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## Article

# Halophilic Plant Growth Promoting Rhizobacteria as Producers of Antifungal Metabolites under Salt Stress

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**Abstract:** Salinity is one of the major factors responsible for the deterioration of soils, making them unsuitable for agriculture. The application of halotolerant and halophilic plant growth promoting bacteria (PGPR: Plant Growth Promoting Rhizobacteria) with biological control activities as an inoculant of cultivated plants offers a biological alternative to the use of agrochemicals, particularly when subjected to salt stress. In this perspective, 70 bacterial strains were isolated from saline soils (sebkha) in arid and semi-arid areas of eastern Algeria. Three isolates were selected based on their ability to produce bioactive molecules allowing them to promote plant growth, such as hydrolytic enzymes, indole acetic acid, HCN, NH<sub>3</sub>, etc. Two of these isolates belong to the genus *Serratia* and the third is a halophilic *Halomonas* bacteria. These bacteria were identified based on the 16S rDNA sequence. Antagonism tests against phytopathogenic fungi were carried out. The identification of the antifungal molecules produced by these bacteria was done by high performance liquid chromatography. These bacteria are capable of inhibiting mycelial development against phytopathogenic fungi with rates reaching 80.67% against *Botrytis cinerea*, 76.22% against *Aspergillus niger* and 66.67% against *Fusarium culmorum* for *Serratia* sp. The strain *Halomonas* sp. inhibited mycelial growth by production of volatile substances of *Aspergillus niger* at 71.29%, *Aspergillus flavus* at 75.49% and *Penicillium glabrum* at a rate of 72.22%. The identification by HPLC of the antifungal molecules produced by these three bacteria reveals that they are polyphenols, which makes these strains the first rhizobacteria capable of producing phenolic compounds. Finally, pot tests to determine the effectiveness of these strains in promoting wheat growth under salinity stress (125 mM, 150 mM and 200 mM) were carried out. The results revealed that a consortium of two isolates (*Serratia* sp. and *Halomonas* sp.) performed best at 125 mM. But, at higher concentrations it was the halophilic bacteria *Halomonas* sp which gave the best result. In all cases, there was a significant improvement in the growth of wheat seedlings inoculated with the bacteria, compared to non-inoculated controls.

**Keywords:** halophilic bacteria; salt stress; biological control; polyphenols; Plant Growth Promoting Bacteria (PGPB)

## 1. Introduction

Soil salinization is a major challenge in arid and semi-arid regions, which seriously affects agricultural production [1]. 20% of total cultivated land and 33% of irrigated agricultural land worldwide are affected [2]. According to the Food and Agriculture Organization (FAO), around 1 billion hectares of the world's land surface are affected by salinization, which represents around 8.7% of the planet's land surface, this causes an estimated 27,3 billion dollars in economic losses [3]. Each year, approximately 10 million hectares of the world's agricultural land are destroyed by salt accumulation [4].

The increase in salinity level has negative repercussions on soil properties and plant physiology leading to deterioration in plant development [5]. Saline soils have high electrical conductivity (EC) and impose ionic toxicity, osmotic and oxidative stress, nutrient deficiency on plants and thus limit water absorption from the soil [2].

Faced with this saline stress, numerous physical, chemical and biological strategies such as plant breeding, genetic engineering of plants and a whole series of agricultural techniques. Recently, the application of rhizobacteria has been developed to improve the plant defense system [6]. The application of PGPR is one of the most potential biological measures for improving agricultural yield. [7,8]. Many studies have shown that PGPR directly or indirectly promotes plant growth. Direct mechanisms refer to the ability of PGPR to fix nitrogen, dissolve organic or inorganic phosphorus, produce phytohormones, such as auxin or indole-3-acetic acid (IAA), and other bioactive molecules that stimulate the growth and development of plants [9,10,11,12,13]. For indirect mechanisms, PGPR participates in preventing the development of phytopathogens, either through competition for space and nutrient occupation, or by producing metabolites such as antibiotics, inducing plant resistance [14,15,16].

To deal with salt stress, in recent decades there has been interest in the application of halotolerant and halophilic bacteria. These PGPRs promote plant growth under salt stress through complex mechanisms, such as enhancing antioxidant enzymes to regulate reactive oxygen (ROS), enhancing the accumulation of intracellular osmolytes to ensure ionic balance and through the accumulation of secondary metabolites [17,18,19].

On the other hand, plant pathogens have a devastating effect on agricultural crops. The use of chemical substances has a negative effect on health and the environment. Therefore, the use of plant growth-promoting rhizobacteria (PGPR) as growth-promoting and plant disease-fighting agents is a better alternative [20].

In this study, we isolated three bacteria from the rhizosphere of halophytic plants from two arid and semi-arid regions of eastern Algeria. These bacteria have been selected for their ability to produce hydrolytic enzymes, hydrogen cyanide, ammonia, indole acetic acid (IAA), etc. After identification, the PGP isolates were tested for their *in vitro* ability to inhibit fungal growth and their ability to enhance the growth of wheat plants under salt stress.

## 2. Materials and methods

### 2.1. Soil Sampling

Sampling was carried out at two sites; Sebkhet Bazer (36°03'16"N 5°40'35"E) and Chott El Hodna (35°25'34"N 4°44'39"E), in Eastern Algeria. 10 soil samples were collected from each site in the rhizosphere of wild halophilic plants.

The samples were collected in sterile plastic bags and then transported to the laboratory at 4°C. 10g of soil from each sample was homogenized in sterile physiological water. 100µl of each suspension were inoculated into culture media for halophiles with a NaCl concentration ranging from 0.5M to 3M in triplicate. After 72 hours of incubation at 28°C, the bacteria were isolated on the same medium supplemented with agar. 70 bacterial isolates were purified then preserved for subsequent studies.

### 2.2. Production of extracellular lytic enzymes:

#### 2.2.1. Cellulase:

The search for extracellular cellulase enzymes was carried out using agar disks 6 mm in diameter containing young bacterial cultures and placed inside Petri dishes containing Carder's medium (1986); composition in g/L: Na<sub>2</sub>HPO<sub>4</sub> (6); KH<sub>2</sub>PO<sub>4</sub> (3); NaCl (0.5); NH<sub>4</sub>Cl (1); Yeast extract (3); CMC (carboxymethylcellulose) (7); Agar (15); at a pH of 7.2 ± 0.02. The boxes are subsequently incubated at 28°C for 8 days [21]. After incubation, a solution of Congo red (0.1%) is added to the surface of the media. After 20 minutes, the surface of the box is flooded with a 1M NaCl solution, then left to stand

overnight. The appearance of a clear halo around the colonies indicates the degradation of the CMC and reflects the presence of an extracellular cellulase.

#### 2.2.2. Amylase:

Discs of young bacterial cultures are placed on starch-based culture medium in order to demonstrate their ability to degrade starch. The medium was prepared according to the method described by Vinoth et al. [22] and whose composition in g/L is: KNO<sub>3</sub> (0.5); K<sub>2</sub>HPO<sub>4</sub> (1.0); MgSO<sub>4</sub> (0.2); CaCl<sub>2</sub> (0.1); FeCl<sub>3</sub> (0.001); soluble starch (10.0); agar (15.0); 7.2 ± 0.02 pH. The boxes are incubated for a period ranging from 48-72 h at 28°C. After incubation, a solution of (lugol) is used to flood the culture medium inside the boxes. After a few minutes of contact, the plates are washed with distilled water, and the appearance of clear zones around the agar discs reflects the presence of extracellular amylase in the medium. Areas where the starch is not degraded appear blackish blue.

#### 2.2.3. Lipase:

Discs of young bacterial cultures are placed on culture medium containing g/l: peptone (10); NaCl (5.0); CaCl<sub>2</sub> 2H<sub>2</sub>O (0.1); Tween 20 (1%, v/v) and Agar (18). The pH is adjusted to 7.4. After 48 hours of incubation at 28°C, a positive result results in the appearance of a clear halo around the colonies [21, 23]

#### 2.2.4. Protease:

In the same way, the search for proteolytic activity is carried out using the culture medium containing in g/l: nutrient agar (23), bacteriological gelatin (10), pH at 7 [21,24]. The presence of extracellular proteases is revealed using the Frazier reagent.

#### 2.2.5. Urease:

According to Christensen [25], the revelation of bacterial urease is carried out by the agar disk method on a medium with a pH of 6.8 prepared as follows: Add (in g) to 950 ml of distilled water: Peptone (1); Glucose (1); NaCl (5); Na<sub>2</sub>HPO<sub>4</sub> (1.2); KH<sub>2</sub>PO<sub>4</sub> (0.8); Phenol red (0.012); Agar (15). After autoclaving, add 50 ml of a sterile urea solution (40%) and pour the medium into Petri dishes. Isolates having produced urease show a pink halo around their colonies.

#### 2.2.6. Chitinase:

The following culture medium is used, it is composed as follows in g/l:

Colloidal chitin (0.8 to 0.6); K<sub>2</sub>HPO<sub>4</sub> (2.7); KH<sub>2</sub>PO<sub>4</sub> (0.3); MgSO<sub>4</sub>·7H<sub>2</sub>O (0.7); NaCl (0.5); KCl (0.5); Yeast extract (0.13); Agar (15). The medium is inoculated by the disk method and incubated at 28°C for a minimum of 7 days. Chitinase activity is manifested by the appearance of a transparent halo around these discs [26,27].

### 2.3. PGPR features:

The isolates with the best scores were tested to select the best performing strains.

#### 2.3.1. Nitrogen fixation:

The bacterial isolates were streaked on nitrogen-free "Burk's N-free" culture medium containing in g/l: glucose (10); NaCl (30); KH<sub>2</sub>PO<sub>4</sub> (0.4); K<sub>2</sub>HPO<sub>4</sub> (0.5); Na<sub>2</sub>SO<sub>4</sub> (0.05); CaCl<sub>2</sub> (0.2); MgSO<sub>4</sub>·7H<sub>2</sub>O (0.005); Na<sub>2</sub>MoO<sub>4</sub> (0.003); agar (15). The development of bacterial colonies demonstrates the capacity of the strain to fix atmospheric nitrogen.

#### 2.3.2. Solubilization of phosphates:

The bacterial isolates were incubated at 28°C for 7 days on modified Pikovskaya medium containing in g/l: glucose (10); Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> (5); (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, (0.5); MgSO<sub>4</sub>·7H<sub>2</sub>O (0.1); KCl (0.2); yeast extract (0.5); MnSO<sub>4</sub>·H<sub>2</sub>O (0.002); FeSO<sub>4</sub>·7H<sub>2</sub>O (0.002); NaCl (50) and agar (15).

#### 2.3.3. Production of ammonia (NH<sub>3</sub>):

The bacterial isolates were inoculated in tubes containing 5ml of peptone water (peptone 20g/l and NaCl 30g/l) then incubated at 28°C for 48h. After incubation, 0.25ml of Nessler reagent is added to each tube and the appearance of a brown to yellow color indicates the production of ammonia.

#### 2.3.4. Production of hydrogen cyanide (HCN):

The production of HCN is determined following the method of Lorck [28]. The bacteria are streaked on nutrient agar supplemented with glycine at a rate of 4.4 g/l. A sterile disk of *whatman* paper is placed on the lid of the Petri dish and impregnated with a solution of sodium picrate (2% anhydrous sodium carbonate in 5% picric acid). The petri dish is sealed with Parafilm and incubated at 28°C for 96 hours. The color change to orange or brown indicates the production of HCN.

#### 2.3.5. Production of indole 3-acetic acid (IAA)

Demonstration of the capacity of each isolate to produce IAA was determined by the Salkowski colorimetric method [29]. Each isolate is inoculated in modified HM culture broth containing 1 mg.ml<sup>-1</sup> and 5% glucose, then incubated with shaking (100 rpm) at 28°C for 3 days. After incubation, 5ml of each culture is centrifuged. 2ml of the supernatant is added with 2ml of Salkowski's reagent (2% FeCl<sub>3</sub> 0.5M in 35% HClO<sub>4</sub>) and incubated in the dark at room temperature for 25 min. optical density is measured at 530 nm.

#### 2.4. Molecular identification:

The selected strains were identified based on the 16S rDNA sequence. DNA transmission was followed by amplification of this gene using the forward primer (356F) 5'ACWCTACGGGWGGCWGC and the reverse primer (1064R) 5'AYCTCACGRACGAGCTGAC. Polymerase chain reactions (PCR) were carried out in a final volume of 20 µl. The reaction mixture contains: 10µl of Xpert Fast Hotstar Mastermix 2x (Grisp), 1µl of DNA sample and 7µl of ultra pure water. The mixture was placed in the T100 PCR thermocycler (BIO-RAD), programmed as follows: initial denaturation at 95°C for 3 min, then 35 cycles each composed of three steps: denaturation at 95°C for 15 s, annealing at 55°C for 15 s, elongation at 72°C for 15 s, then a final elongation at 72°C for 3 min. Aliquots of PCR reactions were resolved on 1% agarose gels stained with ethidium bromide. The purified PCR products were sequenced in one direction on StabVida (Caprica, Portugal) using the previously mentioned primers.

#### 2.5. Antifungal activity

The antifungal activity of the selected bacterial strains was tested against four fungi: *Aspergillus niger*, *Aspergillus flavus*, *Penicillium glabrum* *Fusarium culmorum* and *Botrytis cinerea*.

##### 2.5.1. In-vitro tests:

Directed inhibition of mycelial growth (direct confrontation test):

The direct confrontation method on agar [30,31] was used. A disc of young mycelium (5 days old) from each mushroom is placed in the center of a Petri dish containing PDA (Potato-Dextrose-Agar) medium. 3 disks of a young culture of each bacterial strain are placed 2.5 cm from the fungus. Plates with sterile agar disks served as controls. After 7 days of incubation at 25°C, the percentage of inhibition is calculated according to the following formula: PGI% = (KR-R1) / KR × 100 where KR is the average diameter between the point of deposition of the fungus and the margin of the colony



contained in the control (mm), R1 is the average diameter between the point of deposition of the fungus and the margin of the colony contained in the treated Petri dish (mm) [32,33].

Indirect inhibition of mycelial growth (indirect confrontation test):

This test was carried out against *A. niger*. Bacterial filtrates were prepared by the modified method of Li et al. [34]. The bacterial strains were inoculated on HM agar at 28°C for 24 hours then subcultured in the same liquid medium. Three categories of cultures were prepared. The first pure, containing the bacterial strains alone. The second mixed, where disks of the pathogen (*A. niger*) were added. In the third, the bacterial strains were cultivated in HM culture medium free of glucose. After incubation with shaking (100 rpm) at 28°C for 72 h, these media were centrifuged at 6000 rpm for 30 min, and the supernatant was filtered through a sterile 0.22 µm multi-pore membrane.

Petri dishes containing the PDA medium are inoculated in tight streaks with the spore suspension of the phytopathogen, then sterile paper disks soaked in the filtrate are placed aseptically to test the antifungal activity. Sterile discs soaked in sterile HM medium will serve as controls. The boxes are incubated at 25°C for 2 days. This test is carried out following the modified method of Aghighi et al. [35] and Mahdiah et al. [36].

Indirect inhibition of mycelial growth by production of volatile substances:

The test for inhibition of mycelial growth by production of volatile substances was carried out following the protocol described by Dennis and Webster [37]. A Petri dish containing the HM medium is inoculated in tight streaks with the bacterial antagonist. Another Petri dish containing the PDA medium is inoculated with a 5mm diameter disk of the pathogenic fungus (*A. niger*, *A. flavus*, *P. glabrum* or *F. culmorum*). The bottom of the two Petri dishes containing the seeded culture media are placed face to face and sealed with parafilm. Petri dishes without bacteria are used as a control. The radial growth of the mushrooms is measured after 7 days of incubation and is compared to the control.

## 2.5.2. Characterization of bacterial antifungal metabolites:

The filtrates obtained were freeze-dried at 40°C and 200 KPa. [38]. The residue obtained is dissolved in 50 ml of methanol then adjusted to 100 ml with distilled water. The mixture obtained is centrifuged (1535 × g) for 20 min then made up to 100 ml with a methanol-water solution (50:50 v/v) following the modified method of Kim et al. [39] and N'Guessan et al. [40].

A Hypersil BDS-C18 reversed-phase column (250 × 4.6 mm, 5 µm) was used for chromatographic separations. The mobile phase is composed of 0.2% acetic acid/acetonitrile (ACN) in a gradient, starting with 95% 0.2% acetic acid and ending with 100% ACN. The flow rate was 1.5 ml min<sup>-1</sup> for 30 min.

The injection volume of the SPCs (standard polyphenol compounds) was 20 µl, while that of the samples and its standard solutions was 10 µl. The column temperature was maintained at 30 °C. The UV detection wavelengths were 230-255-280-300 and 355nm, consistent with the UV scanning results [41]

The standards used in this study were: Gallic acid, Hydroxy quinone, Resorcinol, Catechin, 1-2di hydroxy benzene, Syringic acid, Naringenin 7 glucoside, 3 4 5 Trimethoxy benzoic acid, M anisic acid, Hesperidine.

## 2.5.3. In-vivo test:

### Biological test on fruits (apples):

This test is carried out according to the modified protocol of Xiao and Kim [42]. Fresh apples of the "Golden Delicious" variety of approximately the same size and at the same level of maturity were sterilized with bleach (2%) for 2 min, then rinsed three times with sterile distilled water. Four wounds (3 mm wide × 3 mm deep) were made in the equatorial zone of each fruit. Each well is inoculated with 30 µl of the bacterial antagonist (108 CFU/ml for each strain tested) and 30 µl of sterile distilled water for the control [43]. After 2 hours at room temperature, 15 µl of the spore suspension of the pathogen (2×10<sup>4</sup> spores / ml for *Botrytis cinerea*, and 10<sup>5</sup> spores / ml for *Aspergillus niger*) were added. The fruits were incubated at 25°C at 90 to 95% relative humidity for 6 days [44]. The

experiment was carried out in triplicate with 6 fruits in each repetition. Injuries are assessed daily. Disease severity was calculated by the incidence and size of lesions around the wounds. Wound diameters were measured 3 and 6 days after inoculation [45].

## 2.6. Plant Growth Stimulation Tests

### 2.6.1. Potting

Sterilization of the surface of durum wheat seeds (*Triticum durum*, Simeto variety) with 70% ethanol and 3% sodium hypochlorite then rinsed 5 times with sterile distilled water according to the Orhan method [46].

Seed inoculation: CH11, SB6 and SB39 bacteria were cultured in HM medium at 28°C for 18 hours. The bacterial suspensions were centrifuged (6000 rpm/10 min) and the supernatant was discarded. The cells were washed 3 times with 20ml of sterile phosphate-buffered saline (PBS, pH 7.2) then re-suspended in sterile PBS (108 CFU/ml). The sterile seeds are soaked in the bacterial suspensions of the three isolates selected either alone or in combination two by two or all three together for 1 hour. Sterile seeds soaked in sterile PBS served as controls [47].

Growing wheat: wheat seeds are sown in plastic pots filled with sterilized or unsterilized soil, at a rate of three seeds per pot. The pots were divided into batches (9 pots per batch) and each batch was irrigated with the same volume of saline solutions of different concentrations (125, 150 and 200 Mm NaCl) on the 1st day and the 8th day. The other waterings were done with the same volume of sterile tap water (10 ml/pot) to avoid dehydration. The experiment was carried out under semi-controlled conditions with temperatures of 20/25 °C night/day and a photoperiod of 16/8 h light/dark and relative humidity of 60% for two weeks. A block randomization plan was carried out.

### 2.6.2. Evaluation of plant growth factors under salt stress

After 14 days of growth, the seedlings were harvested and the growth of the plants was observed. Thus, the morphological properties of the seedlings were measured under the different experimental growth conditions: the height of the aerial part, the length of the roots, the fresh weight, and dry the aerial part and the roots of the seedlings. The fresh weight was obtained just after harvest. On the other hand, the dry weight is obtained after drying in a hot oven at 45°C until the weight stabilizes.

## 2.7. Statistical analysis

Variance analysis one way (ANOVA) was carried out using the Statistical Package for the Social Sciences (IBM SPSS V.26, USA). The Tukey test is used to compare mean values between treatments at the 5% significance level ( $p = 0.05$ ).

## 3. Results

### 3.1. Researching traits that promote plant growth

In this study, 70 halotolerant bacteria were isolated from saline soils. These bacteria have been studied for their ability to produce enzymes of agricultural interest and promoting plant growth. The preliminary test of enzymatic activities allowed the selection of six named bacterial strains (SB6, CH11, CH12, SB29, SB39, CH42) which produce cellulases, amylases, lipases, proteases, ureases and chitinases. All of these bacteria have the ability to fix atmospheric nitrogen and produce ammonia ( $\text{NH}_3$ ). 53.33% have the power to solubilize inorganic phosphate, and 33.33% produce hydrogen cyanide (HCN). On the other hand, only SB39 and CH42 bacteria have the power to produce small quantities of IAA at a rate of  $2.031 \mu\text{g/ml} \pm 0.01$  and  $1.027 \mu\text{g/ml} \pm 0.1$  respectively. (Table 1)

Table 1. Plant growth promoting traits of selected isolates.

	SB6	CH11	CH12	SB29	SB39	CH42
Cellulase	+	+	++	-	+	+
Amylase	+++	-	-	-	+++	-
Lipase	-	++	-	++	-	+
Protease	+++	+++	+++	+++	+++	-
Urease	-	-	+	-	++	++
Chitinase	+++	+++	+++	+++	++	++
Nitrogen fixation	+	+	+	++	++	++
Phosphate solubilization	++	++	-	+	-	-
IAA (µg/mL)	-	-	-	-		
NH <sub>3</sub>	++	+	+	++	+++	++
HCN	+	-	-	-	+++	-

+++ : high activity    ++ : intermediate activity    + : low activity    - : no activity.

3.2. Molecular identification

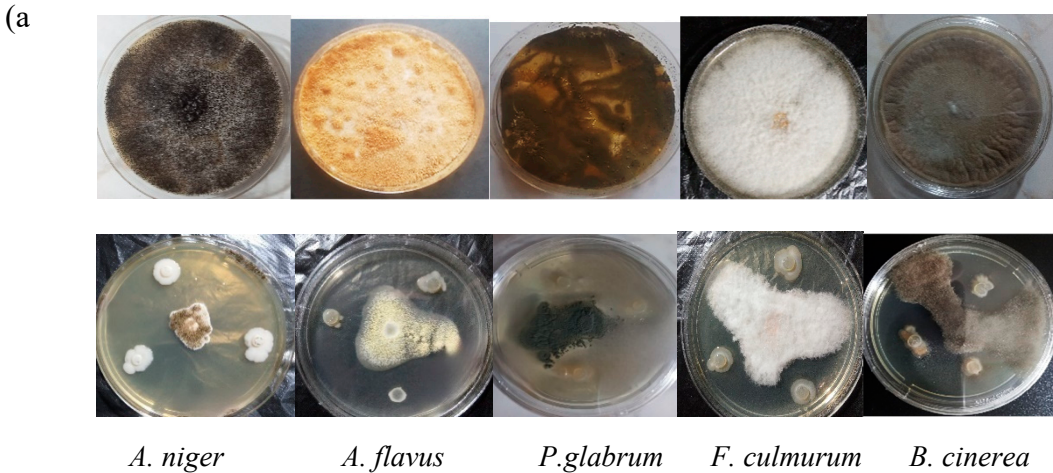
Following the study of the characteristics promoting plant growth, three bacteria (SB6, CH11 and SB39) were selected for molecular identification and further studies.

An analysis of 16S rDNA was carried out. After PCR amplification and gene sequencing, the results showed that the SB6 and CH11 strains are closely related to *Serratia sp* with a similarity rate of 98.84% for SB6, and 98.98% for CH11. On the other hand, strain SB39 gave 98.98% similarity with the halophilic bacterium *Halomonas sp*. Still, these results only allowed an unambiguous attribution of gender.

3.3. Antifungal activity

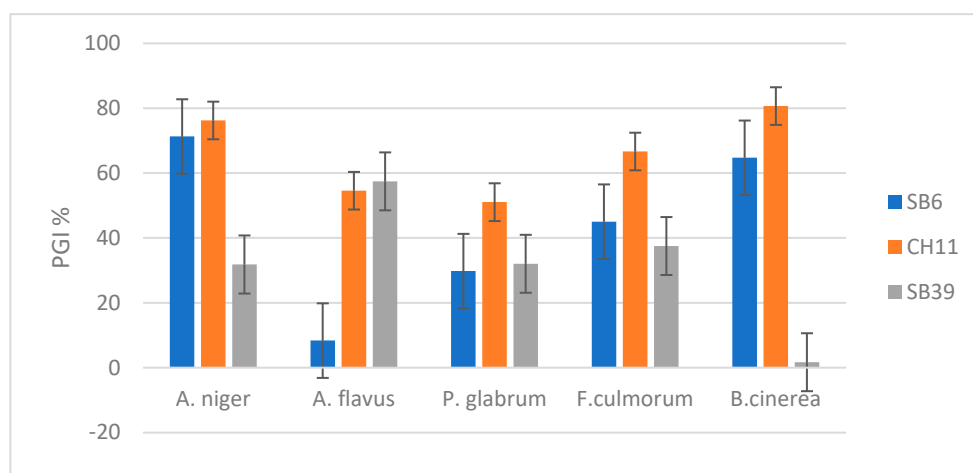
3.3.1. Direct inhibition of mycelial growth

Figure 1.a shows the results of direct comparison on agar of the selected bacterial strains against the four phytopathogenic fungi, namely *Aspergillus niger*, *Aspergillus flavus*, *Penicillium glabrum*, *Fusarium culmorum* and *Botrytis cinerea*. The results show that the CH11 strain obtained the best activity of inhibition rates which vary from 51.04% to 80.67%. The SB6 strain gave an inhibition rate of 71.28% against *A. niger*. On the other hand, isolate SB39 recorded a maximum rate of 57.44% against *A. flavus*. All results show that the fungi were inhibited by isolates SB6, CH11 and SB39 but to different degrees (Fig. 1b).

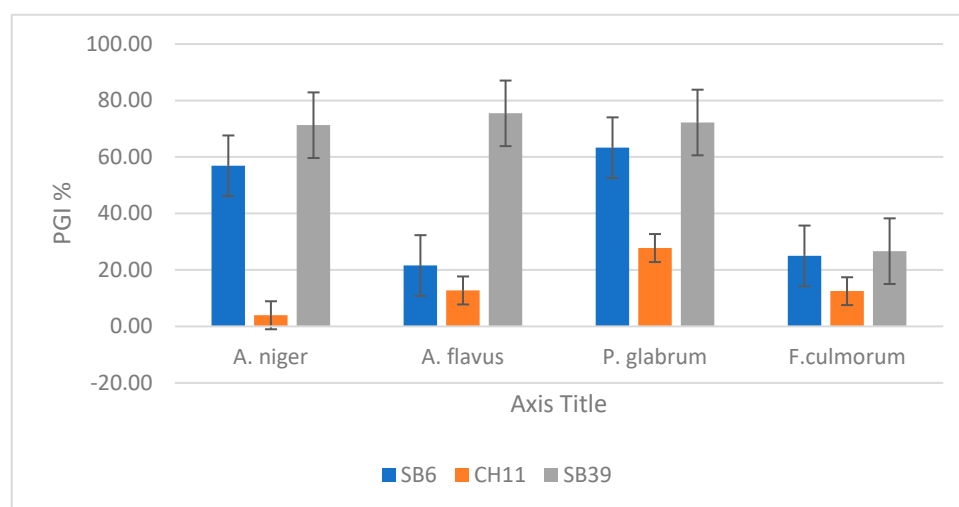




(b)



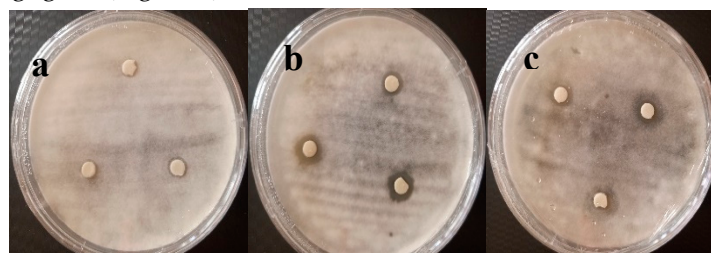
(c)



**Figure 1.** (a) Effect of isolates SB6, CH11 and SB39 on mycelial growth ; (b) Percentage growth inhibition (PGI %) of phytopathogenic fungi by isolates (plate confrontation tests) ; (c) Effect of volatiles substances produced by the three strains on growth inhibition (PGI%).

### 3.3.2. Indirect inhibition of mycelial growth

The results of the effect of the filtrates of the bacterial cultures showed the presence of an antifungal activity against *A. niger* for the CH11 isolate in the three environments (with and without biotic and abiotic stress) which indicates that this isolate produces antifungal molecules in a constitutive manner. As for isolate SB39, the results show that there is an inducible production of antifungal molecules, that is to say only when this bacteria is subjected to biotic stress (presence of the fungus) or abiotic (environment free of glucose). On the other hand, for isolate SB6, the zone of inhibition is very negligible (Figure 2).



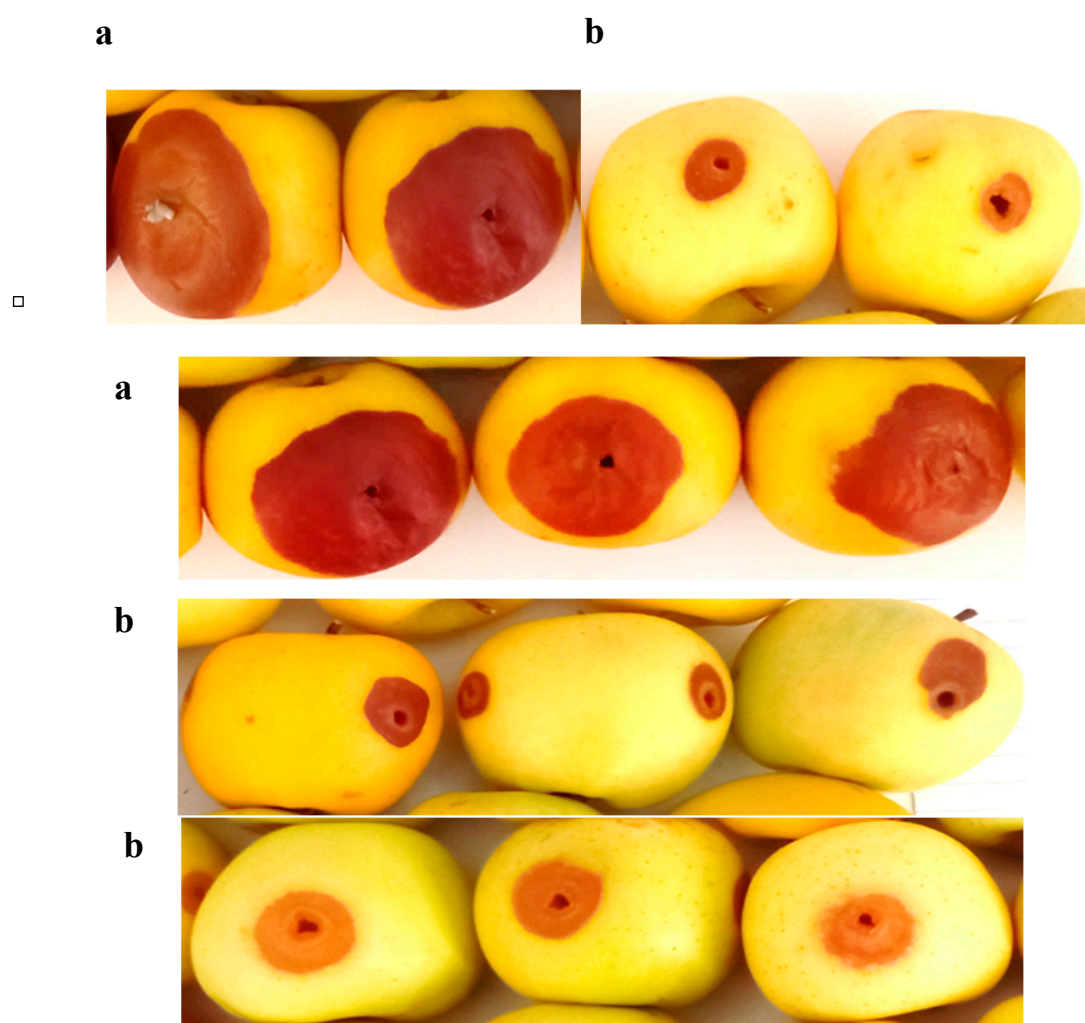
**Figure 2.** Effect of culture broth filtrate on mycelial growth (a. SB6 ; b. CH11 ; c. SB39).

### 3.3.3. Inhibition of mycelial growth by volatile substances

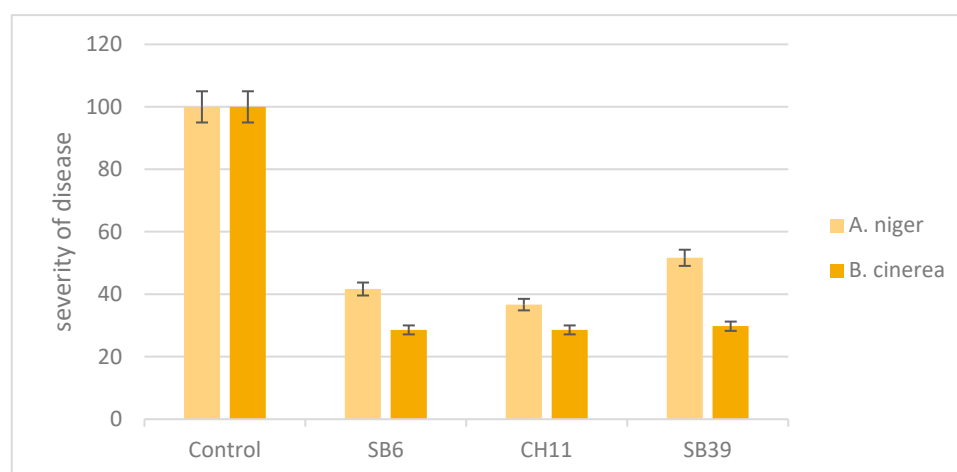
The effect of volatile substances produced by bacteria on mycelial growth was determined by calculating the percentage inhibition compared to the negative control. Volatiles reduced the mycelial growth of phytopathogenic fungi. For *A. niger* and *P. glabrum* the reduction was 71.29% and 72.22% respectively for SB39, 56.93% and 63.33% for SB6 and only 3.96% and 27.78% for isolate CH11. Concerning *F. culmorum* the reduction varied from 12.5% to 26.67% for the three isolates (Fig. 1c).

### 3.3.4. Bioassay on apples

In this experiment, apple fruits that were inoculated with the pathogens (*A. niger* or *B. cinerea*) plus the antagonist developed much smaller areas of infection compared to fruits that were inoculated with the pathogens alone (Figure 3). Thus, the severity of the disease was reduced by more than 50% in the case of *A. niger* and by more than 70% for *B. cinerea* (Figure 4).



**Figure 3.** Effect of fungal mold on apple fruit treated (b) or not (a) with the bacterial antagonist after 6 days of incubation.



**Figure 4.** Efficiency of bacterial isolates (SB6, CH11 and SB39) against *A. niger* and *B. cinerea* in apple fruit.

### 3.3.5. Characterization of bacterial antifungal metabolites

The antifungal molecules produced by the bacteria SB6, CH11 and SB39 were identified by RP-HPLC by matching their retention times to that of the corresponding standard. The quantification of the compounds was determined by comparing their surface area to that of the corresponding standard of known concentration.

The analysis of the filtrates of the culture broths which showed positive antifungal activity allowed the identification of polyphenols produced by the bacterial isolates. The results obtained are represented in Table 2. Thus, the three bacterial isolates produce gallic acid, catechins and syringic acid. In the presence of the pathogen, the SB39 strain is capable of producing gallic acid and syringic acid with concentrations that exceed 20 mg/ml. And it is the only one that produces hydroxyquinone.

**Table 2.** Phenolic compounds identified in the filtrate culture broth and their concentrations.

RRT(min)	Compound name	Broth filtrate (mg/ml)					
		SB6 -	CH11 -	CH11 +	CH11 dep	SB39 +	SB39 dep
3,15 ± 0,05	Gallic acid	13,77	20,79	12,76	13,43	21,56	9,53
3,61 ± 0,00	Hydroxy quinone	-	-	-	-	6,31	-
5,47 ± 0,23	Resorcinol	2,24	2,82	2,29	2,88	5,38	-
6,10 ± 0,17	Catechin	3,81	4,84	4,27	1,29	5,17	3,50
6,23 ± 0,41	1,2-hydroxy benzen	-	8,16	3,13	3,55	-	-
7,21 ± 0,05	Syringic acid	16,65	13,24	18,54	11,72	22,88	12,23
10,63 ± 0,00	Naringenin-7-glucoside	-	-	4,56	-	-	-
11,08 ± 0,00	3,4,5-Trimethoxy benzoic acid	2,51	-	-	-	-	-
11,36 ± 0,01	m-anisic acid	-	1,82	1,01	2,39	-	-
15,07 ± 0,01	Hesperidine	9,69	1,91	-	-	-	-

(-) : in the absence of the phytopathogen ; (+) : in the presence of the phytopathogen ; (dep) : in a depleted culture medium.

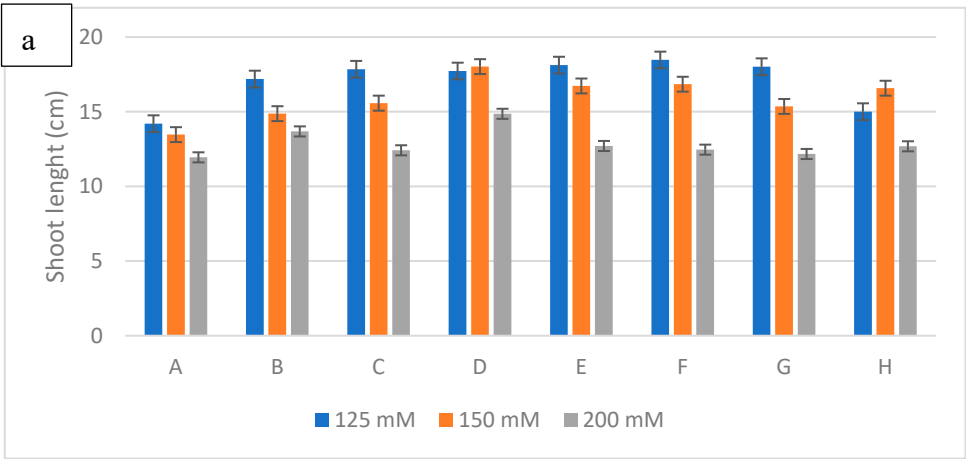
### 3.4. Tests to stimulate the growth of wheat plants subjected to salt stress

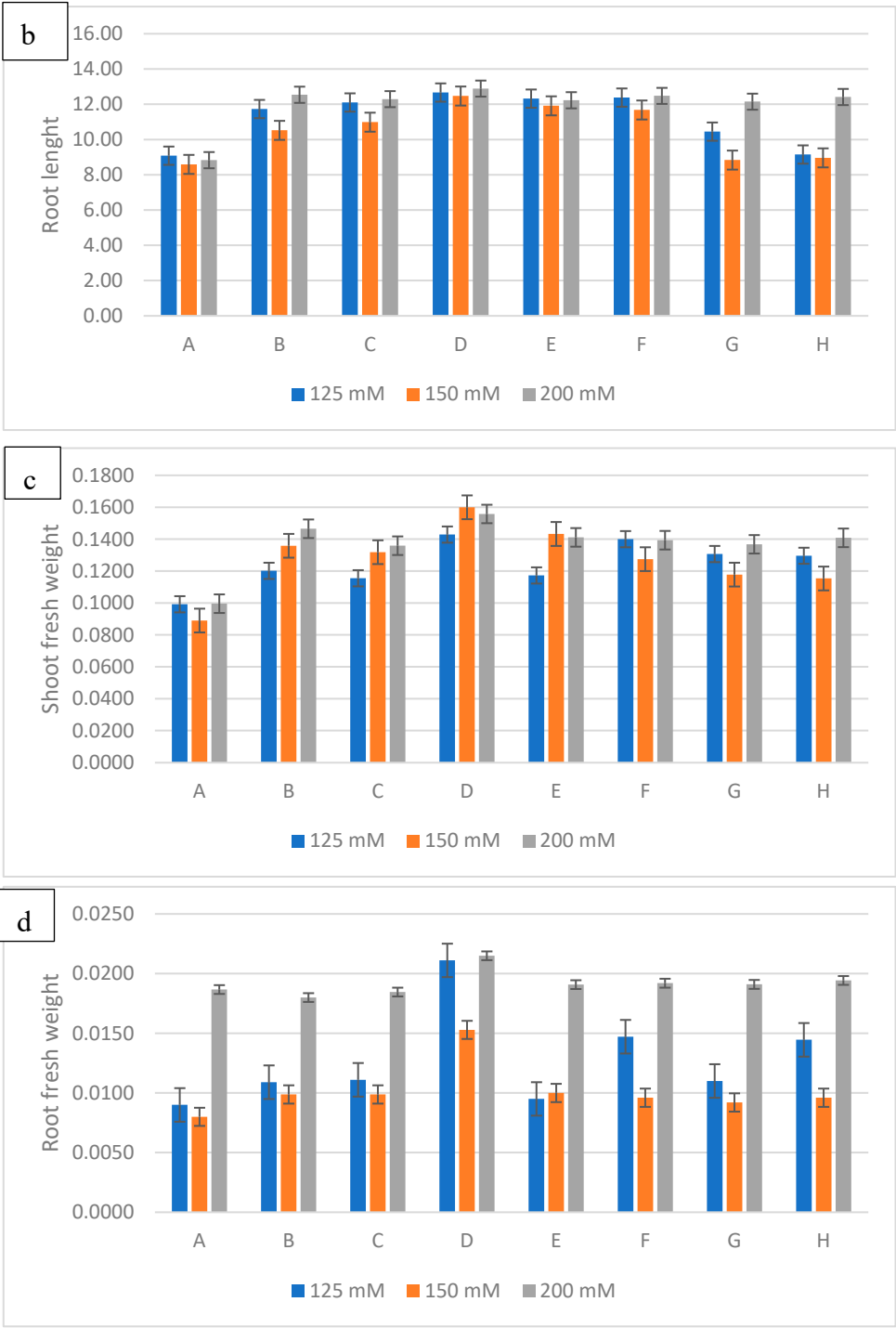
The results of the treatment of wheat seeds with the three bacterial isolates and their different combinations show a significant improvement in the growth parameters considered compared to those obtained with the control under salt stress (Figure 5). To evaluate the harmful effects of salt stress, different concentrations of NaCl were used (125, 150 and 200 mM). The results obtained

indicate that the increase in the NaCl concentration causes a reduction in the length of stems and roots and consequently in their fresh and dry weight in control plants. However, the inoculation of the three bacterial isolates showed a significant attenuation of the harmful effects of salinity (Figure 6). Thus, the inoculation of the bacterial strains caused an increase of 30% for the length of the stems to 125 Mm with l The association of the two strains SB6 and SB39 (*Serratia sp.* and *Halomonas sp.*) and increases of 34% and 24% at 150 and 200 Mm respectively with the strain *Halomonas sp.* Concerning the length of the stems, the best result was obtained with the *Halomonas sp.* strain with an increase of 39%, 45% and 53% for the concentrations of 125 mM, 150 mM and 200 mM.

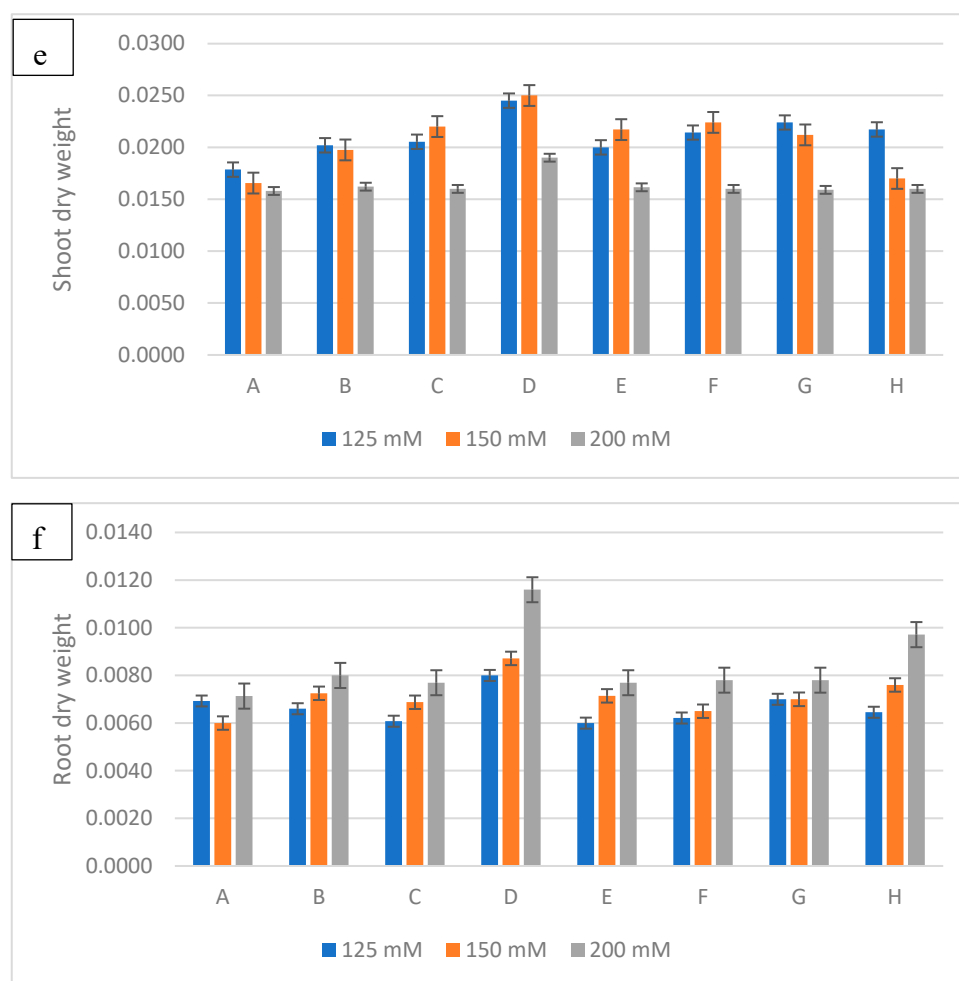


**Figure 5.** Effects of bacterial strains treatment on the growth of wheat seedlings under salt stress conditions (125 mM) 1) Control ; 2) SB6 ; 3) CH11 ; 4) SB39 ; 5) SB6-SB39 ; 6) SB6-CH11 ; 7) CH11-SB39 ; 8) SB6-CH11-SB39.









**Figure 6.** The effect of inoculation with halotolerant rhizobacteria (SB6 : *Serratia* sp; CH11 : *Serratia* sp; SB39 : *Halomonas* sp) on growth (a : shoot length; b : root length ; c : shoot fresh weight; d :root fresh weight; e :shoot dry weight; f :root dry weight) of wheatplants grown under salt stress of 125mM, 150 or 200 mM of NaCl. The bars on the top of the columns indicate standard error of the mean.

#### 4. Discussion

The salinization of agricultural soils constitutes one of the major problems in agriculture which limits plant growth and agricultural productivity by up to 50% [48,49,50]. Around 1 billion hectares of the world's land surface are affected by salinization, which represents around 7% of the planet's land surface [31]. On the other hand, the use of fungicidal chemicals has a negative effect on health and the environment. Therefore, the application of plant growth-promoting rhizobacteria (PGPR) as growth-promoting and plant disease-fighting agents is a better alternative [20,51,52].

In this study, halotolerant rhizobacteria capable of promoting plant growth were isolated. Three bacterial strains were selected and then identified based on the similarity of their 16S rDNA genes. Two of them belong to the genus *Serratia* (SB6 and CH11) while isolate SB39 belongs to the genus *Halomonas*. Bacteria of the genus *Serratia* and *Halomonas* have previously been reported for their plant growth promoting and antagonistic effects against phytopathogens [53,54,55,56].

Antifungal activity was observed in all three bacterial strains. Noting that the *Serratia* sp strain showed better antagonistic potential by direct confrontation on agar. On the other hand, the strain *Halomonas* sp. gave the best results for the inhibition test by production of volatile substances. Similar results were obtained by [57,58,59,60]. According to several studies, one of the mechanisms of action of soil bacteria against phytopathogens is linked to the production of biomolecules (HCN, NH<sub>3</sub>) and lytic enzymes such as cellulase, proteases, chitinases. These enzymes play an important role in

degrading the cell walls of fungi [48,49,50]. The production of HCN by PGPRs played an important role in the biological control of several soil pathogenic fungi, as reported by Azeem al. [64]. And as our results show (Table 1), these bacteria produce, to different degrees, these bioactive molecules which inhibit the growth of fungi. These results are similar to the work reported by [56,60].

One of the problems of harvests is the contamination of products during storage, which leads to significant losses [42]. In our study, the application of the three bacterial strains to apples previously contaminated by phytopathogenic fungi made it possible to limit the expansion of the rot zone induced by these fungi. The results obtained show that there is a significant difference between the tests and the negative control. Furthermore, disease severity after application of the three bacterial antagonists reduced rot in apples contaminated with *A. niger* and *B. cinerea*. Similar results were reported by Sun et al. [59] and Jimtha et al. [65] with bacteria of the genus *Serratia*.

Mixed cultures in liquid HM medium containing the bacterial antagonist and the phytopathogen *A. niger* (biotic stress), or the bacteria alone in a depleted medium (abiotic stress) revealed that the production of antifungal molecules only took place in case of stress in the case of isolate SB39 (*Halomonas* sp.). This means that these molecules are inducible by environmental stress of the bacteria which corresponds to the results obtained by Tabli et al. (2017), who reported that the antifungal activity was only demonstrated when the *Pseudomonas* and *Serratia* strains were grown in the presence of *A. niger*. On the other hand, for the CH11 strain, antifungal molecules are synthesized in a constitutive manner (in the presence and absence of stress).

In order to identify these antifungal molecules, HPLC was carried out. The results reveal that our three bacterial strains have the capacity to produce polyphenols. According to Skorokhod et al. [66], only plants and certain microorganisms are capable of synthesizing the precursors of phenolic compounds. Thus, Livinska et al. [67] studied the production of polyphenols by some lactic acid bacteria in different environments. This present study is the first to demonstrate the capacity of bacteria of the genus *Serratia* and *Halomonas* to produce polyphenols. Endophytes can produce secondary metabolites identical or similar to those of their host [68]. Knowing that *Serratia* and *Halomonas* can be endophytic bacteria, this could explain our results.

Enzymes of microbial origin are considered biofertilizers because they degrade organic polymers, and make essential elements such as nitrogen and phosphorus accessible to the plant [69]. In this study, we selected halotolerant and halophilic bacteria in order to test their beneficial effects on the growth of durum wheat under saline stress. Several studies have shown the beneficial effect of halotolerant and halophilic bacteria on plant growth [46,70,71]. Our studied strains demonstrated the ability to produce hydrolytic enzymes (cellulase, amylase, lipase, proteases, urease and chitinase), to solubilize phosphate, to fix nitrogen and to moderately produce the phytohormone AIA (table 1). The action of microbial enzymes intervenes in the physicochemical of the soil by improving the availability of elements essential for plant growth [72]. Thus, the simultaneous expression of different PGP traits, acting synergistically, promotes plant growth [73]. IAA is a phytohormone involved in root initiation, cell division and enlargement. Therefore, IAA-producing microorganisms increase plant growth and root length, which allows the plant to obtain more nutrients from the soil [74]. A study by Dasele et al. [75] revealed that *Halomonas* sp. had AIA production potential. This corresponds to the results we found where SB39 was the only isolate selected to produce IAA, despite the fact that work reports the ability of bacteria of the genus *Serratia* to synthesize this phytohormone [76,77]. IAA-producing rhizobacteria allow the plant to increase water permeability causing better water absorption in the leaves, thus inducing an improvement in cellular metabolism linked to environmental stress [78,79]. In addition, PGPR can fix atmospheric nitrogen essential to the metabolism of various molecules such as amino acids, enzymes, NADH, chlorophyll, etc. The production of ammonia is also important because it represents another source of nitrogen. Soluble phosphates directly support plant growth because they act as a macronutrient. It notably promotes root development and intervenes in other processes such as the ripening of flowers or fruits, the mobilization of nutrient reserves... Recently, most research has focused on the isolation and study of microorganisms. halotolerant and halophilic for their PGP potential [55,60,80]

Inoculation of halophilic/halotolerant bacteria improves plant growth under salt stress. Thus, the strains selected in this study are capable of synthesizing a large number of these metabolites, which could explain their effectiveness against target pathogenic fungi and the stimulation of wheat growth.

In the results we obtained, we note a significant improvement in the growth stimulated by bacteria under saline stress compared to the control. It is interesting to point out that the consortia gave good results especially at 200 mM NaCl. These results are perhaps due to the synthesis of phytohormones by these bacteria as well as to the improvement in the availability of mineral elements in the soil [81].

These results showed us that NaCl reduces the weight of plant shoots and roots, but that the presence of PGP reduces the harmful effects of stress caused by NaCl. The bacterial strains studied promoted plant growth under salt stress from 120 mM to 200 mM and the results are in agreement with previous studies [46,47,54,82]

## 5. Conclusions

Salinity is a big challenge for agricultural production. The three halotolerant and halophilic strains isolated from *Serratia sp.* (SB6 and CH11) and *Halomonas sp.* (SB39) gave interesting results on the growth of wheat under salinity stress. They have also shown their effectiveness in inhibiting the development of phytopathogenic fungi. These bacteria have the capacity to produce biofertilizers and secondary metabolites such as polyphenols. Based on these results, we can consider these bacteria as effective agents for stimulating plant growth and in biocontrol. It would be interesting to verify these potentialities in vivo and in the field, and to understand the mechanisms used by these bacteria as PGP.

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