Bacillus* spp. from the tomato rhizosphere are effective in the biocontrol of *B. cinerea*. Bu *et al.* (2021) tested the biological control agent '*B. subtilis* L1-21' that was isolated from Citrus plants and found that although this bacterial strain showed an antifungal effect against tomato gray mold pathogen and increased the shelf life of tomato fruits, it failed to improve the fruit quality during the storage period. These finding suggested that the origin of biological control agents contributes significantly in preventing postharvest diseases of tomato fruits as well as in improving fruit quality.

The test for the production of diffusible compounds revealed that all the selected isolates produced diffusible substances, having an inhibitory effect towards *B. cinerea*. The mycelial growth inhibition rates ranged between 80.6 and 100% for the isolates VC-B1, RS-TB3, RH-TB11, RS-T6, RS-TB1, RS-TB4, RH-TB1, RH-TB12,VC-1,VC-5 and VC-13. These values are close to those obtained during the direct confrontation assay. These results are in agreement with those of Kefi *et al.* (2015), who reported that the diffusible compounds released by the tomato stem isolate BT5 reduced the growth of *B. cinerea* by 40%, which is a value close to that obtained during the direct confrontation (42%). This suggests that the diffusible substances emitted by these bacterial isolates are those responsible for the inhibitory activity observed during the direct confrontation assay. Li *et al.* (2015) and Gong *et al.* (2015) are in line with this suggestion and reported that the suppression of pathogenic fungal growth is most likely caused by antagonist bacteria due to secretion of lipopeptides and hydrolytic enzymes that are responsible for hyphal damage. According to Ezrari *et al.* (2021) hyphal damage caused by antagonistic bacteria showed structural changes such as deformations, swelling, and vacuolation of the mycelium. These structural changes were occasionally followed with mycelium disintegration and the release of cytoplasmic contents. The diffusible compounds emitted by the RS-T4 isolate had a weak inhibitory activity, with an inhibition rate equal to 23.03% ± 4.57%. This value is low compared to that obtained during the direct confrontation, which suggests that the RS-T4 isolate produces other antifungal compounds.

Selected bacterial isolates also released volatile compounds that have an inhibitory effect against *B. cinerea* mycelial growth. Inhibition rates varied significantly between isolates. This indicates that the composition of these volatile antifungal compounds varies from one isolate to another. These results agree with those of Rojas-Solís *et al.* (2018), who reported that the variable inhibitory activity of the volatile compounds emitted by the two isolates E25 and CR71 against *B. cinerea*, is due to their different compositions of antifungal volatiles. In the present study, the inhibitory effect of the volatile compounds emitted by all the isolates except RS-T4, is lower than that of the diffusible compounds. These results are in agreement with those of Hernández-León *et al.* (2015), who demonstrated that the volatile antifungal compounds produced by four bacteria isolated from the rhizosphere of *Medicago* spp. have less antifungal power than their diffusible products.

Nevertheless, the volatile compounds emitted by the RS-T4 isolate have greater inhibitory activity against *B. cinerea* than its diffusible compounds. This is in agreement with Hmouni *et al.* (1999), who reported that some bacteria that are antagonistic to phytopathogenic fungi are more active through the emission of volatile compounds than diffusible ones.

The HCN production test revealed that nine among the 12 selected bacterial isolates produced hydrogen cyanide. According to Devi *et al.* (2018), HCN is well known to be harmful to microorganisms. This antimicrobial compound acts on the cells of fungal pathogens by blocking the cytochrome oxidase enzyme of the respiratory chain.

Although *in vitro* agar assay is an important first step for screening a large number of isolates against postharvest pathogens, *in vivo* tests are needed in order to verify the effectiveness of potential candidates (Fravel, 1988). The *in vivo* screening assay on tomato fruits using the fruit acupuncture method showed a significant reduction of gray mold disease severity when compared to untreated control where severity of the disease was 100%. In this experiment, the culture suspension of bacterial isolates injected at a concentration of 10^8 ufc/ml into the wounds, followed by the injection of the pathogen spore solution after 4 hours resulted in variable percentages of disease reduction, ranging from 37.92 to 93.14%. These results are consistent with those of Bu *et al.* (2021) who tested different concentrations of the bacterial culture suspension of *B. subtilis* L1-21 against *B. cinerea* on artificially wounded tomatoes. In this study, Bu *et al.* (2021) demonstrated that different concentrations of *B. subtilis* L1-21 (10^8, 10^6 and 10^4 cfu/ml) had different inhibitory effects on the pathogen and high concentrations resulted in low disease occurrence which led to greater disease control effects.

In the present study, the most effective strains *in vivo* were isolated from the vermicompost sample followed by isolates from tomato rhizospheric soil, then from the rhizoplane. The effectiveness of these strains could be a result of their ability to colonize the wounds successfully. Indeed, Bu *et al.* (2021) indicated that the ability of a biological control agent to efficiently colonize a host plant is a crucial element in disease resistance and stability throughout the long-term. The mechanism of action involved in such biocontrol activity is the competition for both available space and nutrients (Taqarort *et al.*, 2008; Bu *et al.*, 2021). The release of several antagonistic metabolites including antibiotics, lipopeptides, polykeones, chitinase, proteases, and other degrading enzymes, may also be responsible for the biological control activity of bacterial isolates (Caulier *et al*., 2019; Wu *et al*., 2019). Chitinase has been shown to be implicated in the fungal cell wall degradation (Parafati *et al.*, 2015).

Recently, investigations on antifungal volatile organic compounds (VOCs) against postharvest diseases have gained more interest around the world because of the enclosed storage and transportation conditions (Arrarte *et al.*, 2017; Alijani *et al.*, 2019). Li *et al.* (2012) reported that volatiles produced by *Streptomyces globisporus* JK-1 prevented *B. cinerea's* mycelial growth and decreased tomato gray mold incidence from 100% to 35.8%. In fact, VOCs produced by antagonists prevent postharvest fungal disease by inhibiting hyphal growth, increasing plant growth, and developing systemic disease resistance (Sharifi and Ryu, 2016; Alijani *et al.*, 2019; Calvo *et al.*, 2020).

Conclusions

The present study showed that the selected biological control agents isolated from different substrates are effective against *B. cinerea in vitro* and on tomato fruits. The use of such biological control agents could be a substantial replacement for synthetic fungicides in the management of tomato postharvest gray mold.
The strains VC-1, VC-5, RS-T4, RS-TB3, RS-TB1, VC-B1, RS-TB4, RS-T6, RH-TB11 and VC-13 have the potential to suppress postharvest gray mold on artificially wounded tomato fruits. The efficacy of these biocontrol agents against *B. cinerea* may be related to their ability to produce a range of extracellular hydrolytic

enzymes, diffusible and volatile bioactive compounds. Moreover, the potential of selected bacteria to produce spores, as demonstrated by the heat treatment, could be exploited for their storage, mass production, encapsulation and subsequent application prior to harvesting in the field or under greenhouse conditions, as well as during trials involving large-scale storage. Further research should focus on molecular identification, VOCs and extracellular hydrolytic enzyme analysis to develop new strategies for commercial applications.

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by L.A., M.E., B.A., and M.A.H. The manuscript was written by L.A. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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