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Phenotypic and molecular characterization of plant growth promoting Rhizobacteria isolated from the rhizosphere of wheat (*Triticum durum* Desf.) in Algeria

Benmati Mahbouba[1,2]*, Le Roux Christine[3], Belbekri Nadir[1,2], Ykhlef Nadia[1,2] and Djekoun Abdelhamid[1,2]

[1]Université Constantine1, Faculté des sciences de la nature et de la vie. Constantine Algérie.
[2]Laboratoire de Génétique Biochimie et Biotechnologie Végétales, équipe biotechnologie végétales, Constantine 25000, Algérie.
[3]CIRAD, UMR LSTM, F-34398 Montpellier Cedex 5, France.

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Several strains were isolated from rhizosphere of wheat in Eastern Algeria. All the 10 strains (Azo4, Azo5, Azo6, Azo7, Azo8, Azo10, S1, S2, S3 and S9) were identified; based also on the characteristics which are not only morphological, characteristics such as shape but physiological ones: optimal temperature, pH optimum and NaCl tolerance. These isolates were screened *in vitro* for their growth promoting traits like production of indole acetic acid (IAA), ammonia (NH_3) and solubilize phosphate. The bacterial strains have an optimum growth pH equal to 6.8; in a broad temperature range (28 and 37°C). The molecular identification was done by simple PCR to amplification for 16S rDNA gene using primers AZ16S-D. Some strains (Azo4, Azo5, Azo6, Azo7, Azo8 and Azo10) have high identity with genus *Azospirillum* which indicates that these isolates belong to the *Azospirillum* genus, specially to species of *Azospirillum brasilense* A nested PCR approach performed with degenerate primers was used to amplify *nif*H gene fragments from the bulk DNA. The amplification product bands at the expected *nif*H gene fragment size are about 370 bp. Some isolates (S1, S2, S3, Azo4, Azo5, Azo6, S9 and Azo10) were considered by the nitrogen-fixing gene *nif*H detection of their genome. We managed to highlight the contribution between *A. brasilense* and durum wheat with inoculation experiments performed under greenhouse conditions showed that all *A. brasilense* (six strains) have a good report for growth of roots and plants and improve the production of durum wheat (*Triticum durum* var: GTA). These data demonstrate the importance of the test especially in programs to improve the quality of wheat in Algeria.

Key words: Rhizobacteria, indole acetic acid, ammonia, 16S rDNA, *Azospirillum brasilense, nif*H.

INTRODUCTION

Durum wheat is produced in many arid and semi arid regions of the world (Bogole et al., 2011); in Algeria, durum wheat has been extensively cultivated for many centuries; the species encountered a large diversification

*Corresponding author. E-mail: benmati.m@gmail.com. Tel: +213)561 22 68 10.

Abbreviation: PGPR, Plant growth promoting rhizobacteria, **PCR,** polymerase chain reaction, **LB,** medium Luria bertani; **bp,** base pairs; **IAA,** indole-3-acetic acid.

implied by the great diversity of climates that led to great genetic diversity of the species (Boudour et al., 2011). The term PGPR "plant growth promoting rhizobacteria" was first used by Joseph W. Kloepper in the late 1970s and has become commonly used in scientific literature (Vessey, 2003). It has been shown that PGPR were applied to the various cultures to promote growth, the emergence of seeds and to increase crop yields, and some have been commercialized (Dey et al., 2004; Herman et al., 2008; Minorsky, 2008). These rhizobacteria can improve the host by promoting the growth by biocontrol of plants diseases and sustainable agriculture. PGPR activity has been reported for strains belonging to several genera, such as *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia* and *Bacillus* (Kloepper, 1993; Glick and Bashan, 1997). Several mechanisms have been proposed to explain how PGPR stimulate plant growth. These can be classified as direct or indirect mechanisms (Verma et al., 2010).

A direct mechanism occurs when metabolites or compounds synthesized by the bacteria were supplied to the plant such as phytohormones, or when bacteria facilitate the absorption of plant nutrients in the environment such as nitrogen. In the case of the indirect mechanism, bacteria protect plants against fungal pathogens of the soil or harmful bacteria (Kloepper, 1993; Glick, 1995; Lugtenberg et al., 2004). The immediate response to inoculation with PGPR in rhizosphere varies among bacteria, plant species, soil type, inoculum density and environmental conditions (Okan et al., 1997). Bacteria of the genus *Azospirillum* are Gram-negative and have different ways to fix nitrogen through partnerships with non-leguminous plants (cereals and grasses) from different geographical origins (Renato et al., 1995). *Azospirillum* strains that can be used as biofertilizers provide plants with nitrogen, help with phosphate solubilization production of phytohormones such as auxin, and regulate substances or sederophore (Bashan et al., 2004). The genus *Azospirillum* is characterized as a Gram negative, aerobic, nitrogen-fixing, and rod-shaped a-proteobacterium. *Azospirillum* species (spp.) have been isolated from rhizosphere soils and the roots of various plants around the world (Shime et al., 2011). Some strains of *Azospirillum* are reported as plant growth promoting rhizobacteria (PGPR) that can increase crop growth and yield (Okon and Labandera-Gonzalez, 1994; Okon and Vanderleyden, 1997).

A total of 15 species have been described in the genus *Azospirillum*: *A. amazonense* (Falk et al., 1985), *A. brasilense* (Helsel et al., 2006), *A. canadense* (Mehnaz et al., 2007a), *A. doebereinerae* (Eckert et al., 2001), *A. halopraeferens* (Reinhold et al., 1987), *A. irakense* (Khammas et al., 1989), *A. largimobile* (Ben Dekhil et al., 1997), *A. lipoferum* (Tarrand et al., 1978), *A. melinis* (Peng et al., 2006), *A. oryzae* (Xie and Yokota, 2005), *A. palatum* (Zhou et al., 2009), *A. picis* (Lin et al., 2009), *A.* were mainly isolated from soil and commonly in association with grasses and cereals (Kirchhof et al., 1997). However, the isolation of *Azospirillum* from plants and rhizosphere depends on the methods used in the laboratory and requires the use of selective media and morpho-physiological tests (Tarrand et al., 1978) (based for example on cell morphology, colony color, and the ability of nitrogen fixation); hence the diversity of isolates can be obtained (Han and New, 1998).

The advantage of the recent sequencing and DNA finger printing technology has facilitated the detection and identification of target microbes in the process of isolation in selective media and therefore reduced the morpho-physiological tests (Stead et al., 2000). The use of techniques for detection, identification and quantification of microorganisms based on PCR can be completed by a dependent culture and biochemical methods. The developments of specific species of *Azospirillum* were studied by Stoffels et al. (2001) by analysis of fluorescence *in situ* hybridization. However, this method can only detect physiologically active bacteria, and can not detect all the members who belong to the genus *Azospirillum*. At that time, they adopted specific oligonucleotides (primers) for the genus *Azospirillum* based on the conservation of the 16S rDNA gene sequence of *Azospirillum*. Using PCR amplification, primer sequences were quickly identifying the members of the genus (Shih et al., 2011). For evaluation of nitrogen-fixing populations in the environment, analysis of *nif*H, the gene encoding nitrogenase reductase, due to the vast phylogenetic differences among nitrogen fixers, the sequences of *nif*H genes have diverged considerably (Zehr et al., 1989), and even the DNA sequences encoding conserved protein regions may differ due to codon redundancy for most amino acids. The design of universal nifH primers requires a high degree of DNA sequence degeneracy and may result in reduced specificity during PCR amplification. However, the use of more sophisticated amplification protocols may make it relatively simple to study the complexity of nitrogen-fixing microorganisms in a given ecosystem.

The objective of the present study was to evaluate the properties and specificity of the strains isolate at different levels: (i) phenotypic and morphological identification of isolates; (ii) analysis of the molecular diversity and detection of gene *nif*H; and (iii) evaluation of inoculation between the *A. brasilense* and the durum wheat under controlled conditions.

MATERIALS AND METHODS

Isolation of bacterial strains and culture conditions

The bacterial strains were isolated from the rhizosphere of durum wheat grown in the fields of several regions in Algeria (Ain El bey Constantine, El Khroub-Constantine (zoneA), El Khroub-Constantine (zoneB), El Khroub-Constantine (El Baraouia zone1), El Khroub-Constantine (El Baraouia zone2), Mila, Souk Ahrass, Setif, Batna and Tebessa), in the period of 2010 and 2011. Samples are collected at a depth of 20 to 30 cm, transported to the laboratory at low temperature (4°C) where they are processed for

the isolation of bacteria. The soil was left in NaCl buffer (0.85%) and diluted with water distilled and inoculated into tubes containing 5 ml of semi-solid medium (nitrogen free medium, NFB) with 0.05 g of yeast extract. After 48 h of incubation at 30°C, a loop of culture was transferred to fresh semi solid NFB medium (Dobereiner, 1995). The colonies were transferred on the solid medium NFB containing yeast extract, and also on the medium Congo red (0.5%), and incubated for 48 h at 30°C.

Phenotypic and morphological characterization of the strains isolated

Morphology and Gram staining was determined by microscopic observation (Myoungsu et al., 2005). Several parameters have been used to identify strains (carbon sources, different pH). Culture of the 10 isolates was performed on solid NFB medium and incubated at 27, 32, 40, 45 and 50°C. The change of growth and coloration were observed after 72 h of incubation.

Resistance of bacteria towards salinity

The medium NFB was prepared with different concentrations of NaCl (100, 200, 300, 400, 500, 600, 700 mM). The 10 isolates were inoculated separately and incubated for 48 h. The level of growth was measured by spectrophotometer at 620 nm.

Effect of PGPR strains

Production of IAA

Bacteria were grown overnight in liquid Luria-bertani (LB) medium in darkness at 30°C. Bacterial cells were recovered by centrifugation at 8000x *g* for 10 min. 1 ml of the supernatant was mixed vigorously with 2 ml of Salkowski's reagent, and incubated at room temperature in the dark for 30 min. The absorbance at 535 nm was measured (Benizri et al., 1998; Fischer et al., 2007). Development of Pink color indicated IAA production (Gangoir et al., 2012).

Production of ammonium

The isolated bacteria were tested for their capacity to produce ammonia in peptone water. Fresh culture of bacteria was inoculated in 10 ml of peptone water in tubes and incubated for 48 to 72 h at 36 ± 2°C. After 72 h, Nessler's reagent (0.5 ml) was added to bacteria suspension. Development of brown to yellow color was positively test for ammonia production (Yadav et al., 2010).

Solubilization of phosphorus

Solubilization was detected by the formation of transparent halos surrounding the bacterial colonies on a medium (NFB) containing insoluble phosphate (PO_4) after 5 days of incubation at 30°C (Ashrafuzzaman et al., 2009).

Amplification and sequencing of 16S rDNA

This part of the work was performed in the laboratory LSTM in Montpellier France. For 16S rDNA amplification, they resuspended a single colony in 20 µl of sterile water and heated to 95°C for 10 min to allow cell lysis. 2 µl of the lyses cell suspension was used for polymerase chain reaction (PCR) (Renato et al., 1995). The PCR was done as follows: initial denaturation at 96°C for 3 min followed by 35 cycles consisting of a 30" denaturation at 95°C, 30" at annealing temperature of 55°C, followed by a Az16S-D (Forward, 5'CCGCGGTAATACGAAGGGGGC3', and Az16S-D Reverse, 5'GCCTTCCTCCGGCTTGTCACCGGC3') primer extension at 72°C. Then, the PCR products were run on a 1% agarose gel (Sigma, France) in TAE buffer with a DNA size standard (Eurogentec Smartladder). The size of the amplificon by the primers was determined to be 670 bp (Shime et al., 2011). Amplified fragments were purified with a QIAquick Gel Extraction Kit (Qiagen, France). Sequencing was performed using a BigDye Terminator Cycle Sequencing kit (Perkin Elmer Applied Biosystems, Foster City, USA) and reactions were analyzed on an automated DNA sequencer (Applied Biosystems model 310, Perkin Elmer Applied Biosystems).

The 16S rDNA sequences of *Azospirillum* spp. strains were corrected using the sequence viewer 4Peaks program from Mackintosh B.V. For multiple alignments and for phylogenetic tree building, the SeaView program version 4 was used (Gouy et al., 2010). This interface drives the Clustal Omega program and includes the BioNJ distance-based tree reconstruction method (Gascuel, 1997).

Search for the presence of the *nif*H gene

A loop ful of rhizobacteria cells was suspended in 20 µl of sterile water in an Eppendorf tube for preparing DNA for the PCR. The cell suspension was boiled in water for 5 min for lysing the cells. Cell debris was removed by centrifugation at 13,000 rpm for 1 min at room temperature, and 2 µl of the supernatant was used as a template for PCR. Fragments of *nif*H genes were amplified by using nested PCR. The first PCR was performed with the forward primer *nif*H (for A) (GCIWTITAYGGNAARGGNGG) and the reverse primer *nif*H (rev) (GCRTAIABNGCCATCATYTC). The second PCR nested was performed with the forward primer nifH (forB) (GGITGTGAYCCNAAVGCNGA) and the same reverse primer *nif*H (rev) according to Widmer (1999). The first PCR was performed for 40 cycles with a 25-ml reaction mixture containing 2 ml of purified bulk DNA was done as follows: denaturation for 11 s at 94°C and for 15 s at 92°C, annealing for 8 s at 48°C and for 30 s at 50°C, and extension for 10 s at 74°C and for 10 s at 72°C.

A final 10-min extension step at 72°C was performed after the cycling steps and before the samples was maintained at 4°C. The nested reaction was performed for 35 cycles with a 100-ml reaction mixture containing 2 ml of the first PCR product as the template. The quality and quantity of the amplification products were analyzed on 2% ultraPure agarose gels with a DNA 1 Kb ladder (Eurogentec Smartladder).

Seed sterilization and germination

Seeds of wheat (*Triticum durum* Desf.), collection GTA/DUR, with similar size and weight were washed with distilled water three times and soaked in alcohol of 70% for 20 s. The extra alcohol was removed and seeds were washed with sterilized water. Seeds were then sterilized for 15 min using sodium hypochlorite 3% and rinsed with sterilized water 5 times. The sterilized seeds were soaked in distilled water for 3 h and 15 of them were grown in sterilized Petri dishes for 48 h at 20°C in darkness.

Seedlings inoculation with different strains of *Azospirillum brasilense*

Six isolates including Azo4, Azo5, Azo6, Azo7, Azo8 and Azo10

were selected for the experiment. The bacterial suspension was prepared in LB medium. After germination, wheat seedlings were inoculated wit 3 ml of bacterial inoculums. After 3 h, the extra inoculums were removed from tubes and the tubes were rinsed with distilled water. After the phase of inoculation, the germination seed were transferred into the pots containing sterile soil and n-free nutrient solution (10 g Malat, 1 g KH_2PO_4, 0.2 g $MgSO_4$ $7H_2O$, 0.2 g NaCl, 0.005 g $FeSO_4$ $7H_2O$, 5 g Mannitol, 100 mg yeast extract (Azlin et al., 2005) and uninoculated seeds are irrigated also with n-free nutrient solution. The experiment was placed in the greenhouse under conditions controlled (temperature 25°C, 16 h photoperiod and humidity 30%). After three months of growth for greenhouse experiment, different parameters were measured to determine growth of plants: plant length (cm), plant weight (g), roots length (cm), roots weight (g), thousand seeds weight (g), rate of total chlorophyll (Spad Unit) and determination of nitrogen content in leaves wheat of durum (mg/ml).

Statistical analysis

For each of the durum wheat studied and each parameter analyzed, all the data collected were subjected to a one way analysis of variance (ANOVA). When *Azospirillum* strain factor had a significant effect on a given parameter, the means of the different treatments were ranked into homogeneous groups according to NEWMAN-KEULS test. All the statistical analyses were performed using Excel STAT Pro 2012.

RESULTS

Isolation of bacterial strains and culture conditions

Ten strains were isolated from the rhizosphere of different regions in eastern Algeria (Table 1). All isolated S1, S2, S3, Azo4, Azo5, Azo6, Azo7, Azo8, S9 and Azo10 were tested and observed (colony, color and shape). They show that all strains (S1, S2, S3, Azo4, Azo5, Azo6, Azo7, Azo8, S9 and Azo10) are Gram-negative (Table 1).

We noted that the 10 different strains have shown a formation of white veil in the subsurface environment and on the solid medium, small white colonies appeared as dense mucosa. This result was demonstrated in Table 1. The result showed in Table 1 found that strains isolated have a good growth in the five different temperatures used (27, 32, 40, 45 and 50°C), but some strains (Azo8, Azo10, S3 and S9) do not have growth at 50°C. Thus, some isolates (S1, S2, Azo5, Azo7, Azo8, S9 and Azo10) have a good growth and tolerate different pH levels (5.5, 6.0, 6.8, 7.0 and 7.5). However, isolates S3, Azo4 and Azo6 do not tolerate pH 5.5. This result was shown in Table 1. Concerning the use of different nitrogen source, we notice that the majority of strains use mannitol, fructose, sucrose and lactose; but Azo5 does not use sucrose and S3; and S9 does not use Lactose (Table 1).
The growth of the bacteria in the presence of different concentrations of NaCl shows that the strains tolerate up to 300 mM NaCl. We noticed that strains S1, S2, Azo4, Azo5, Azo7, Azo8 and S9 were very tolerant to the highest concentration of NaCl (Figure 1), but the growth rate was slightly lower compared to the other strains (S3 and Azo10) (Figure 1).

We also noted that among the 10 selected isolates, S1, S2, Azo5, Azo7, Azo8 and Azo10 had a good growth at concentration of NaCl equal to 400 mM.

Effect PGPR of strains

Isolated S1, S2, S3, Azo4, Azo5, Azo6, Azo7, Azo8, S9 and Azo10 were tested for their ability to produce IAA, ammonium and solubilization of phosphate. Results in Table 2 demonstrate the ability of isolates to synthesize IAA and ammonia with a production rate that changes from one strain to another, and can also solubilize phosphate.

Molecular characterization of isolates

Based on morphological and phenotypic characterization, several similar isolates were selected to facilitate the identification of *Azospirillum* by simple PCR. The distributions of isolates gave six strains of the genus *Azospirillum* and are very close to the species *A. brasilense*; other belongs to different genera of *Pseudomonas*, *Aeromonas* and *Stenotrophomonas.* About 670 bp fragments of the 16s rDNA gene was sequenced with primers Azo16S-D (Figure 2); then, the sequence obtained was analyzed using Blast search. Isolated Azo4, Azo5 and Azo6 showed high identity (98 to 100%) with *Azospirillum* genus. The phylogenetic position of the isolate is shown in Figure 3. It indicates that this isolate is clustering with the *Azospirillum* genus, especially to species of *A. brasilense*. *Azospirillum* members and the inter-genera phylogenetic difference were very limited.

Amplification of nifH gene fragment by using a nested PCR approach

The results were shown in Figure 4. The majority of strains amplification gives bands at expected nifH gene fragment size of approximately 370 ≈ 380 bp (Figure 4). We observed in Figure 4 the lanes: 1, 2, 3, 4, 5, 6, 9 and 10 corresponding to S1, S2, S3, Azo4, Azo5, Azo6, S9 and Azo10. They give a good result to show a band in 370 to 380 bp according to DNA ladder. However, lanes 7 and 8 corresponding to Azo7 and S8 do not give a good result, they give unclear bands.

Effect of inoculation with Azospirillum brasilense on the different plants parameters of durum wheat (Triticum durum Desf. var: GTA/dur*) under greenhouse*

The results of the greenhouse experiments reported in

Table 1. The sites of the strains isolated and morphological and physiological characterization of isolates

Strain	Isolation site	Surveying point	Physiological characterization										Different nitrogen source					Morphological characterization		
			Temperature					Ph												
			27	32	40	45	50	5.5	6.0	6.8	7.0	7.5	M	S	F	L	G	Shape	Color	Gram
Azo4	El Khroub-Constantine (zone B)	36°15′49.31″N 6°39′19.37″E	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Rod	White	Negative
Azo5	El Khroub-Constantine (El Baraouia zone 2)	36°16′29.86″N 6°40′27.64″E	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Rod	White	Negative
Azo6	Ain El bey -Constantine	36°16′59.63″N 6°36′59.63″E	+	+	-	+	+	-	+	+	+	+	+	+	+	-	+	Rod	White	Negative
Azo7	Setif	36°10′59.91″N 5°26′13.51″E	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Rod	White	Negative
Azo8	Batna	35°32′16.97″N 6°04′47.69″E	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Rod	White	Negative
Azo10	Tebessa	35°25′52.84″N 8°03′12.71″E	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Rod	White	Negative
S1	El Khroub-Constantine (zoneA)	36°12′52.93 ″N 6°36′57.03″E	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	Rod	White	Negative
S2	El Khroub-Constantine (El Baraouia zone1)	36°15′49.97″N 6°39′10.62″E	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	Rod	White	Negative
S3	Mila	36°21′49.44″N 6°09′40.83″E	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	Rod	White	Negative
S9	Souk Ahras	36°17′34.87″N 7°55′24.73″E	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Rod	White	Negative

+, Presence; -, absence; M, mannitol; S, sucrose; F, fructose; L, lactose; G, glucose.

Table 2. IAA production and phosphorus solubilization by PGPR isolates.

Strain N	Production of IAA	Solubilization of phosphate
Azo4	++	Solubilization
Azo5	++	Solubilization
Azo6	+++	Solubilization
Azo7	++	Solubilization
Azo8	+++	Solubilization
Azo10	++	Solubilization
S1	+	Solubilization
S2	++	Solubilization
S4	++	Solubilization
S9	+	Solubilization

+, Low production; ++, average production; +++, good production.

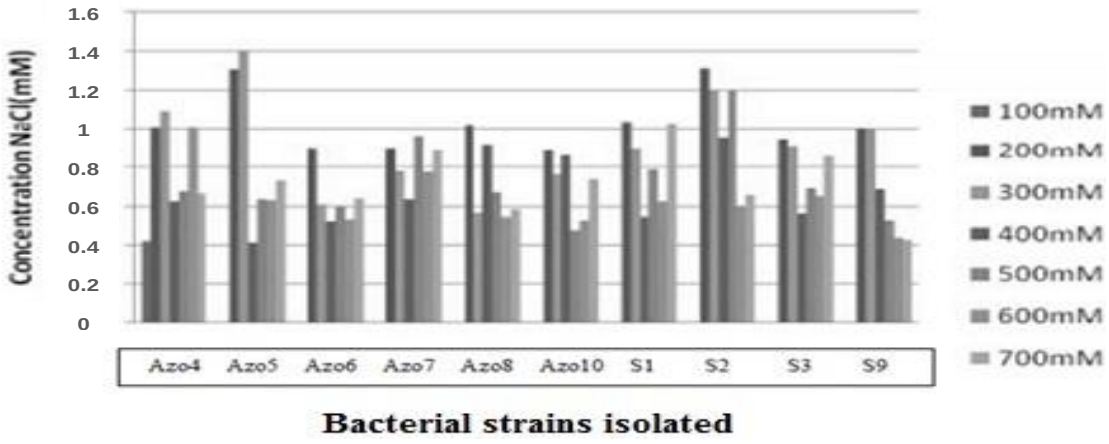

Figure 1. The effect of different concentrations of NaCl (mM) on growth of isolates. Histogram was based on the growth of different strains in media containing different concentrations of NaCl ranging from the lowest concentration (100 mM) to the highest concentration (700 mM).

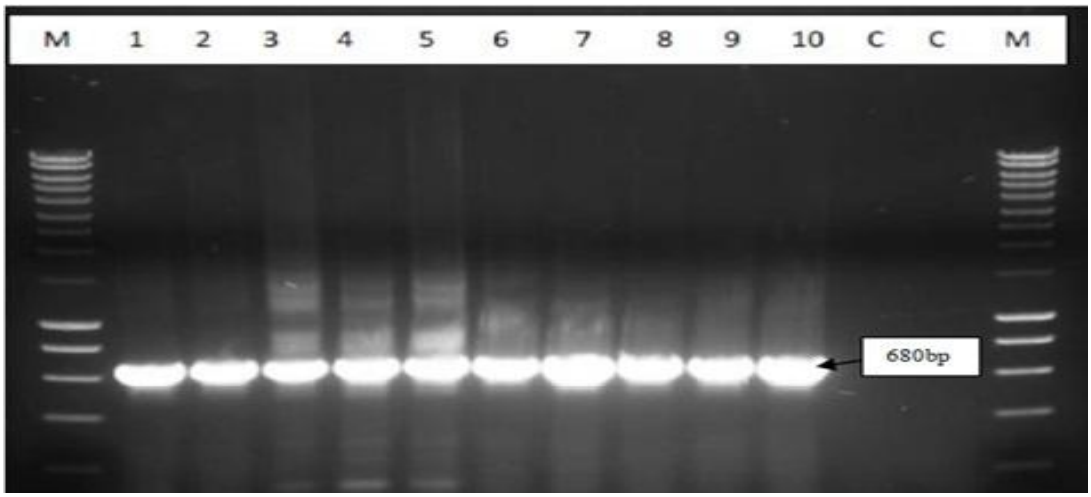

Figure 2. Gel of 16S rDNA PCR products generated (received) by the primers rhizobacteria isolated. PCR products were resolved in 1% agarose gel and stained with ethidium bromide. Lane M, size marker (Smart ladder 1 Kb); lanes 1 to 10, bacterial isolates; lane C, negative control.

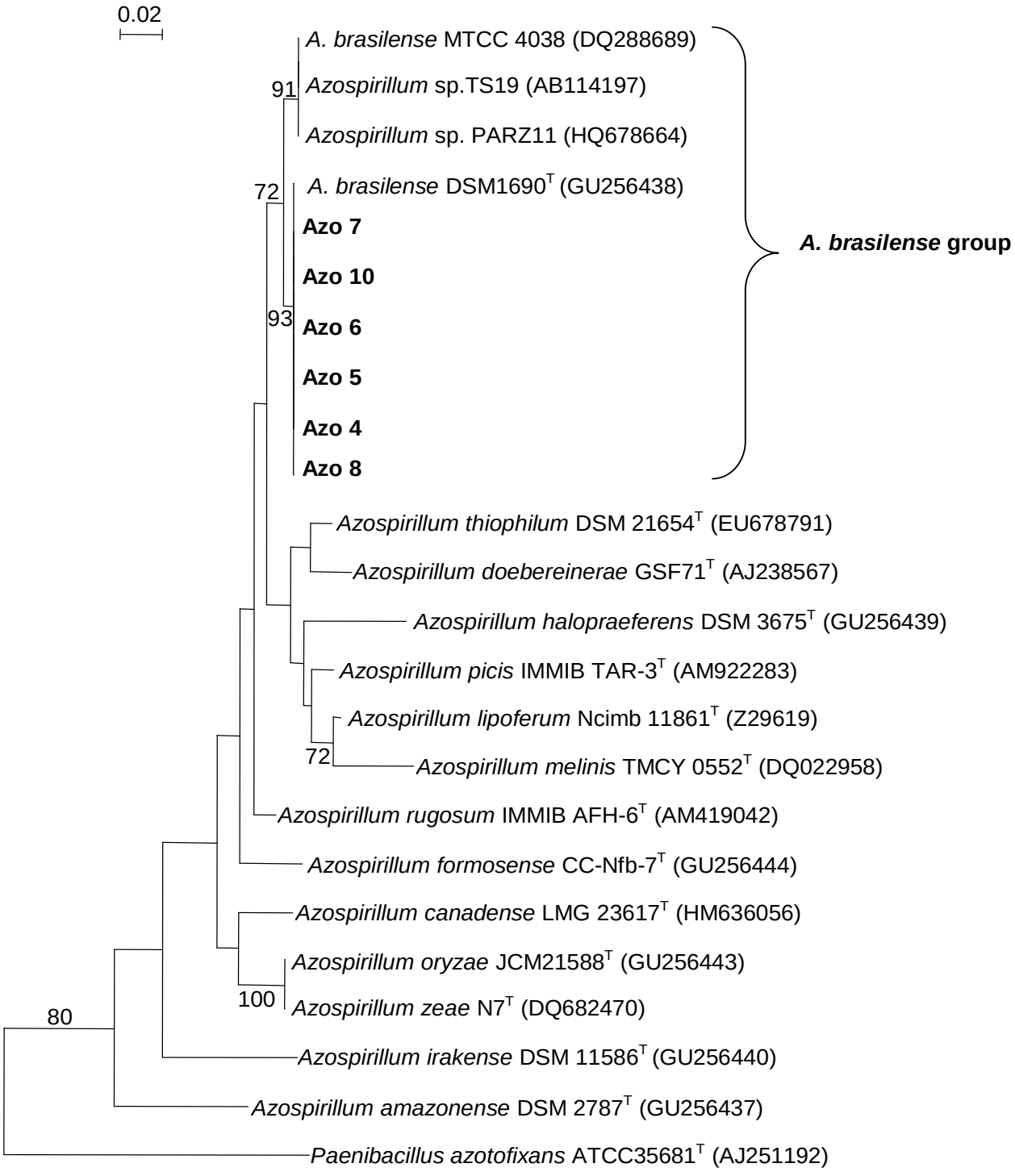

Figure 3. BioNJ phylogenetic tree based on partial 16S rDNA sequences of *Azospirillum* spp. strains (isolated from durum wheat rhizosphere), reference and related strains. The type strains are indicated with the letter "T". Only bootstrap probability values greater than 70% (1000 replications) are given at the branching points. Gaps were not considered. Scale indicated 2% sequence divergence. *Paenibacillus azotofixans* was chosen as an outgroup.

Table 3 show that the difference in parameters measured between plant inoculated and uninoculated plant control the plant length (cm) and highest result was obtain with strain Azo6 (85.39 ± 0.00 cm) and an average value was observed with Azo4, Azo5, Azo7 and Azo10 (83.75 ± 0.02 and 80.19 ± 0.00 cm) but plant control uninoculated

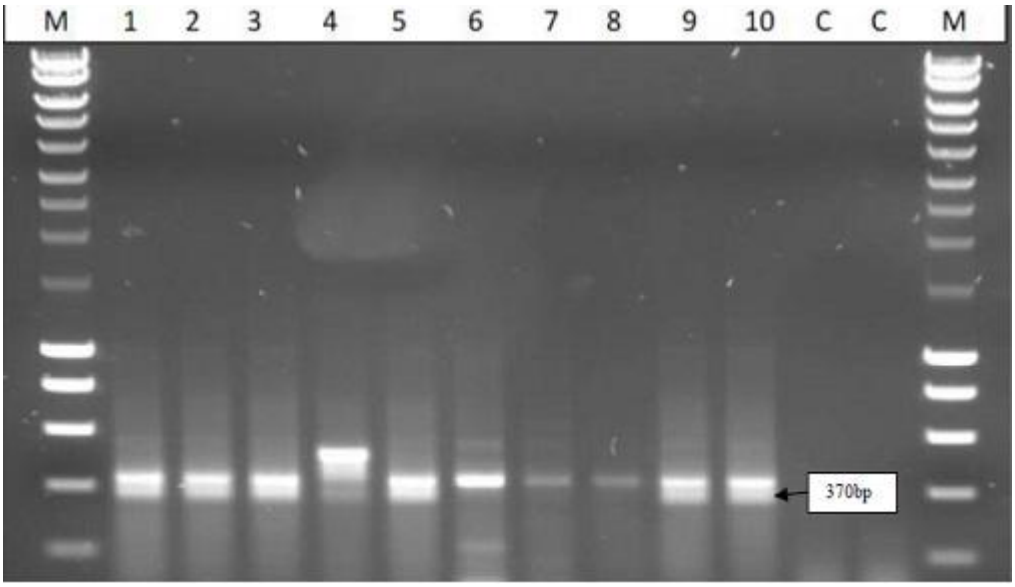

Figure 4. The gel of nifH nested PCR amplification of a *nif*H fragment of 370 bp using the universal primers *nif*H (for A) and reverse (rev) and *nif*H (for B) and reverse (rev). M: ladder; lanes 1-10, *Azospirillum* (isolated from durum wheat); C: negative control.

Table 3. Evaluation of durum wheat growth under the various treatments.

Treatment	Plant length (cm)	Plant weight (g)	Roots length (cm)	Roots weight (g)	Thousand seeds weight (g)
			Wheat Durum: GTA		
Control	73.40±0.01^{Ga}	1.73±0.00^{Ga}	36.05±0.06^{Gb}	0.54±0.01^{Da}	64.20±0.03^{CDa}
Inoculated Azo 4	80.19±0.00^{Fa}	1.92±0.00^{Ea}	39.03±0.01^{Da}	0.65±0.01^{Ca}	64.36±0.02^{Fa}
Inoculated Azo 5	83.69±0.00^{Db}	1.96±0.00^{Ca}	38.32±0.03^{Fa}	0.74±0.01^{Ba}	65.24±0.04^{Da}
Inoculated Azo 6	85.39±0.00^{Aa}	2.06±0.01^{Aa}	39.43±0.04^{Ca}	0.83±0.02^{Aa}	67.26±0.02^{Ba}
Inoculated Azo 7	82.52±0.02^{Ea}	1.94±0.01^{Da}	41.37±0.01^{Aa}	0.73±0.02^{Ba}	68.13±0.01^{Aa}
Inoculated Azo 8	85.12±0.04^{Ba}	2.03±0.01^{Ba}	40.57±0.00^{Ba}	0.65±0.02^{Ca}	65.82±0.02^{Da}
Inoculated Azo 10	83.75±0.02^{Ca}	1.89±0.00^{Fa}	38.38±0.00^{Ea}	0.64±0.03^{Ca}	64.84±0.02^{Ea}

	Chlorophyll total rate (Spad unit)	Nitrogen total (mg/ml)
	Wheat durum: GTA	
Control	33.96±0.03^{Cb}	0.14±0.00^{Db}
Inoculated Azo 4	47.63±0.00^{Ba}	0.52±0.00^{AB}
Inoculated Azo 5	48.70±0.02^{Aa}	0.47±0.01^{Ba}
Inoculated Azo 6	47.43±0.01^{Ba}	0.46±0.01^{Ba}
Inoculated Azo 7	47.50±0.00^{Ba}	0.53±0.05^{Aa}
Inoculated Azo 8	48.60±0.00^{Ab}	0.40±0.01^{Ba}
Inoculated Azo10	47.36±0.01^{Ba}	0.44±0.02^{Ca}

The mean values are those of 7 parameters measured on plants for pot experiments.
^{A,B,C}separation index homogeneous groups test by Newman-Keuls. Values ± SD accompanies by same latter (a and b) are not significantly different according to Fisher's least significant different test (P=0.05).

length was very low (73.400 ± 0.012 cm). For plant weight (g); we note that all strains have good effects and plant inoculated with Azo6 and Azo8 were better than the other plants inoculated (2.06 ± 0.01 and 2.03 ± 0.01 g) but the uninoculated control plants was very low (1.73 ± 0.00 g). For roots, we note all the strains have good effects of elongation, plant inoculated with Azo7 (41.37 ± 0.01 cm) have a better effect in plants than the other

strains. However, the uninoculated control plants have small roots (36.05 ± 0.06 cm) when we compare with the plants inoculated with strains *A. brasilense.* The results of the total nitrogen reported in Table 3 show that the plants inoculated with Azo4 and Azo7 produced more nitrogen (0.52 ± 0.00 mg/ml, 0.53 ± 0.01 mg/ml) than the other plants inoculated three months after inoculations. However, uninoculated control plants produced no more nitrogen (0.14 ± 0.00 mg/ml).

Chlorophyll total content of plants of durum wheat (GTA) inoculated with *A. brasilense* (six strains: Azo4, Azo5, Azo6, Azo7, Azo8 and Azo10) and the uninoculated control plant was reported in Table 3. The results obtained are different; we note that chlorophyll total content is higher in plants inoculated with *A. brasilense*. Note that the highest content was recorded in plants inoculated with strains Azo5 and Azo8 (48.70 ± 0.02 and 48.60 ± 0.00 Spad Unit). However, the uninoculated control plants produce no more chlorophyll total (33.96 ± 0.03 Spad Unit). The yields were estimated with thousand seeds weight (g). We note that all the strains gives good results and the plant inoculated with Azo7 have good effect to enhanced growth and yields (68.13 ± 0.01 g) when compared with uninoculated plant (64.202 ± 0.034 g) (Table 3). In contrast, no significant difference (at $P = 0.05$) between strains was for weight (68.13 ± 0.01 g) of biomass higher with 94%.

DISCUSSION

Azospirillum spp. has been given much attention as important PGPR (Okon and Vanderleyden, 1997; Bashan et al., 2004). Despite their importance in agriculture, the isolation of *Azospirillum* bacteria from agricultural samples greatly depends on laborious conventional methods using selective media and morpho-physiological tests (Caceres, 1982; Han and New, 1998; Baldani et al., 2005). The formation of the fine colonies is a characteristic of *Azospirillum* spp. (Dobernier and Day, 1976) showed growth under micro-aerophilic conditions on semi-solid agar and suggests that this method was particularly used to study the substrates and growing conditions for nitrogen fixation. For different strains, the observed color was white colonies; this result was similar to the study of Lakshmi et al. (1985) who showed that the bacterial colonies obtained from different isolates are not colored. Ekert et al. (2001) has stated that all these characteristics are very similar to *Azospirillum*. The features that distinguish are species to another, based on its ability to use more sugar and some growth in the detail genetic optimum occurs at 30°C and pH between 6.0 and 7.0. Kreig (1984) reported that organic acids are used to support growth and also support nitrogen fixation of all species of *Azospirillum*. However, the strains have very different abilities for sugar utilization; Goebel et al. (1984) showed that *A. brasilense* can not grow and fix nitrogen with most sugars, and *A. lipoferum* actually used glucose and sucrose *A. amazoneuise* uses.

It was suggest that the chemotaxis of organic acid sugars and amino acids is important for the establishment of bacteria root associations contributing to the growth of *Azospirillum* in their ecological niche. The results obtain about strains growth on medium with different concentrations of NaCl are similar to result demonstrated by Usha et al. (2011) and indicating in the presence of 700 mM NaCl that the increase in growth rate was purely osmotic and not the result of specific ions present in high concentration. It has been proven that the mode of action of many PGPR is to increase the availability of nutrients for the plant, it is mainly the trais PGPR, as the production of IAA and solubilization of phosphates, than some strains that have promote the growth of wheat. Richardson (2001) showed that phosphate solubilization in the rhizosphere is the most common mode of action implied by PGPR increasing the availability of the host plant. Examples of associations include *Azotobacter chroococcum* and wheat, *Bacillus circulans* wheat and *Pseudomonas chlororaphis* and *P. putida* and soybeans (Vessey, 2003). On the other hand, production of IAA by PGPR increased root growth and root length, resulting in greater root surface which allows the plant to access more soil nutrients (Vessey, 2003). The presence of IAA and related compounds has been demonstrated for many diazotroph, for example, *Acetobacter diazotrophicus*, *Azospirillum* sp. *Azotobacter*, *Paenibacillus polymyxa* (Dobbelaere et al., 2003). Moreover, these isolates have the capacity to produce ammonia.

Mirza and collaborators (2001) have found that the production of IAA and ammonium by PGPR varied between different species and strains, and was influenced by the state of culture, growth stage and substrate availability. On the other hand, phosphorus is a major nutrient element; most of the soil phosphorus is present as insoluble phosphates and can not be used by plants. The ability of bacteria to solubilize phosphate was of great interest to agricultural microbiologists to improve plant growth. These reports indicate that this species is a phylogenetically close relative of *Azospirillum* from a different habitat. Moreover, it remains unclear whether the primers can discriminate *Azospirillum* bacteria from other major nitrogen-fixing bacteria such as *Azotobacter* and *Klebsiella*. Taken together, the specificity and sensitivity of Az16S-D primers were highly satisfactory for the detection of *Azospirillum* bacteria even in contaminated cultures, indicating their applicability to identify *Azospirillum* from agricultural samples. Moreover, the current study adopted simple bacterial-cell boiling and standard (inexpensive) Taq DNA polymerase for DNA extraction and amplification, respectively, indicating the easy, low cost applicability of PCR tests with Az16S-D primers.

The isolation of *Azospirillum* bacteria from agricultural samples involves the repeated uses of selective media and

and morpho-physiological tests, which cause selection bias. Han and New (1998) reported that *Azospirillum* bacteria with low nitrogen fixation ability were missed in the conventional isolation process involving nitrogen-free media. The PCR detection of *Azospirillum* isolates by Az16S-D primers is considered to reduce the uses of the media and tests and consequently to accelerate the isolation while decreasing the selection bias. This study developed a rapid and simple genus-specific PCR method using Az16S-D primers to facilitate the detection and identification of *Azospirillum* isolates within populations of rhizosphere bacteria. The PCR method developed would probably serve as a useful tool for isolating a variety of indigenous *Azospirillum* bacteria from agricultural samples to discover novel strains of PGPR and to analyze the diversity and ecology of *Azospirillum* populations (Mcgilloway et al., 2002; Mirza et al., 2006). Further studies are required to develop a rapid, easy and selection bias-minimizing *Azospirillum* isolation technique involving the current PCR method, as well as a real-time PCR quantification technique based on the Az16S-D primer sequences.

In order to increase the specificity and sensitivity of ni*f*H detection, we developed a nested PCR approach based on previously described conserved primer target sites (Kirshtein et al., 1991; Ueda et al., 1995). As shown in Figure 3, this approach improved both the sensitivity and the specificity of *nif*H amplification. PCR amplification of gene sequences has proved to be a powerful tool, but if applied to mixtures of related genes, it may be biased by two known factors. One factor is the potential for preferential amplification of certain sequences, which prevents quantitative correlation of sequence abundance in the DNA sample and sequence abundance in the PCR product. The analysis of *nif*H PCR products from samples is a powerful tool for assessing the presence and diversity of nitrogen-fixing microorganisms in ecosystems. Although, this approach does not directly allow evaluation of functional aspects of the nitrogen-fixing populations in a sample, structural information on the gene pool and the potential for nitrogen fixation may be assessed. The differences are consistent with reports that the nitrogen fixation activities associated with forest litter and logs are greater than the nitrogen fixation activities associated with soil (Hope et al., 1997).

Nitrogen availability in soil may limit or regulate degradation processes (Berg, 1986; Berg et al., 1994; Dawson, 1992) and controlled nitrogen fixation may provide nutrient levels that allow optimal mineralization activities. The correct taxonomic classification of organisms in the environment may be determined by isolation and polyphasic characterization (de Lajudie et al., 1994). Durum wheat Inoculated with *A. brasilense* increase the total dry plant weight, the amount of nitrogen, the thousand seeds weight and *A. brasilense* also affects roots length and increased yield. These results were similar to Bashan and Levanony (1991) who describe that these bacteria have a direct influence on root length, number of secondary roots, increase the elongation zone, root volume, dry weight and increased yield. In general, these diazotroph are reported to improve root growth and function, often leading to increased uptake of water and mineral nutrients. Plant inoculation with *A. brasilence*, for example, promoted greater uptake of NO_3, K and H_2PO_4 in sorghum, wheat and setaria (Zavalin et al., 1998; Saubidet et al., 2000). The beneficial effects of inoculating durum wheat with *A. brasilense* give higher yielding wheat when compared with uninoculated durum wheat. Our result is confirmed by Martin et al. (2008). Several strains of *A. brasilense* and *A. lipoferum* have been used successfully as crop inoculants to increase yield (Dobbelaere et al., 2001; Okon and Labandera-Gonzalez, 1994).

In conclusion, the population of Rhizobacteria isolated from soil mainly cereals (wheat) is identical and dominated by the genus *Azospirillum*. Many strains of these genus are able to grow in an environment with high concentrations of NaCl, and can also synthesize IAA, ammonium and solubilize phosphate, thus confirming the potential for tolerance of PGPR from Algerians soil. One of the recommandations is the evaluation of inoculation between the *A. brasilense* and the durum wheat under controlled conditions and in the field. In future, these strains could be effectively used as biofertilizers for wheat (*T. durum* Desf.) in Algeria.

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