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Biological Control xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Plant growth promoting and inducible antifungal activities of irrigation well water-bacteria

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ARTICLE INFO

Keywords:
Pseudomonas
Serratia
Biocontrol
Biological Control Agents
Plant Growth Promoting Bacteria (PGPB)

ABSTRACT

Different bacterial groups in irrigation well water are strongly implicated in soil health and plant development. Herein, 48 bacterial strains were isolated from agricultural well water in northern Algeria. Among them, four strains were selected based on their antifungal potential and their ability to express Plant Growth Promoting traits such as Indole Acetic Acid (IAA), hydrolytic enzymes, siderophores etc. The isolates were identified as *Pseudomonas* sp. (B, D and N strains) and *Serratia* sp. (C strain) by 16S rRNA gene sequencing. Mycelial growth inhibition against *Botrytis cinerea* and *Aspergillus niger* ranged from 60 to 90% for the four strains. Moreover, volatiles compounds emission by the isolates resulted in Plant Growth Inhibition values ranging from 13 to 50%, specifically against *B. cinerea*. Impressively, the strains' antifungal activity showed high inducibility as it was obtained only by the filtered supernatants from bacterial cultures previously in contact with the fungus. Finally, a greenhouse assay, carried out to determine the strains' efficacy in promoting plant growth and protecting seedlings under *Pythium aphanidermatum*-infected soil, revealed that the strain N markedly enhanced pea germination (+250%) and fresh weight (+43%) and tomato fresh weigh (+10%). The results constitute an attempt for better use of the bacterial functional diversity from irrigation wells in sustainable agriculture.

1. Introduction

The worldwide demographic explosion, together with environmental degradation, has the unfortunate consequence that global food production may soon become insufficient to feed the world's population. It is therefore essential to increase agricultural productivity within the next few decades (Glick, 2012). Both biotic and abiotic stresses in agriculture such as drought, salinity, elevated temperature, metal toxicity, nutrient deficiencies and plant diseases caused by fungi, bacteria, viruses, and nematodes are more to more aggressive and constitute serious problems affecting final yields of the most consumed crops (El Khoury and Makkouk, 2010; Borges et al., 2014). Thus, achieving sustainable crop production to protect the environment and ensure enough food requires strategic measures to manage these ecological stresses such as water scarcity and climate change, commonly related to pathogens development and aggressiveness (Itier, 2008; Haggag et al., 2015). However, better understanding of the mechanisms underlying plant resistance/tolerance-related characters is of a great help to develop fruitful new agricultural strategies (Borges et al., 2014).

In the last few decades, the application of chemical fertilizers, pesticides, herbicides and fungicides was the dominant tool to enhance plant growth and to control pathogens propagation. However, such approaches have led to soil degradation and resistance emergence in plant-pathogen populations (Pal and McSpadden Gardener, 2006). In addition, the harmful effects of such chemicals on both human health and the environment obliged researchers to seek for secure and eco-friendly alternatives (Reuveni, 1995; El Khoury and Makkouk, 2010). Certain soil bacteria, commonly described as Plant Growth Promoting Bacteria (PGPB), are able to enhance plant yield and to control phytopathogens, constituting the most widely studied and increasingly used tool in modern agriculture. In future, PGPB are expected to replace chemical fertilizers, pesticides and artificial growth regulators that have numerous side effects on sustainable agriculture (Tsegaye et al., 2017).

PGPB can directly affect plant metabolism through nitrogen fixation, mineral solubilization, plant hormones modulation and plant tolerance improvement to abiotic stresses (drought, salinity, nutrient

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http://dx.doi.org/10.1016/j.biocontrol.2017.10.010
Received 6 August 2017; Received in revised form 5 October 2017; Accepted 20 October 2017
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Please cite this article as: Tabli, N., Biological Control (2017), http://dx.doi.org/10.1016/j.biocontrol.2017.10.010

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deficiency etc.). PGPB also participate indirectly in preventing phytopathogens development either by competition for space and nutrient occupation or by producing antibiotic metabolites, inducing systemic resistance in the whole plant (Niranjan Raj et al., 2006; Ahmadzadeh and Tehrani, 2009; Suresh et al., 2010; Tri Wahyudi et al., 2011; Beneduzi et al., 2012). Bacteria belonging to genera *Acetobacter, Achromobacter, Arthrobacter, Azoarcus, Azospirillum, Pseudomonas, Serratia, Anabaena, Acetobacter, Clostridium, Enterobacter, Flavobacterium, Frankia, Rhizobium* and others are the most used as PGPB for several vegetal species (wheat, rice, pea, chickpea, tomato etc.). In addition, the use of PGPB in the biocontrol of phytopathogenic fungi (*Botrytis*, *Aspergillus*, *Mucor*, *Phytophthora* etc.) is not less adopted (Elad et al., 1996; Limura and Hosono, 1998; Loqman et al., 2009; Glick, 2012; Talibi et al., 2014; Tabli et al., 2014). Unfortunately, as the results obtained in laboratory are not necessarily ensured *in vivo*, the unpredictable character of the most *in vitro*-selected PGPB oblige both scientists and farmers to seek for more competent isolates that are able to ensure better results in soil, a more complex environment than *in vitro* substrates (Przemieniecki et al., 2015).

As water is the most limiting factor for agricultural production, water scarcity could potentially lead to lands abandonment due to economic losses (EEA, 2009). From a microbiological point of view, some bacteria in irrigation well water may constitute potential source of significant metabolic richness with high ability to interact with plants, leading to yields improvement, soil restoration and pathogens biocontrol (Bensidhoum et al., 2016). In addition, PGPB are isolated from different natural sources including soil, plants and mineral waters (Ramadan et al., 2015; Bensidhoum et al., 2016). Herein, we selected four multi PGP- and antagonistic-treats bacteria from an irrigation well water in Northern Algeria. The bacteria were screened for their ability to produce metabolites such as hydrogen cyanide (HCN), ammonia, indole acetic acid (IAA), hydrolytic enzymes, siderophores etc. After identification, the multi-PGP isolates were tested for their *in vitro* ability to inhibit fungal growth and sporulation. The PGP ability of the selected strains was then verified on tomato and pea plants and biocontrol properties against pathogens was confirmed on pea plants growing on *Pythium aphanidermatum*-infected soil.

2. Materials and methods

2.1. Water sampling

Bacteria were isolated from water samples of an irrigation well located in the region of Djebira in Bejaia, northern Algeria (36°41′59.2″N; 5°04′28.8″E). The samples were transported to the laboratory at 4 °C and bacterial isolation was immediately performed on Plate Count Agar medium (PCA). Thus, the samples were serially diluted (up to 10^{-6}) and each dilution served as inoculum for three PCA plates (1 ml/Petri dish). After incubation (72 h at 30 °C), the phenotypically different colonies were purified on the same medium and conserved for further studies.

2.2. Fungal strains and growth conditions

The plant pathogenic fungi used in this work were *Botrytis cinerea* BC1, kindly provided by the laboratory of Mycology (University of Bejaia-Algeria), and *Aspergillus niger* ATCC 9642, available at the Institute of Bioscience and Bio-Resources (IBBR)-CNR, Italy. The two fungal strains were revivified and purified on Potato Dextrose Agar (PDA) medium before use.

2.3. Selection procedure and antifungal activity

Initially, 48 bacterial isolates, obtained from water samples were tested for their antifungal potential against the aforementioned fungal strains, using agar diffusion method as described by Sagahón et al. (2011). Thus, 5 mm diameter PDA plugs, containing young mycelium of either *B. cinerea* or *A. niger*, were taken from actively growing colonies (4 days old) and then deposited in the plate center of the same medium. 10-μL overnight cultures of each bacterial strain, growing on Luria Bertani broth, were spotted at 1.5 cm from the plate edge (three spots/plate). The plates were sealed with parafilm and incubated for 7 days at 21 ± 2 °C (*B. cinerea*) and 25 ± 2 °C (*A. niger*). Controls without bacteria were used and the experiment was done in duplicates. At the end, the percentage of mycelial growth inhibition (PGI) was calculated according to the following formula: PGI% = KR-R1/KR × 100 (KR is the distance in mm covered by the fungi in the control plate; R1is the distance in mm between the fungi and the bacterial colony) (Soylu et al., 2005).

Four isolates, selected following the previous test, were phenotypically characterized (cell/colony shape, Gram, catalase, oxidase, mobility) (Guiraud and Galzy, 1980). They were then phylogenetically identified based on their 16S rRNA sequences and used for further studies.

2.4. Spore's germination inhibition

The selected strains' ability to inhibit fungal spore's germination was screened by mixing 20 μL of the spore suspension (10^6 spores/mL) with equal volumes of 24 h-old bacterial culture grown in LB medium (10^8 CFU/mL). The mixtures were kept in Eppendorf tubes containing 1 mL of 5% glucose solution, prepared in sterile distilled water. The tubes were incubated at 21 ± 2 °C (*B. cinerea*) and 25 ± 2 °C (*A. niger*) for 24 h. Control tubes with only fungal spores were prepared and the experiment was repeated in triplicate. The spores' germination success was hemocytometrically measured by determining the percentage of spores germination inhibition (SGI%) (Sadfi-Zouaoui et al., 2008).

2.5. Antifungal volatiles production

The production of volatile compounds implicated in the strains' antagonistic activities was assayed using the protocol described by Dennis and Webster (1971). A bottom portion of PDA-containing plates was inoculated with 5 mm disc of the pathogen fungi (*B. cinerea* or *A. niger*). A similar bottom portion was streaked with the antagonist. Both bottom plates were placed face to face and sealed with parafilm. Plates without bacteria were used as control. The fungal radial growth was recorded, 7 days later, as compared to the control.

2.6. Metabolic and functional characterization

2.6.1. Hydrolytic enzymes secretion

Hydrolytic enzymes production by the selected strains were tested using agar disk methods. The bacterial strains were initially grown on PCA plates for 48 h at 30 °C. Five mm agar discs, containing the obtained cultures, were used to determine cellulolytic (Carder, 1986), esterasic (Carrim et al., 2006), lipolytic (Carrim et al., 2006), chitinolytic (Kopečný et al., 1996), proteolytic (Carrim et al., 2006), amylolytic (Vinoth et al., 2009) and ureasic (Christensen, 1946) activities.

2.6.2. Phosphate solubilization

The ability of the four strains to solubilize tricalcium phosphate was tested on Pikovskaya's agar medium, as described by Peix et al. (2001). After 3 days of incubation (30 °C), phosphate-solubilizing bacteria generate transparent halos around colonies.

2.6.3. Siderophores production

The bacterial isolates were assayed for siderophores production on the Chrome Azurol-S agar medium, as described by Schwyn and Neilands (1987). After incubation (30 °C/48–72 h), the development of yellow to orange halo around the colony indicated siderophores

production by the strain.

2.6.4. Indole Acetic acid (IAA) synthesis

IAA production was determined using the protocol originally proposed by Bric et al., (1991). Therefore, the strains were incubated under agitation (100 rpm; 30 °C; 3 days) in LB tubes supplemented with 0.5% glucose and 500 µg/mL L-tryptophan. Five mL of each culture was centrifuged (9000g; 20 min) and 2 ml of Salkowski's reagent (2% 0.5 M FeCl3 in 35% perchloric acid) was mixed with an equal volume of the culture supernatant. The mixture was then incubated in darkness at room temperature for 25 min. IAA production was spectrophotometrically measured (530 nm) and quantified by extrapolation on a standard curve previously prepared with pure IAA solutions.

2.6.5. Hydrogen cyanide (HCN) production

HCN production was detected according to the method of Lorck (1948). A nutrient agar was supplemented with filter-sterilized glycine solution (4.4 g/L) before to be casted in Petri dishes. The plates' covers were upholstered by Whatman paper impregnated with sodium picrate solution (5% picric addition of 2% anhydrous sodium carbonate acid). Fresh bacterial culture (100 µL) was flooded on the agar using sterile swabs then Petri dishes were sealed with parafilm and incubated at 30 °C for 96 h. The transformation of the yellow color on Whatman paper (initially provided by the solution of sodium picrate) to orange or brown color indicates HCN production by the strain.

2.6.6. Ammonia (NH_3) production

Freshly grown cultures of the selected strains were inoculated into peptone water-containing tubes (5 mL) and incubated at 30 °C for 48 h. After incubation, 0.25 mL Nessler's reagent was added to each tube. The mixture's color transformation to brown or to yellow indicates ammonia production by the tested bacteria (Cappucino and Sherman, 1992).

2.6.7. Phylogenetic identification

Total genomic DNA from the four bacterial strains was isolated using the PureLink® Genomic DNA Mini Kit (Invitrogen/Thermo Fisher Scientific, USA). Amplification of 16S rDNA was carried out using Phusion Green Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, USA) with 25 ng of genomic DNA as template and the universal primers 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Edwards et al., 1989; Stackebrandt and Liesack, 1993). PCR were performed with the following conditions: one cycle at 98 °C for 30 s, 35 cycles at 98 °C for 10 s, 54 °C for 30 s, and 72 °C for 45 s, and a final cycle at 72 °C for 7 min. After amplification, reaction products were analysed on 1.0% agarose gel. DNA sequencing was carried out on both strands using the amplification primers at Eurofins Genomics (Ebersberg, Germany). The obtained sequences were subjected to BLAST analysis on the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned using the MUSCLE program contained in the CLC Main Workbench 7.8.1 software package (QIAGEN Aarhus, Denmark). Maximum likelihood (ML) phylogenetic analysis was performed using the program MEGA 6.0 with the best nucleotide substitution model identified for the data set (K2 + G + I) (Tamura et al., 2013). Statistical support for the different internal branches was assessed by bootstrap resampling (1000 bootstrap replications). Finally, bootstrap consensus trees were constructed.

2.7. Antifungal activity and induction assays

2.7.1. Induction and antifungal activity assays

The strains' antifungal activity was evaluated against *A. niger*. It was plated on PCA agar and incubated at 28 °C until sporulation. To obtain extracellular filtrates with antifungal activity, bacteria were inoculated on LB broth, incubated (30 °C/180 rpm) then collected 24 h later. Thereafter, the bacterial suspensions were added in holes placed on PCA plates where the fungus spores were scraped. The bacterial suspensions were withdrawn from the holes after two days of incubation at 28 °C and the samples were centrifuged at 8000 rpm. The obtained cell free supernatants (extracellular fraction) of each isolate were sterilized by filtration, using a 0.22-µm pore diameter membrane, and then 300 µL of the filtrate was added in holes placed on PCA plates where the fungus spores were scraped. The plates were incubated at 28 °C for 72 h. The filtrates with antifungal activity were those that inhibited the growth of the fungus. The same experiments were also performed on the extracellular fractions obtained from the bacterial strains cultured without co-incubation with the fungus, as a control.

2.7.2. Preparation of intracellular and surface-membrane protein fractions

The preparation of intracellular and surface-membrane protein fractions was performed according to Gogliettino et al. (2010) with some modifications. The bacterial suspensions, withdrawn after the co-incubation experiments, were centrifuged at 8000 rpm for 45 min and the pellets were re-suspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM PMSF). Cells were lysed by sonication (four cycles with a 60-s pulse and 60 s off) using a Soniprep (B. Braun Labsonic U) and unbroken cells were removed by centrifugation (13,000 rpm at 4 °C for 30 min). The supernatant represents the intracellular fraction. The surface-membrane protein fraction was prepared by re-suspending the cell pellets in 50 mM Tris-HCl (pH 7.5) buffer and solubilizing it by incubation for 15 min at 37 °C in the presence of 1% Triton X-100. Insoluble material was removed by centrifugation (13,000 rpm at 4 °C for 30 min) and the supernatants (membrane fraction) were harvested. The intracellular and membrane fractions were sterilized by filtration, using a 0.22-µm pore diameter membrane, and then the filtrates (30 µg from each fraction) were added in three holes placed on PCA plates where the fungus spores were scraped. The plates were incubated at 28 °C. The same experiments were also performed on the intracellular and surface-membrane protein fractions obtained from the bacterial cells cultured without co-incubation with the fungus. Protein concentration was determined by the Bradford assay (Bradford, 1976).

2.8. In vivo assays

2.8.1. Plant growth promotion effect in tomato

Thirty days old tomato [*Solanum lycopersicum* L.; cv. Naxos F_1 (Nunhems)] seedlings were transplanted in 8 L-plastic pots containing steam-sterilized soil inoculated or not with the bacterial cell suspension. Bacterial inoculum was prepared in 1 L LB broth-containing bottles that were incubated under agitation (150 rpm) for 48 h at room temperature. The obtained culture was then centrifuged (8000 × *g* for 5 min) and the pellet was re-suspended in water. Cell suspension (OD_{600nm} = 0.6) served as inoculum. *Pseudomonas protegens* strain *CHA0*, kindly provided by Christoph Keel, University of Lausanne, Switzerland, and *Pseudomonas* sp. strain *DSMZ 13134,* from the commercial product Proradix® (Sourcon Padena, Germany) were included in the experiments as reference strains. Inoculation was carried out one day before transplanting by drenching 60 mL inoculum per liter of soil. A randomized complete block design with four blocks (12 plants/pot) was used. Twenty days after transplanting, fresh weight of the aboveground parts of tomato seedlings was measured

2.8.2. Control of Pythium Damping-off in pea

Pea (*Pisum sativum* L. cv. Sprinter) seeds were sowed in 8 L-plastic pots containing steam-sterilized soil inoculated with *Pythium aphanidermatum* IPSP-GB556 strain and bacterial cell suspension. Pots inoculated only with pathogen but not bacteria, or non-inoculated, served as the controls. *P. protegens* CHA0 and *Pseudomonas* sp. DSMZ 13134 were included in the experiment. Inoculum of *P. aphanidermatum* was prepared according to Bates et al. (2008) and used for soil inoculation (5% v/v). Inoculum preparation and inoculation procedures were carried out as described above. A randomized complete block design with

four blocks (20 seeds/pot) was used. Sixteen days after sowing, the percentage of plant emergence and the whole plant fresh weight were measured.

2.9. Statistical analysis

A binomial model (GENMOD procedure) was used to analyze plant emergence data, and multiple comparisons of means was based on least-squares means statistics ($P < .05$), using SAS/STAT v. 9.0 (SAS Institute, Cary, NC). All other data were subjected to one-way ANOVA for column analysis ($P < .05$), using Graph Pad Prism v. 6.01.

3. Results

3.1. Mycelial growth inhibition

In this study, 48 bacterial strains were isolated from agricultural well water and screened based on their ability to antagonize the phytopathogen fungi *B cinerea* and *A niger*. Among these, four isolates designated B, C, D and N, revealed to be the most effective strains against the two tested phytopathogens. Specifically, B and N strains showed the highest antifungal activity against *A. niger* mycelia (about 90%), while about 68% growth inhibition was achieved by the isolates C and D (Fig. 1A). In addition, the isolate D exhibited 82.5% antagonistic effect against *B. cinerea*, while the isolates N, C and B resulted in PGI ranging from 80% (N) to 63% (B) against the same fungus (Fig. 1B).

3.2. Spores germination inhibition

The germination success of *A. niger* spores was reduced by 20% in the presence of the tested strains, while 100% of the untreated spore suspensions (control) succeeded germination (Fig. 2A). In addition, the four strains completely inhibited *B. cinerea* spore's germination (100% inhibition), whilst only about 10% of spores' germination failure was observed in absence of bacteria (Fig. 2B).

3.3. Phenotypic and phylogenetic identification

The four strains appeared rod-shaped at the microscope, showing the appearance of a bacillus; they were Gram negative, motile, and positively reacted to catalase and oxidase tests. Moreover, the identification of the selected bacteria was determined by amplification and sequencing of the 16S rRNA genes. Specifically, the B, D and N sequences obtained the best BLAST hits with strains belonging to the *Pseudomonas* genus, while, the sequence of the isolate C showed the highest matches belonging to *Serratia* genus. Moreover, the phylogenetic analysis revealed that 16S rRNA gene sequences of the isolates B, D and N formed a distinct cluster with *Pseudomonas protegens* CHA0 (99.93% of sequence identity). On the other hand, the isolate C displayed the highest sequence identity (99.86%) with both *Serratia quinivorans* strain LMG 7887 and *Serratia quinivorans* strain 4364. Anyway, despite the strong homologies identified for the 16S rRNA genes of the four isolates, this analysis only allowed the unambiguous attribution of the genus.

3.4. Detection of plant growth promoting traits and antagonistic properties

The four strains produce protease, lipase, chitinase, esterase, and urease enzymes but no amylases, and the strain C was the only one able to degrade tricalcium phosphate. Concerning the siderophores and ammonia synthesis, positive signals were recorded for all the strains under investigation. Moreover, lower HCN amounts were detected in the presence of the two isolates B and D as compared to C and N. Otherwise, IAA amounts, produced in Tryptophan-supplemented LB medium, ranged from 2.40 μg/ml ± 0.65 for the strain C to 5.09 μg/ml ± 0.65 for the strain D (Table 1).

3.5. Volatiles substances synthesis

The effect of bacterial volatile compounds on mycelial development on Petri plates was determined by measuring the percentage of growth inhibition as compared to a negative control (in the absence of bacteria). The reduction in *A. niger* growth induced by the volatiles production ranged from 8% to 22% for the isolates B, N, C and D (Fig. 3A). Moreover, the strain C showed the best inhibitory result against *B. cinerea* through volatiles emission (50.8%), whilst the isolates D and N significantly reduced *B. cinerea* propagation by 25.8 and 21.3%, respectively (Fig. 3B).

3.6. Localization of the antifungal compounds and induction assays

Petri dish-based assays were carried out to investigate the localization of antifungal compounds produced by the selected bacterial strains (B, C, D and N), using *A. niger* as fungus model. Firstly, the extracellular filtrates obtained from 24 h-old bacterial culture of the four strains were spotted on Petri dishes, scraped with fungal spores, but no inhibition of fungal development was detected (Fig. 4, *upper panel*). Therefore, in order to verify if the antifungal activity was confined to specific cell compartments, the surface-membrane and the intracellular protein fractions were prepared and tested. Again, no growth inhibition was evidenced, suggesting that these bacteria could exert their antifungal activity only in presence of the fungus (data not shown).

Therefore, we set up induction experiments, exposing the bacterial cultures to the fungus for possible induction of the antifungal activity compounds. The bacterial suspensions were withdrawn from holes made in agar plates containing the fungus and centrifuged to collect the cell-free supernatant and cell pellets. Impressively, the extracellular and surface-membrane preparations showed significant antifungal activities

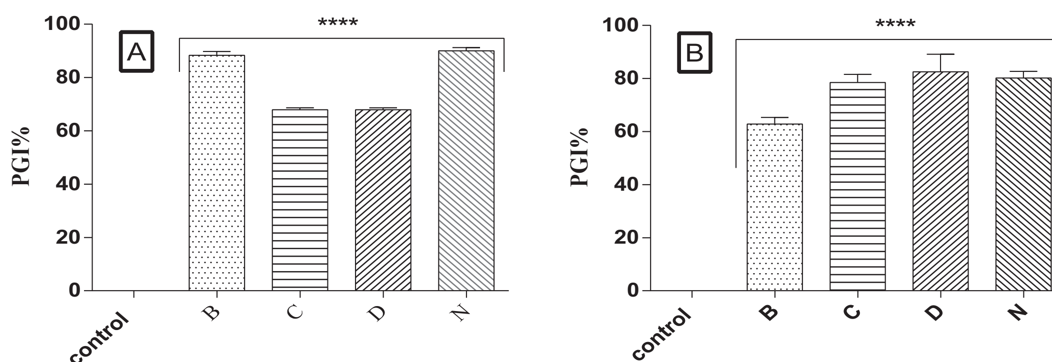

Fig. 1. Mycelial growth inhibition (percentage) of A. *niger* (A) and B. *cinerea* (B) by the antagonistic strains B, C, D, and N in Petri dual culture test. ****: significant difference (p < .0001).

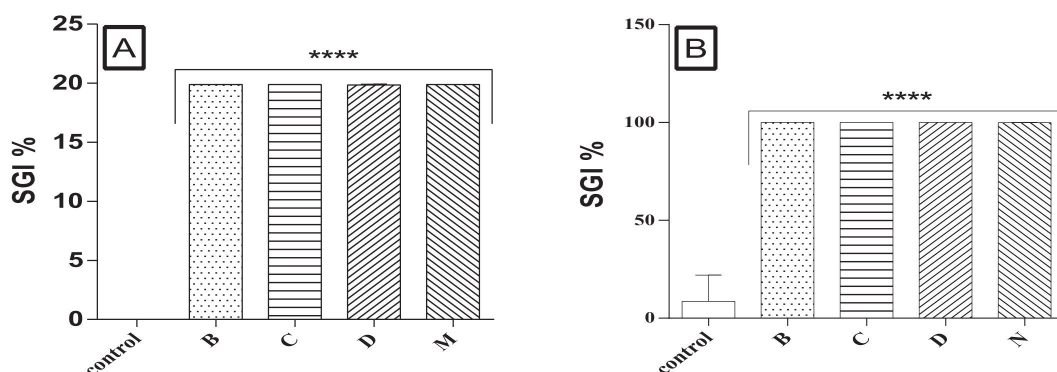

Fig. 2. Spore's germination inhibition (%) of A. *niger* (A) and B. *cinerea* (B) by the antagonistic strains B, C, D, and N in Petri dual culture test. ****: significant difference (p < .0001).

Table 1
Plant growth promoting and antagonistic traits of the analyzed isolates.

	B	C	D	N
Protease	+	+	+	+
Lipase	+	+	+	+
Cellulase	–	+	+	–
Chitinase	+ +	+ + +	+ + +	+
Esterase	+ + +	+ + +	+ + +	+ + +
Amylase	–	–	–	–
Urease	+ +	+ + +	+	+ + +
Siderophores	+ + +	+ + +	+ + +	+ + +
Phosphate solubilization	–	+	–	–
IAA (μg/mL)	3.32 ± 0.25	3.21 ± 1.14	5.09 ± 0.65	2.40 ± 0.65
NH3	+ + +	+ + +	+ + +	+ + +
HCN	+	+ + +	+	+ + +

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

when tested on the fungus, confirming our suggestion about the inducible character of their antifungal activity (Fig. 4, *down panel*). In addition, the extracellular fractions obtained from all the strains under investigation, were able to affect consistently the spore production of the fungus, being the N strain the most efficient, as shown in Fig. 4.

3.7. Plant growth promotion effects on tomato plants

PGP effects exerted by bacterial strains were evaluated in pot-grown tomato by measuring shoot fresh weight 20 days after soil inoculation. The strain C significantly increased tomato fresh weigh (+15%) as compared to non-inoculated controls (Fig. 5). The other bacteria, including the two commercial reference strains (CHA0 and DSMZ 13134), increased tomato growth by 2–10%, as compared to the control.

3.8. Control of Pythium Damping-off in pea

The bacterial strains were evaluated for the control of *Pythium* damping-off in pea by determining plant emergence 16 days after sowing in soil inoculated with *Pythium aphanidermatum*. The *Pythium* infection drastically reduced pea emergence from 83.3% in non-inoculated control to only 7.5% in pathogen-inoculated soil (Fig. 6A). All bacterial strains, except D, reduced *Pythium* damping-off compared to control, but such reduction was statistically significant only for the strain N. In soil inoculated with N, plant emergence was 26.3% i.e. a sevenfold increase compared to pathogen-inoculated control. Effectiveness of the strain N was superior to the references strain *CHA0* and *DSMZ 13134*, which increased plant emergence by 4.3- and 2-fold, respectively. The strains B and C determined non-significant increase in plant emergence (2.7- and 3.7-fold, respectively).

In pathogen-inoculated soil, emerged plants also showed a 54% weight reduction (0.23 g/plant) compared to non-inoculated control (0.50 g/plant) (Fig. 6B). Increase in plant weight was observed upon bacteria inoculation, that raised by 43% and 48% in soil inoculated with N and *CHA0*, respectively, although such an increment was not statistically significant compared to pathogen-inoculated control. In pots inoculated with the other strains, a null or negative variation in weight was observed.

4. Discussion

Using chemical fungicides in agriculture causes serious environmental pollution and health concerns. In addition, their efficiency is continually decreasing due to emergence of resistant pathogens (Handelsman and Stabb, 1996; Zhu et al., 2007). Thus, the application of antagonistic microorganisms or their active metabolites as an ecologically sustainable alternative to chemicals constitute a non-negligible approach to follow (Haggag and Mohamed, 2007).

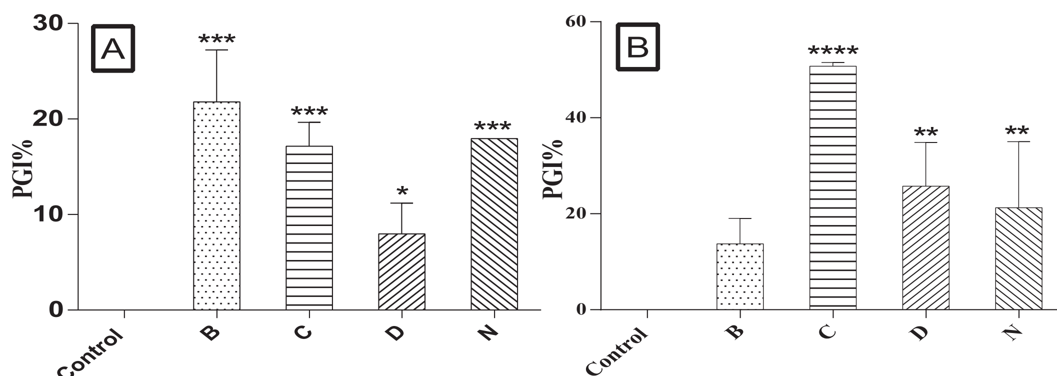

Fig. 3. Effect of volatiles substances produced by the four strains on growth inhibition (PGI%) of *A. niger* (A) and *B. cinerea* (B).

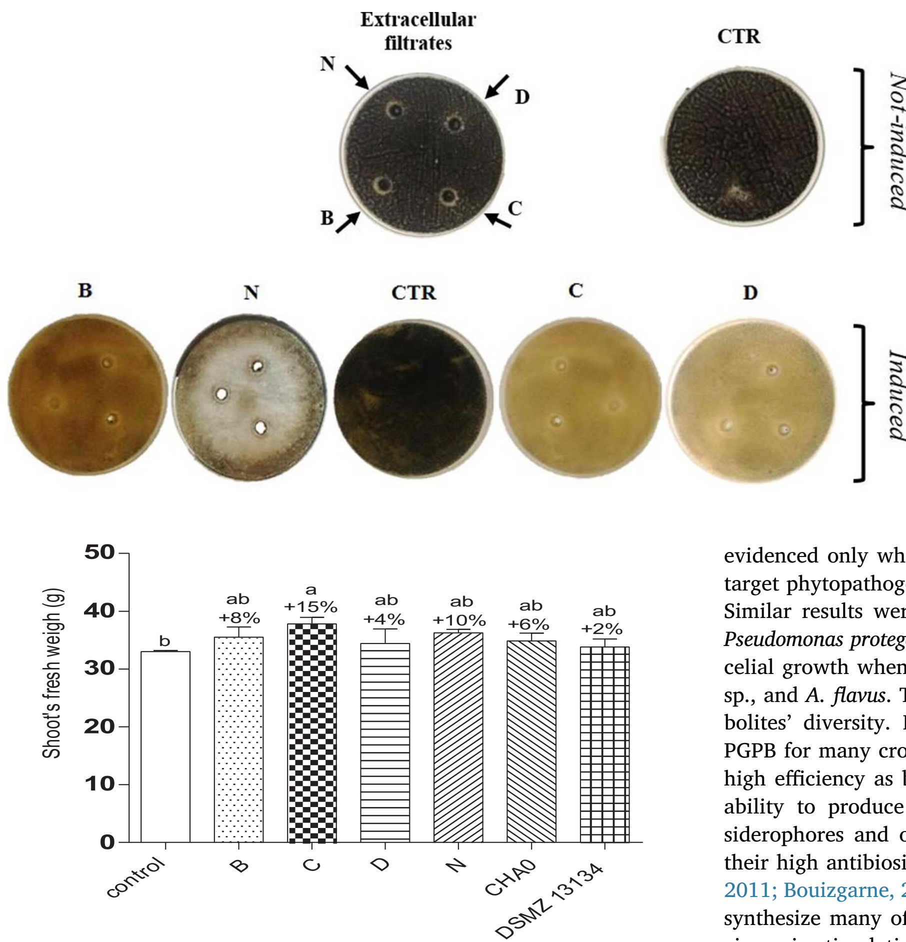

Fig. 4. Inducible antifungal activity obtained with culture supernatants of the strains B, N, C and D. The supernatants were obtained from spots of 24 h bacterial cultures in presence of the fungus *A. niger*.

Fig. 5. Growth promoting effect on pot-grown tomato plants. *Pseudomonas protegens CHA0* and *Pseudomonas* sp. *DMSZ 13134* were included as reference strains. Bars with different letters are significantly different according to LSD test ($p < .05$).

In this work, we selected four strains, three of which were identified as belonging to the *Pseudomonas* genus (B, N and D), while the isolate C was molecularly identified as belonging to the genus *Serratia*. Such bacteria were already reported to be effective against plant pathogens (Bensidhoum et al., 2016; Rai et al., 2016; Kamil et al. 2007; Khare et al., 2010) by competing for niches and nutrients, but also by stimulating plant defenses and producing fungal toxic compounds (Timmusk, 2003; De Vleesschauwer and Höfte, 2007).

All the selected strains showed an efficient fungal growth inhibition *in vitro* against *A. niger* and *B. cinerea*. Interestingly, this activity was evidenced only when the bacteria were grown in the presence of the target phytopathogens suggesting a clear induction effect of the fungi. Similar results were obtained by Rai et al. (2016), reporting that a *Pseudomonas protegens* (strain RhiNA) exerted potent inhibition of mycelial growth when confronted to the fungi *B. cinerea, A. niger, Mucor* sp., and *A. flavus*. They attributed this capacity to the produced metabolites' diversity. Bacteria belonging to *Pseudomonas* are known as PGPB for many crops (potato, radish, sugar, beet, lettuces etc.). Their high efficiency as biostimulants and biocontrol agents is due to their ability to produce antibiotics, phytohormones, hydrolytic enzymes, siderophores and other metabolites of agricultural interest, ensuring their high antibiosis, competition, and parasitism (Tri Wahyudi et al., 2011; Bouizgarne, 2013). The strains selected in this study were able to synthesize many of these metabolites, which could explain their efficiency in stimulating tomato and infected pea seedlings.

For many years, extracellular enzymes secretion has been associated with biocontrol abilities of the producing bacteria. Thus, some bacterial enzymes are able to destroy fungal spores, affecting their germinability and inhibiting their mycelial growth (Bouizgarne, 2013). Bacteria such as *Enterobacter*, *Erwinia*, *Bacillus*, *Burkholderia*, *Stenotrophomonas* and *Rhizobium* have been reported to be effective against several phytopathogenic fungi. However, *Pseudomonas*-belonging bacteria are the most widely studied in biological agents' development (John Rusagara Nzungize et al., 2012; Rai et al., 2016). Gohel et al. (2006) mentioned that chitinase- and protease-producing microorganisms might cause fungal cell wall lysis, contributing to pathogen elimination. Otherwise, Suresh et al. (2010) and Raaijmakers et al. (2002) reported that the PGP ability of *Pseudomonas fluorescens* is also due to HCN, siderophores, protease, antimicrobials, and phosphate solubilizing enzymes synthesis.

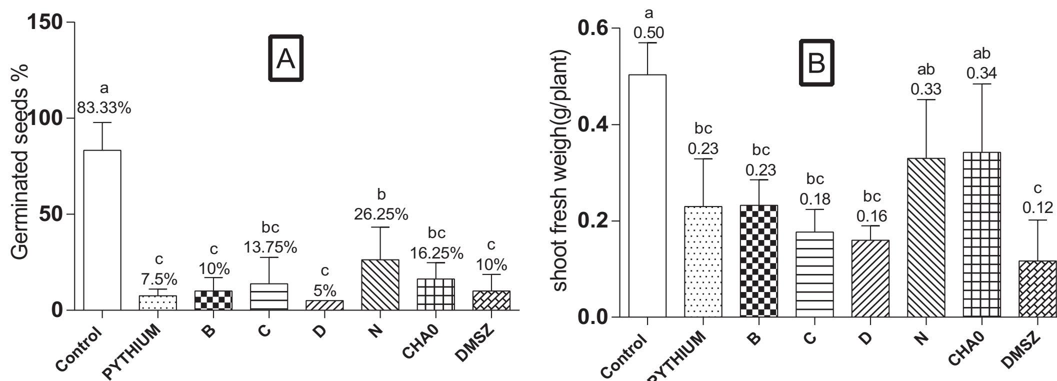

Fig. 6. Control of *Pythium* damping-off in pea: plant emergence (A) and weight (B). *Pseudomonas protegens CHA0* and *Pseudomonas* sp. *DMSZ 13134* were included as reference strains. Bars with different letters are significantly different according to the least-squares means statistics (A; $p < .05$) or LSD test (B; $p < .05$).

Siderophores, HCN, pyrrolnitrine, phenazine and 2, 4-diacetyl phloroglucinol, lytic enzymes (chitinase, cellulase, protease etc.), and other antibiotics production by bacteria belonging to the genus *Pseudomonas,* and their efficiency against fungal pathogens are widely reported (Raaijmakers et al., 2002; Al-Hinai et al., 2010; Suresh et al., 2010; Bensidhoum et al., 2016). In addition, Islam et al. (2012) justified the inhibitory effect of some antagonistic pseudomonads to their ability to inhibit the integral functioning of some fungal cell wall components, altering the fungal membranes permeability.

Siderophores are low molecular weight ligands that are produced by organisms as iron-scavenging agents when available forms of iron are limited (Hofte and Bakker, 2007; Saha et al., 2015). They provide competitive advantages to the producer organism for iron absorption (Suresh et al., 2010). According to Bensidhoum et al. (2016), bacterial siderophores production indirectly stimulates plant growth through phytopathogens inhibition and competition for ferric iron resources in soils. Furthermore, Zhu et al. (2007) reported the presence of negative correlation between chlamydospores germination and inoculation by *Pseudomonas* sp. or its pyoverdine-type siderophore.

In this study, pea seeds germination in the presence of *Pythium aphanidermatum* was enhanced by +83% after inoculation by *Serratia* sp.-C comparing to the non-treated control. It also stimulated tomato growth by 15%. Such PGP and biocontrol activities of *Serratia* species could be explained by its ability to produce IAA, siderophores and to solubilize tricalcium phosphate. Brurberg et al. (1996) reported that bacteria belonging to the genus *Serratia* are known to be highly efficient for chitin degradation. They produce up to five different chitinolytic enzymes upon induction with chitin. Chitin-degrading enzymes inhibit fungal growth by hydrolyzing their chitin-containing cell walls (Kamil et al., 2007; Velusamy and Kim, 2011). According to Velusamy and Kim (2011), strong correlation was observed between bacterial chitinolytic potential and their *in vitro* fungal mycelium lysis. In addition, volatile compounds such as ammonia and HCN could be implicated in mycelial growth inhibition (Trivedi et al., 2008; Vanitha and Ramjegathesh, 2014). Consistently with Islam et al. (2012), microbial organic volatiles are implicated in initiating some biochemical changes of plants metabolism, hence inhibiting protein synthesis. Moreover, Ramette et al. (2003) reported that HCN production by *Pseudomonas fluorescens* is recognized to be effective in root diseases biocontrol, inhibiting the terminal cytochrome-C oxidase in the fungal respiratory chains and causing severe damages to the fungal metabolism.

IAA is by far the most common as well as the most studied phytohormone. It affects plant roots elongation, nodulation process, cell division and differentiation, and stimulates seeds and tubers germination (Maleki et al., 2010). IAA also controls vegetative growth, photosynthesis, pigment formation and biosynthesis of various metabolites, providing plant growth stimulation and resistance to stressful conditions (Glick, 2012). Ponmurugan and Gopi (2006) reported the role of IAA-producing *Pseudomonas* in enhancing pea roots development and hence, other plant growth parameters. Quantitatively, IAA is the most naturally occurring auxin produced by bacteria. In addition, a positive correlation was reported by Basharat et al. (2009) concerning *in vitro* auxin production by soil bacteria and their ability to promote plant growth under different circumstances. However, Baca and Elmerich (2007) reported that plants response to IAA-producing bacteria depends on several parameters, such as the plant species and the produced amount.

Soil-born oomycete pathogens such as *Pythium* cause damping-off and root rot diseases, damaging young seedlings of several horticultural and vegetable crops under both greenhouse and field production systems (Khabbaz and Abbasi, 2014. In addition, *P. aphanidermatum* is the most widespread and aggressive species of *Pythium* genus, being implicated in severe losses of the most economically important crops (cereals, leguminous, legumes) (Levenfors, 2003; Agrios, 2005; Nwaga et al., 2007; Dimova et al., 2010; Parveen and Sharma, 2015). It infects seeds, seedlings, plant stem, fruits and roots, inducing seeds pre- or post-emergence damping-off, seedlings general stunting, leaf fall or plant death (Rankin and Paulitz, 1994; Uzuhashi et al., 2010; Múnera and Hausbeck, 2016). In addition, Parveen and Sharma (2015) reviewed that *Pythium* species are not host-specific. Their resistant zoospores, able to infect growing roots, are able to germinate rapidly due to exudates or volatiles exposure from seeds or roots. Thus, chemically treated crops can be exposed to repeated infections waves throughout the cropping season (Nzungize et al., 2011).

According to Vanitha and Ramjegathesh (2014), applying biocontrol agents against *Pythium aphanidermatum* can indirectly promote plant growth for a long time by persisting in soil, thus, giving them several advantages as compared to chemicals. The study of Kraus and Loper (1992) showed that a *Pseudomonas* strain (pf-5) significantly increased cucumber seedlings emergence (± 60%) under *Pythium ultimum*-infested soil. They explained that by the strains' ability to compete for nutrients, but also by their antifungal metabolite secretion in soil. The same authors reported that a siderophore deficient *Pseudomonas* lost its ability to inhibit *Pythium* fungus. In addition, several *Serratia* species are known as chitinolytic bacteria. Bacteria with such character are considered as efficient biocontrol agents for various diseases, including those caused by *Pythium* species (Velusamy and Kim, 2011).

Applying soil bacteria that are able to compete oospores in germination for soluble carbon and nitrogen sources may reduce oospores germination and disease level. Such pathogen growth inhibition could be also attributed to antibiosis, parasitism or chitinase production, leading to fungus cell wall degradation through mycelium lysis (El-Mohamedy and El-Mougy, 2009).

In pre-emergence damping-off, seed may be decayed or rot under soil surface directly after infection when moisture penetrates the seed coat or when the radical begins to extend, resulting in poor and uneven seedlings stand (Khabbaz and Abbasi, 2014). The strong antagonistic activity of the isolate *Pseudomonas* sp.-N against *Pythium* could be attributed to siderophores, protease, chitinase and IAA production (Khabbaz and Abbasi, 2014; Nzungize et al., 2012). Similar results were obtained with *Pseudomonas strains* when applied as biocontrol agents for pea protection against *Pythium* damping-off and *Aphanomyces* root rot (Abdel-Kader et al., 2012).

5. Conclusion

Irrigation well water could constitute an important source of bacteria with high agricultural interest. The three *Pseudomonas* sp. B, D, and N strains and the *Serratia* sp. C strain exhibited remarkable *in vitro* PGP and antagonistic traits. They also proved high efficiency in inhibiting fungal spores' germination and mycelial development. The *Pseudomonas* sp. -N revealed interesting ability to promote tomato and pea growth under both normal and *Pythium*-infected soils, as compared to the two commercial PGPB *P. protegens* CHA0 and *Pseudomonas* sp. DMSZ 13134. These attempts to understand the bacterial functional diversity in irrigation well water constitute an important contribution to develop new biological agent for plant growth enhancement and phytopathogens' biocontrol.

Acknowledgments

Authors are grateful to Professor Houali Karim, head of the faculty "Biological and Agronomic Sciences, University of Tizi Ouzou, Algeria", for the internship financed by his faculty.

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