Rhizospheric solutions: *Pseudomonas* isolates counter *Botrytis cinerea* on tomato

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Abstract

Gray mold caused by *Botrytis cinerea* causes serious losses in more than 200 crop species worldwide. The necrotrophic fungus sporulates to effect a grey covering on leaves, stems and flowers. *B. cinerea* is controlled by chemical synthetic fungicides, endangering human and environmental health. Synthetic fungicides stimulate emergence of pathogen resistance. Organic alternatives which may be present or introduced into the edaphic environment are suitable solutions to control outbreaks. This study was done in order to elucidate the mode of action involved in the control of *B. cinerea* using fluorescent *Pseudomonas* isolates from tomato roots. The results show that all 76 isolates inhibit fungal growth during *in vitro* bioassay using dual culture technique. Five isolates of *Pseudomonas* (Q6B, Q13B, Q7B, Q14B and Q1B) cause significant inhibition levels ranging from 65 to 73%. These isolates inhibit fungal growth in both fruits and leaves. Each isolate tested produced antifungal metabolites (siderophores, hydrogen cyanide and enzymes). Results of this study show that all tested *Pseudomonas* isolates have a strong efficacy in biological control against *B. cinerea* and can be used for environmentally sustainable control.

Keywords: *Pseudomonas; B. cinerea*; rhizospheric solutions; biological control; antifungal; tomato

Solutions rhizosphériques : Isolats de *Pseudomonas* contre *Botrytis cinerea* de la tomate

Résumé

La moisissure grise causée par *Botrytis cinerea* provoque des dégâts sur plus de 200 espèces de cultures dans le monde. *B. cinerea* sporule pour former une pourriture grise sur les feuilles, les tiges et les fruits. Pour lutter contre *B. cinerea,* des fongicides synthétiques sont utilisés. Ces derniers mettent en danger la santé humaine et environnementale en plus de la résistance qu'ils peuvent occasionner chez les souches de *B. cinerea*. Les alternatives écologiques sont des solutions appropriées pour contrôler la moisissure grise tout en maintenant l'équilibre environnemental. L'objectif de cette étude est d'évaluer l'effet des isolats de *Pseudomonas* issus de la rhizosphère de la tomate sur *B. cinerea.* Les résultats ont montré que les 76 isolats testés inhibent le développement de *B. cinerea in vitro*. Cinq isolats de *Pseudomonas* (Q6B, Q13B, Q7B, Q14B et Q1B) ont provoqué des niveaux d'inhibition significatifs allant de 65 à 73%. Par ailleurs, ces isolats ont également inhibé *B. cinerea* sur les feuilles et le fruit de la tomate. Pour tenter d'élucider les mécanismes d'action, les cinq isolats ont montré une production des métabolites antifongiques tels que les sidérophores, le cyanure d'hydrogène et d'autres enzymes. Les résultats de cette étude ont montré que les isolats de *Pseudomonas* Q6B, Q13B, Q7B, Q14B et Q1B ont une forte efficacité dans la lutte biologique contre *B. cinerea* et peuvent être utilisés pour une lutte écologique durable.

Mots clés : *Pseudomonas* ; B. *cinerea* ; rhizosphère ; contrôle biologique ; antifongique ; tomate.

حلول من الجذور: تأثير سلالات *Pseudomonas* على الفطر *Botrytis cinerea* في شجيرات الطماطم

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ملخص

يسبب العفن الرمادي الناجم عن *Botrytis cinerea* خسائر على من 200 نوع من المحاصيل حول العالم. تشكل ابواغ فطر *B. cinerea* غطاء رماديا على الأوراق والسيقان والفواكه، وتستعمل المبيدات الفطرية الاصطناعية للسيطرة على العفن الرمادي *B. cinerea* غير أنها تهدد صحة الإنسان والبيئة، وتودي أيضًا الى ظهور المقاومة لدى فطر *B. cinerea* و تعتبر البدائل العضوية حلولا مناسبة للتحكم في العفن الرمادي مع الحفاظ على التوازن البيئي. تهدف هذه الدراسة الى تقييم تأثير سلالات *Pseudomonas* من جذور الطماطم على *B. cinerea*. وقد أظهرت النتائج أن 76 سلالة تم اختبارها تمنع تطور فطر *B. cinerea* في المختبر. تسبب السلالات الخمس من *Pseudomonas* (Q6B، Q13B، Q7B، Q14B و Q1B) مستويات كبح تتراوح من 65 و 73٪. كما أن هذه السلالات تمنع تطور العفن الرمادي على أوراق وفواكه الطماطم، وأظهرت جميع السلالات الخمس قدرة إنتاج المستقلبات المضادة للفطريات (حامض الحديد، سيانيد الهيدروجين والإنزيمات). بينت نتائج هذه الدراسة أن السلالات Q6B، Q13B، Q7B، Q14B و Q1B لها فعالية عالية في المكافحة البيولوجية لفطر*B. cinerea* مسبب العفن الرمادي ويمكن استخدامها للتحكم البيئي المستدام.

الكلمات المفتاحية: *Pseudomonas*؛ التحكم البيولوجي؛ *Botrytis cinerea* ؛ طماطم

Introduction

Tomatoes are one of the most cultivated crops in Mediterranean greenhouses (Abou Hadid, 2013). In Morocco, tomatoes grown in greenhouses cover approximately 16.000 ha with 1.5 million tons/year of production. These are considered as an essential crop grown in the Souss Valley (Ait Hou *et al.,* 2015). Tomato productivity suffers constraints and risks from increased pathogenic agents. The main objective of Morocco's Green Plan is to improve yield up to 142% without expanding production areas. To achieve this and other objectives, it is imperative to reduce damage caused by pest and diseases.

The fungal plant pathogen *Botrytis cinerea* is the causal agent of gray mold disease on tomato, apple, strawberry and a range of other economically important crops (Price, 1979; Sharma *et al.,* 2009; Jurick *et al.,* 2017; Rguez *et al.,* 2020). Gray mold causes huge losses in protected tomato crops (Ni and Punja, 2019). It infects the flowers, fruits, leaves and stems before harvest (Borges *et al.,* 2014; Chen *et al.,* 2019). Gray mold is recognized as an important postharvest disease on fresh-market tomatoes (Mari *et al.,* 1996). Gray mold may be controlled by chemical fungicides (Eckert and Ogawa, 1988; Rupp *et al.,* 2017), although there is increasing international concern over the heavy use and application of these products on crops due to their harmful effects on human and environmental health. Additionally, emergence of pathogen resistance is incited by chemical fungicides (Chung *et al.,* 2009). Elad *et al.,* (1992) reported that *B. cinerea* developed a rapid resistance to specific fungicides including benzimidazoles, dicarboximides and sterol biosynthesis inhibitors. Halime *et al.,* (2019) reported a high level of resistance of *B. cinerea* to Fenhexamid in Moroccan tomato greenhouses. Moreover, chemical products cause soil pollution and have detrimental effects on humans (Martínez-Romero *et al.,* 2008). In order to find a safe fungicidal control, alternative strategies have been considered.

Biological control offers an alternative to synthetic fungicides and has become a well established practice over recent decades (El-Shatoury *et al.,* 2020; Syed Ab Rahman *et al.,* 2018). Non-phytopathogenic bacteria of rhizospheric, endophytic or halophilic environments have been frequently reported to protect plants against phytopathogens (Van Loon *et al.,* 1998; Magnin-Robert *et al.,* 2007; Compant *et al.,* 2010; Verhagen *et al.,* 2011). Bacterial genera such *Bacillus, Pseudomonas,* and *Enterobacter*; fungi belonging to *Pythium,* and *Trichoderma* genus and actinomycetes have an interesting biocontrol potential (Lange *et al.,* 1993; Chernin *et al.,* 1995; Amkraz *et al.,* 2010; Gao *et al.,* 2018). Further, Sadfi-Zouaoui *et al.,* (2007b) reported that a function of plant growth-promoting rhizobacteria (PGPR) is to reduce phytopathogenic infections of host species directly via biocontrol mechanisms. PGPR also assist plant hosts indirectly by induction of systemic resistance, additional phytohormone or anologue pathways or by changes in nutrient status (Van Loon *et al.,* 1998; Zamioudis and Pieterse, 2012; Qessaoui *et al.,* 2019a). *B. Cinerea* has been controlled by both fungi and bacteria (Elad 1985; Loqman *et al.,* 2009; Mónaco *et al.,* 2009).

Researchers report direct mechanisms of bacterial biocontrol to be: antagonistic; via production of antibiotic compounds; in competition for nutrients; through siderophore-mediated competition for iron and/or production of extracellular enzymes (Lavicoli *et al.,* 2003; Meziane *et al.,* 2005; Gao *et al.,* 2018; Qessaoui *et al.,* 2019b). In indirect interactions, rhizobacteria reduce disease by inducing or priming plant defense mechanisms, which lead to a state of phytopathogenic resistance, commonly termed induced systemic resistance (ISR) in the whole plant (Conrath *et al.,* 2002; Verhagen *et al.,* 2010). ISR has been demonstrated in different plant species against various disease vectors when bacteria and phytopathogens remained spatially separated (Hoffland *et al.,* 1995; Van Loon *et al.,* 1998; Lavicoli *et al.,* 2003; Meziane *et al.,* 2005). It has been reported that local and systemic resistance against *B. cinerea* can be induced by *Acinetobacter*, *Bacillus* and *Pseudomonas* spp. (Magnin-Robert *et al.,* 2007; Verhagen *et al.,* 2011; Vignatti *et al.,* 2020). The aim of the present study is to investigate the effect of *Pseudomonas* isolates on *Botrytis cinerea* and to elucidate the mechanism of their antagonistic activity.

Material and Methods

Isolation of bacteria from tomato roots

Isolation was performed as described by Qessaoui *et al.,* (2019a). Samples of rhizospheric soil with roots were collected from tomato greenhouses of the experimental farm at the Regional Agricultural Research Center of Agadir. Bacterial isolates were isolated from the rhizosphere (RS), rhizoplane (RH) as well as from the endorhizosphere (ER) (Amkraz *et al.,* 2010; Qessaoui *et al.,* 2019a). RS, RH and ER extract samples were diluted and dilutions were spread on King B medium (King *et al.,* 1954), to isolate and quantify fluorescent *Pseudomonas* under UV (Amkraz *et al.,* 2010). Three replicates were made for each extract, all being incubated at 26^{o}C for 48 h. Results were expressed as colony forming units per gram (CFU g-1) of dried rhizospheric soil or of fresh roots for rhizoplane and endorhizosphere. Fluorescent colonies were purified by streaking and were stored at -80°C in 40% glycerol (Parke *et al.,* 1986; Kaur *et al.,* 2007; Qessaoui *et al.,* 2019a).

Isolation of *B. cinerea* from tomato plants

Isolation of *B. cinerea* was made from infected fruit and vegetative tissues of tomato plants. Cultures were grown on Potato Dextrose Agar (PDA) after which *B. cinerea* was selected, purified and characterized. Isolate characterization was carried out at the Plant Protection Laboratory of INRA-Agadir, Morocco.

In vitro selection of antagonistic fluorescent *Pseudomonas* against *B. cinerea*

In vitro evaluation of *Pseudomonas* isolates was carried out using dual culture technique on PDA (Kaur, 2003). A heavy inoculum was applied as a band of 1.5 cm length equidistantly on three opposite edges of the agar medium in Petri plates using an inoculation loop. A mycelial disc of 5 mm diameter from a 7 day-old culture of *B. cinerea* was placed at the center of the Petri plate. Three replications were made for rhizobacteria. Plates containing the pathogen alone served as control. Plates were

incubated for five days at 25°C (Kaur*et al.,* 2007). After incubation, the mycelial growth inhibition percentage (MGIP) was calculated using the following expression.

$$\text{MGIP} = \frac{r1-r2}{r1} \times 100 \quad (1)$$

Where *r1* is radial growth of the fungus in the control and *r2* is radial growth of the fungus in the treated plates (Chaurasia *et al.,* 2005; Berrada *et al.,* 2012). The bacterial isolates showing maximum zones of inhibition were selected for further studies.

Characterization of fluorescent *Pseudomonas* isolates

Five selected bacterial strains were subjected to biochemical testing for characterization (Fluorescence production, motility, oxidase, arginine dehydrogenase, catalase, leven production, nitrate reduction and gelatin liquefaction) (Falkow, 1958; Stanier *et al.,* 1966; Bossis *et al.,* 2003). These isolates were further characterized based on a partial rpoD gene sequence using the primers PsrpoD FNP1 (5′-TGAAGGCGARATCGAAATCGCCAA-3′) and PsrpoDnprpcr1 (5′-YGCMGWCAGCTTYTGCTGGCA-3′) (Qessaoui *et al.,* 2019a).

***In vivo* screening of antagonists for antifungal activity against *B. cinerea* on tomato fruits**

Preparation of tomato fruits

Red and uniform (57-67 mm) tomato fruits were collected from unsprayed plants growing as organic crops at the experimental farm of INRA, Agadir, Morocco. The fruits were surface-sterilized by soaking in aqueous sodium hypochlorite (2%) for 5 min (Sadfi-Zouaoui *et al.,* 2007b a). They were thoroughly rinsed with sterile distilled water, dried, and two small wells (3 mm in diameter and 3 mm in depth) were made in each fruit with a sterile needle.

Inoculation of tomato fruits

Fresh cultures of the pathogen and fluorescent *Pseudomonas* antagonists were used for each experiment. In evaluation of antagonistic activity, ten fluorescent *Pseudomonas* strains were grown for 48 h in King B medium and adjusted to 10^8CFU/ml. Conidial suspensions of *B. cinerea* kept in Sabauroud broth medium (10g/l peptone; 20g/l glucose) were adjusted to 10^5 spores mL^{-1}. Twenty microliters of the bacterial suspensions were inoculated into the wells of fruits. After drying for one hour in a sterile area, wells further inoculated with 20 µL of conidial suspension. Fruit inoculated with the pathogen alone was considerated the positive control; while fruits inoculated with distilled water constituted negative controls. All fruits were stored at 20°C for 7 days in autoclaved transparent plastic bags. The mycelial growth inhibition percentage on treated fruits (MGIPF) was calculated in the following way:

$$\text{MGIPF} = \frac{R1-R2}{R1} \times 100 \quad (2)$$

Where *R1* is lesion diameter recorded in positive control fruits and *R2* is lesion diameter in treated fruits.
Nine tomato fruits were used per treatment (Janisiewicz and Roitman, 1988).

Effectiveness of *Pseudomonas* to control gray mold on tomato leaves

Tomato leaves excised from organically-grown plantlets (8-week-old) were floated abaxial side down on the buffer surface (2 mM MES pH 5.9, containing 0.5 mM $CaCl_2$ and 0.5 mM K2SO4), in the presence of each bacterial isolate at 10^7 CFU/ml. Controls consisted of leaves incubated with the buffer. After 20 h, the leaves were rinsed with sterile distilled water, patted dry and placed in Petri plates, the adaxial side facing a wet absorbing paper. One needle-prick wound was applied to each leaf, and the fresh wounds were covered with 5µl drops of the *B. cinerea* conidial suspension (10^5conidia/ml). The experiment was done with seven leaves excised from four plants for each isolate. Each experiment was repeated three times. Disease development was measured as the average diameter of lesions formed 7 days after inoculation with *B. cinerea*. Percent protection was defined as reduction in lesion diameter relative to the control. The mycelial growth inhibition percentage on treated leaves (MGIPL) was calculated using the following formula:

$$\mathrm{MGIPL} = \frac{D1-D2}{D1} \times 100 \qquad (3)$$

Where *D1* is lesion diameter recorded in positive control fruits and *D2* is lesion diameter in treated fruits.

Production of volatile antifungal compounds (VOCs)

Effects of volatile organic compounds produced by the ten selected fluorescent *Pseudomonas* on the growth rates and activity of fungi were assessed according to Fiddaman and Rossall (1993). The antagonism of *Pseudomonas* was evaluated on King B medium plates. After incubation for 48h, the lid of each Petri dish was replaced by a plate containing PDA medium with a 6mm plug of *B. cinerea*. The two plates were sealed with parafilm. Controls were prepared without bacteria in the bottom plate. Petri dishes were incubated at 25°C, and observations were recorded after 5 days (Kumari and Khanna, 2014). The percentage of growth inhibition by VOCS (PIVOC) was calculated in the following way:

$$\mathrm{PIVOC} = \frac{r1-r2}{r1} \times 100 \qquad (4)$$

Where *r1* and *r2* are radial growth of the fungus in the control and in treated plates respectively.

Hydrogen cyanide production

To determine the production of HCN, *Pseudomonas* isolates were streaked onto KB agar plates supplemented with glycine (4.4 g l^{-1}). Petri plates were inverted and a piece of filter paper impregnated with 0.5% picric acid (yellow) and 2% sodium carbonate was placed on their lids. Petri plates were sealed with parafilm and incubated for 96 h at 28 °C. After incubation a change in the filter paper colour (from orange to brown) of the filter paper indicated the production of HCN (Bakker and Schippers 1987).

Spore germination assay

Germination of spores and growth of germ tubes of *B. cinerea* were assayed by mixing a 40µl drop of the spore suspension (10^6 spore/ml) prepared in Sabauroud broth by the method of Elad (1985) with an equal volume of isolate filtrate in wells of sterile depression slides. The slides were incubated at 20°C for 18h in sterile Petri plates containing filter paper moistened with distilled water. After incubation, percentage of spore germination inhibition and development of germ tubes were calculated as compared to the control. At least 100 spores in three observation fields were examined for each isolate. The numbers of germinated and non-germinated spores were counted using a light microscope at 400x magnification and the length of germ tubes were measured with an ocular micrometer. Spores were considered to be germinated when germ tubes were twice the spore length (Xiao *et al.,* 2008).

Siderophore production

Production of siderophores was determined by the method of Schwyn and Neilands (1987), using CAS reagent (chrome azurol S; Fluka Chemika, Buchs, Switzerland). Isolates were grown on CAS agar plates supplemented with 2% glucose, 0.5% L-glutamic acid (neutralized), and 5 ppm biotin. The presence of orange haloes was recorded up to 7 days after incubation.

Proteolytic activity

Protease activities of *Pseudomonas* strains were determined according to the method reported by Jha *et al.,* (2009). Skim milk agar was used (5 g/l pancreatic digest of casein, 2.5 g/l yeast extract, 1.0 g/l glucose, 100 ml/l of 7% skimmed milk solution and 15 g/l agar). Bacterial cells were spot inoculated and incubated for 48h at 28 °C. After incubation, plates were observed for the zone of clearness around the colony. Proteolytic activities were indicated as negative with no clear zone around the cells and positive with a zone of clearness (Smibert and Krieg, 1994).

Chitinolytic activity

To detect chitinolytic activity on the plates, cells were streaked on a mixture of synthetic medium (SM) and nutrient broth (3:1), supplemented with colloidal chitin (0.2%) solidified with 1.5% agar (Chernin *et al.,* 1995). After incubation at 30 °C for 5 days, the plates were treated with Congo red solutions (0.03%). Enzymatic activity was noted with appearance of clear zones (Elshafie *et al.,* 2012).

Cellulase activity

Cellulase production was determined by using the M9 medium agar amended with 10 g/l of cellulose and 1.2 g/l of yeast extract (Cattelan *et al.,* 1999), after 8 days of incubation at 28°C the appearance of a clear halo indicated cellulase production.

Statistical analysis

The mycelial growth inhibition percentage (MGIP) was calculated for each *Pseudomonas* isolate. The data were subjected to Analysis of Variance test (ANOVA) using Statistical software, STATISTICA (Ver. 6). Any difference mentioned is significant at P< 0.01 using the Newman–Keuls multiple range test.

Results

Isolation of *Pseudomonades* from tomato roots

Bacterial populations were quantified in the samples collected from the tomato greenhouse of the experimental farm. Of the bacteria, 76 *Pseudomonas* were isolated, of which 56.58% (43 isolates) were isolated from the rhizoplane, 28.95% (22 isolates) from the endorhizosphere and 14.47% (11 isolates) were from the rhizosphere.

In vitro selection of *B. cinerea* antagonistic *Pseudomonas*

All 76 fluorescent *Pseudomonas* isolates were tested against the *B. cinerea* strain in Petri plates containing PDA medium. All isolates showed activity against *B. cinerea* ranging between 10.92 % to 73.63% inhibition (**Table 1**). The results showed that out of the 76 isolates tested against *B. cinerea* in dual culture under *in vitro* conditions, fifty-four showed an antagonistic potential (MGIP>25%) against *B. cinerea*. Variation in inhibition potential was observed though the inoculum load was the same for all isolates (**Table 1**). Among fifty-four isolates, five bacteria Q6B, Q13B, Q7B, Q14B and Q1B showed a maximum percent inhibition of *B. cinerea* with (MGIP>65%) (**Figure 1**).

Table 1: In vitro screening of bacterial isolates based on mycelial growth inhibition of B. *cinerea*

Soil part	Number of antagonistic isolates	Antagonism of isolates (MGIP>25%) (%)	Inhibition potential (%)
Rhizosphere	11	82	10.92 - 65.48
Rhizoplane	43	63	11.67 – 70.59
Endorhizosphere	22	72.73	16.88 – 73.63

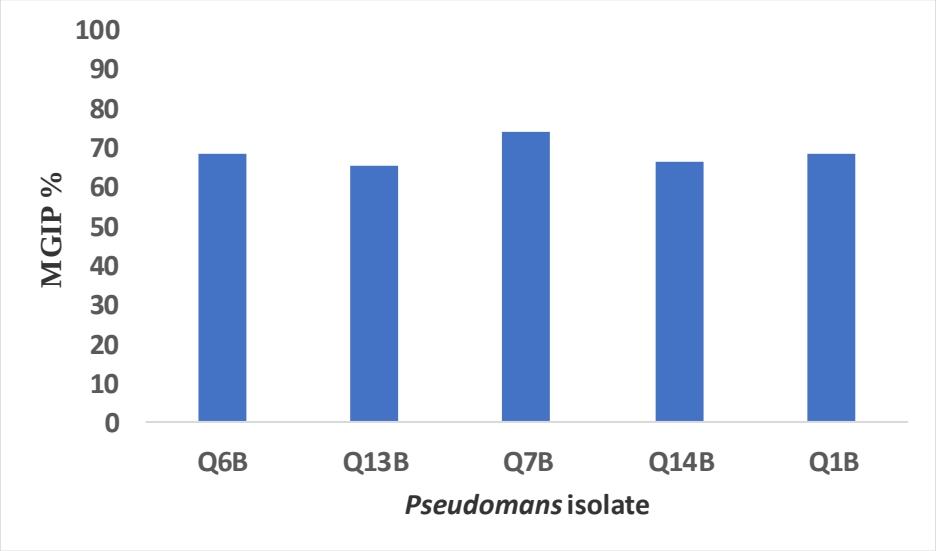

Figure 1: Effect of five *Pseudomonas* isolates on the mycelial growth of *B. cinerea* (P<0. 01; Newman-keuls test)

Pseudomonas isolates characterization

Q6B, Q13B, Q7B, Q14B and Q1B isolates were Gram-negative and produced fluorescence manifested by a diffusible yellowish-green pigment, under ultraviolet light (360 nm), in KB medium. Q6B was negative in the motility test; the other tested isolates were positive in motility, oxidase, and arginine dehydrogenase. Q7B isolates were negative for catalase and leven production. Q14B and Q1B were positive for nitrate reduction while others were negative. Q6B and Q13B were positive in terms of gelatin liquefaction (**Table 2**).

Molecular characterization using an rpoD gene sequence confirmed the five isolates as *Pseudomonas* sp. (Qessaoui *et al.,* 2019a).

Table2: Biochemical characteristics of five *Pseudomonas* isolates

	Gram	Fl	Ox	Mt	Ca	N	Arg	L	Gl	Glu*	Suc*	Man*
Q6B	-	+	+	-	+	-	+	+	+	Ox	-	-
Q13B	-	+	+	+	+	-	+	+	+	ox/fr	-	-
Q7B	-	+	+	+	-	-	+	-	-	Ox	+	-
Q14B	-	+	+	+	+	+	+	+	-	Ox	-	-
Q1B	-	+	+	+	+	+	+	+	-	Ox	-	-

Fl = fluorescence; Ox = oxydase; Mt = Motility; Ca = catalase; N= nitrate; Arg = arginine;
L = leven; Gl = Gelatin; Carbon source (Glu = glucose; Suc = sucrose; Man = mannitol; ox: oxidation; fr: fermentation

In vivo screening of fluorescent *Pseudomonas* antagonists of *B. cinerea* on tomato fruits and leaves

These five tested *Pseudomonas* showed antagonistic activity against *B. cinerea* on tomato fruits compared to controls, with 97% inhibition after 7 days of inoculation (**Table 3**). MGIPF ranged from 53% to 97% for Q14B and Q6B respectively. These isolates kept the fruits almost intact (**Figure 2**).

The five *Pseudomonas* isolates were screened for their effectiveness to control *B. cinerea* using detached leaves and effectively reduced disease development on leaves compared to the non-bacterized control (**Table 3**). The reductions of necrotic lesions (MGIPL) varied from 63 to 95% for isolate Q1B and Q6B respectively.

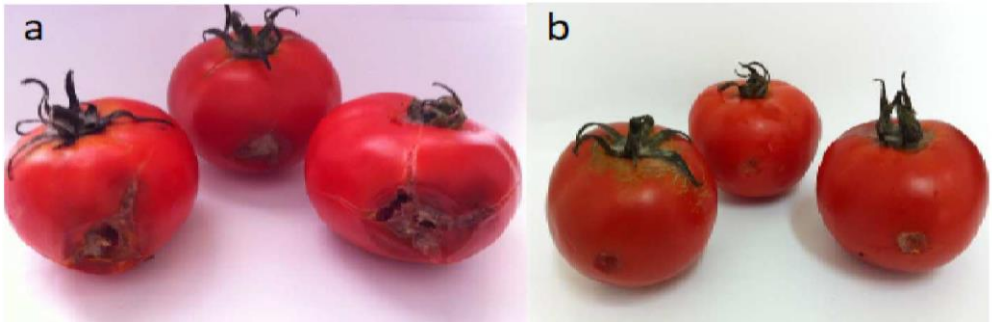

Figure 2: *In vivo* tests of antagonists to *B. cinerea* on tomato fruits (a) Positive Control; (b) Treated fruits

Table3: Protection of tomato fruits and leaves against *B. cinerea* by five *Pseudomonas* isolates

Isolate	Source	MGIPF(%)	MGIPL(%)
Q6B	Rhizoplane	97.88 ± 2.29^{cd}	95.12 ± 12.90^{c}
Q13B	Rhizoplane	63.77 ± 7.10^{abc}	86.84 ± 23.52^{c}
Q7B	Endorhizosphere	80.94 ± 7.37^{abdc}	85.37 ± 31.82^{c}
Q14B	Endorhizosphere	53.62 ± 8.67^{ab}	78.06 ± 21.39^{c}
Q1B	Endorhizosphere	86.55 ± 6.89^{bcd}	63.77 ± 41.68^{bc}

*Values indicate mean values (±S. D.) followed by different letters are significantly different within a row or column at P<0. 01 according to Newman-keuls test

The results showed that the five *Pseudomonas* isolates significantly inhibited *B. cinerea*. Moreover, the *Pseudomonas* isolated from endorhizosphere and rhizoplane were more potent than isolated ones from the rhizosphere (**Table 3**).

Production of volatile antifungal compounds (VOCs)

The experiement related to VOCs indicated that tested *Pseudomonas* isolates produced effective volatile products against *B.cinerea* under *in vitro* conditions. A total inhibition of the *B. cinerea* was observed during the five days of incubation (**Table 4**) (**Figure 3**). Inhibition was in accordance with the results of the inhibition in PDA medium, on tomato fruit and leaves (**Table 3**).

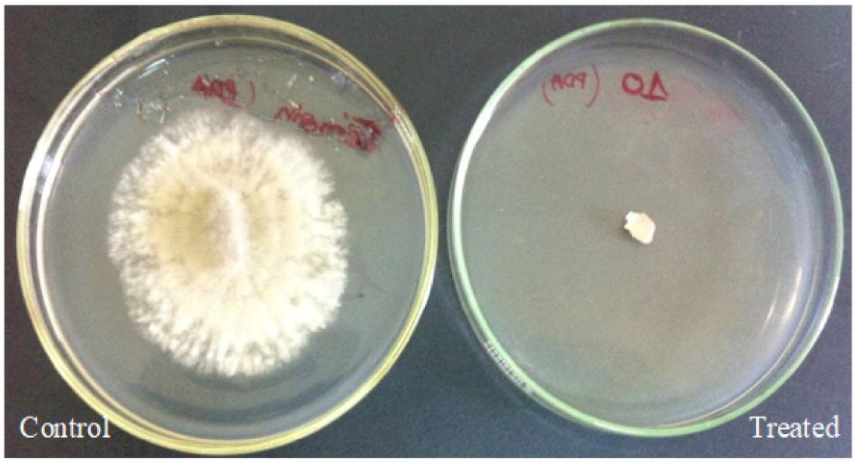

Figure 3: Antagonistic effect of volatile antifungal compounds on growth of *B. cinerea*

Production of hydrogen cyanide (HCN)

All five antagonistic *Pseudomonas* isolates showed the production of HCN as indicated by the discoloration of the filter paper from orange to brown (**Table 4**, **Figure 4**).

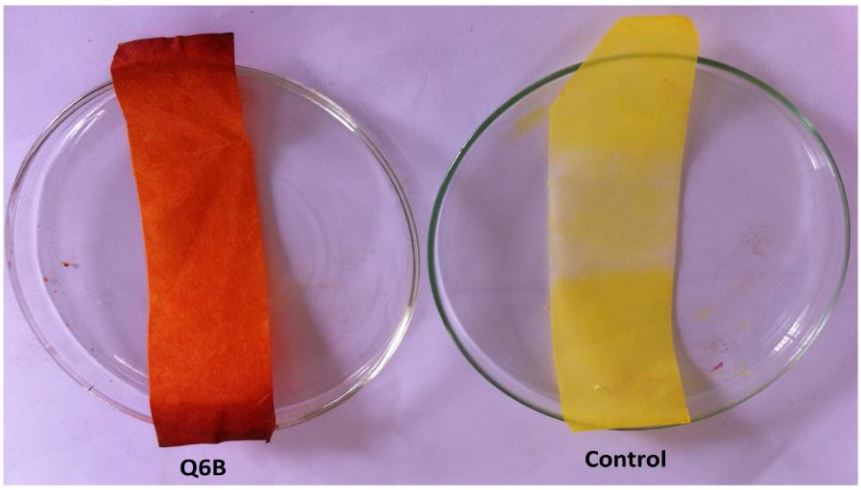

Figure 4: Production of HCN on King B agar by antagonistic *Pseudomonas* isolates

Spore germination assay

The germination (germ tube growth) of *B. cinerea* spores was inhibited by *Pseudomonas* strains filtrate after 18 h of incubation compared with the control. The percentage of spore germination inhibition ranged from 33 to 59%. The germ tube inhibition percent ranged from 40 to 75% (**Table 4**).

Table 4: Effect of five *Pseudomonas* isolates on spore and tube germination, of *B. cinerea.* and their capacity to produce HCN, siderophores and enzymes

Isolate	Spore germination inhibition (%)	Germ tube inhibition %	PIVOCs	HCN Production		Chitinase	Protease	Cellulase	Siderophores
				P	Colour				
Q6B	59.33±3.61^{c*}	75.00±0.29	100%±0.00^{a}	++	D-b	+	+	+	+
Q13B	35.33±6.56^{ab}	50.00±0.58	100%±0.00^{a}	++	D-b	+	+	+	+
Q7B	35.66±1.53^{a}	60.00±0.58	100%±0.00^{a}	++	D-b	+	+	+	+
Q14B	33.00±10.69^{a}	50.00±0.58	100%±0.00^{a}	++	D-b	+	+	+	+
Q1B	43.33±10.39^{ab}	40.00±1.00	100%±0.00^{a}	++	D-b	+	-	+	+

Values indicate mean values (±S. D.) followed by different letters are significantly different within a row or column at P<0. 01 according to Newman-keuls test; P: picric acid test; D-b: dark brown

Siderophore production

All five isolates produced siderophores, which manifested by the presence of an orange halo around colony (**Table 4**).

Lytic enzymes

The protease activity was shown by the isolates (Q6B, Q13B, Q7B and Q14B). while the chitinase and the cellulose activities were shown by all the five selected isolates (**Table 4**).

Discussion

Five *Pseudomonas* isolates Q6B, Q13B, Q7B, Q14B and Q1B, obtained from the soil rhizospheric of tomato, have shown an efficient and significant ability to protect tomato plants against *B. cinerea*. Obtained results agreed with those that report that *Pseudomonas* is of major use in the biocontrol of pathogens (Kaur *et al.,* 2007; Gao *et al.,* 2018; Dutta *et al.,* 2020, Chaouachi *et al.,* 2021). Isolates of the bacterial strain sourced from greenhouse grown tomato plant roots showed significant antifungal activity against *B. cinerea.* Additionally, isolates showed significant antifungal activity against *B. cinerea* in tomato fruit and leaves as well.

The cell-free supernatant of *Pseudomonas* strains had an inhibitory effect on conidia germination, germ tube elongation and mycelial growth of *B. cinerea*. The latter agrees with previous reports in which it was found that rhizobacteria can reduce pathogenic infections directly by competition for space and/or nutritional competition (niche exclusions) and secretion of antibiotics (Lugtenberg and Kamilova, 2009; Mitter *et al.,* 2013). Bioactive compounds which have been secreted include lipopeptides, 2,4diacetylphloroglucinol and phenazine-1-carboxylic acid (Paulin *et al.,* 2017; Jaaffar *et al.,* 2017).

The current study revealed the mechanism of action of *Pseudomonas* isolates against *B. cinerea*. the These isolates were able to produce HCN and siderophores. The current study concurs with Chang *et al.,* (2007) who reported that the supernatant of *Bacillus cereus* QQ308 inhibited spore germination and germ tube elongation of *Fusarium oxysporum*, *F. solani*, and *Pythium ultimum*. Bryk *et al.,* (1998) reported that *Erwinia herbicola* directly inhibited spore germination of both *B. cinerea* and *Penicillium expansum* in liquid culture. Products may act on the spore germination or on the length of the germ tube. Other bioactive metabolites produced by the selected strains played crucial roles in inhibiting *B. cinerea*, such as volatile antifungal compounds. In the current study, production of VOCs and HCN in the *in vitro* tests resulted in a complete inhibition of *B. cinerea*. Ramette *et al.,* (2003) reported that the microbial production of HCN is an important antifungal trait in the control of root infecting fungi. In the same context, Kumari and Khanna (2014) reported that the plant growth promoting rhizobacteria isolate (15B) significantly inhibited growth of *F. oxysporum* sp.*ciceri* by producing VOCs, resulting in 64.2% inhibition compared to controls. Experimentation of the the current study illustrated that all five strains produce lytic enzymes including proteases, cellulases and chitinases. It has been shown that some enzyme producing bacteria are able to destroy oospores of phytopathogenic fungi (El-Tarabily, 2006) and affect the spore germination and germ-tube elongation of phytopathogenic fungi (Sneh, 1984; Frankowski *et al.,* 2001).

Conclusion

A combination of different mechanisms play an important role in inhibition of *B. cinerea in vitro* as well as on the plant parts; fruits and leaves. Potential production of volatile and diffusible antagonistic metabolites infers that selected *Pseudomonas* are potential antagonists against a range of phytopathogenic fungi that infect tomato and other crops. *Pseudomonas* hold great potential as biopesticidal alternatives to chemical fungicides in reducing the damage of fungal disease. *Pseudomonas* isolates of this study have an ecological role and a key utilization in sustainable agriculture systems, which additionally benefits ecosystem sustainability.

Future investigations are recommended in both biochemical and molecular approaches in order to refine both broad and specific mathematic control for the benefit of ecosystem service returns. It is suggested that investigation of mechanisms and pathways are made. These may proceed from ascertaining key qualities of bacterial genomes and work towards sequence studies leading towards quantitative and qualitative measurement of the factors involved (Kearsey and Pooni 1996). With use of specific genetic and biochemical / biological and mathematic refinement we look to unveil relationships with the vast range of members of edaphic communities in order to maximize agroecological productivity, ecosystem services and soil diversity potentials alike.

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Conflicts of interest/Competing interests
There are no known conflicts of interest associated with this publication.

Author contributions
- R. Qessaoui and R. Bouharroud designed the study, conducted the experiment and provided the original version of manuscript.
-B. Chebli, E. H. Mayad., M. Alouani, J. N. Furze and T. Paulitz contributed subjectively and corrected the final version of the manuscript.
- H. Lahmyed and A. Ajerrar provided support during antifungal tests.

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The manuscript has been read and approved by all named authors.

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