

E. Nabti¹
 M. Sahnoune²
 S. Adjrad²
 A. Van Dommelen³
 M. Ghoul⁴
 M. Schmid⁵
 A. Hartmann⁵

Research Article

A Halophilic and Osmotolerant *Azospirillum brasilense* Strain from Algerian Soil Restores Wheat Growth under Saline Conditions

Dedicated to Prof. Dr. Wolfgang Babel on the occasion of his 70th birthday

¹University of Bejaïa, Laboratory of Nutrition and Food Science, Targa Ouzemmour, Bejaïa, Algeria.

²University of Bejaïa, Laboratory of Ecology and Environment, Targa Ouzemmour, Bejaïa, Algeria.

³KU Leuven, Centre of Microbial and Plant Genetics, Heverlee, Belgium.

⁴University of Setif, Laboratory of Bacterial Ecology, Setif, Algeria.

⁵GSF-Forschungszentrum für Umwelt und Gesundheit, Abteilung Mikrogen-Pflanzen Interaktionen, Neuherberg/München, Germany.

A new bacterial isolate (NH) from salt-affected soil was identified as *Azospirillum brasilense* using phenotypic analyses and 16S rDNA-based phylogeny. This isolate showed resistance towards 3,4-dehydroproline and optimal growth at 200 mmol/L NaCl, tolerating salt stress of 300 mmol/L NaCl in the absence of osmoprotectants and up to 600 mmol/L NaCl in the presence of glycine betaine and *Ulva lactuca* extracts. This effect was enhanced with extracts of the marine algae *Ulva lactuca*. *A. brasilense* strain NH can produce auxin indole acetic acid under saline conditions. The hypothesis was tested that the inoculation of this osmotolerant rhizosphere strain could improve the growth of wheat under saline stress conditions. Normal wheat growth was restored in the presence of both 150 mmol/L and 200 mmol/L NaCl after inoculation with *A. brasilense* NH. Under saline conditions, its effect of promoting plant growth of wheat was significantly superior to that of *A. brasilense* Sp7, the non-halotolerant type strain. *A. brasilense* NH restored wheat growth at elevated salt concentrations in pot and field experiments even better in the presence of osmoprotective *Ulva lactuca* extracts.

Keywords: Fertilization, Plants, Saline conditions, Soil

Received: May 8, 2007; *revised:* May 29, 2007; *accepted:* June 5, 2007

DOI: 10.1002/elsc.200720201

1. Introduction

In many countries, the tendency of further increases in aridity, salinity and chemical pollution seems unavoidable. Hence, the development and sustainability of profitable agricultural systems are seriously threatened. A key to resolve this problem could reside in the selection and improvement of osmotolerant biofertilizer strains (e.g., *rhizobia* and *azospirilla*), improvement of management practices and suitable plant cultivar selection. Plant growth promotion by root associated microbes is an important issue for the achievement of sustainability in agriculture, because it provides the possibility of improving plant growth and performance by using natural biological resources. It has repeatedly been shown in many countries

around the world that the application of biofertilizers like plant growth promoting rhizobacteria (PGPR), e.g., *Azospirillum* sp., resulted in comparable yields despite less nitrogen fertilizer being applied [1, 2]. This is mostly due to the improved root development and uptake of nutrients and water by PGPR-inoculated plants. The effects of *Azospirillum* on plant growth have been intensively studied, as reviewed by several authors [3–5].

The performance of agriculture under less favorable, e.g., under semiarid or saline conditions, presents major challenges in many countries. In addition to a proper supply of nutrients, dominating constraints for plant growth are the lack of water and salinity of soils. Sustainable and cost-effective plant growth becomes even more important due to the need of energy efficient plant growth for biomass and bioenergy production, especially in soils of lower quality, in addition to food production. Therefore, this study focused on the characterization of a halophilic and osmotolerant PGPR and the stimulation of wheat growth under saline conditions.

In *Azospirillum* spp., the degree of osmotolerance is positively correlated with lower efficiencies to use amino acids and betaines for growth as sole carbon and nitrogen sources [6] and

Correspondence: A. Hartmann (anton.hartmann@gsf.de), GSF-Forschungszentrum für Umwelt und Gesundheit, Abteilung Mikrogen-Pflanzen Interaktionen, Ingolstädter Landstr. 1, D-85764 Neuherberg/München, Germany.

the ability to take up and accumulate choline or glycine betaine as osmoprotectants [7–9]. Mutants of *Azospirillum brasilense* Sp7 and Cd resistant towards 3,4-dehydroproline (DHP), exhibiting improved osmotolerance, have been selected under NaCl-stress in the laboratory [10]. It was shown that extracts of the marine algae *Ulva lactuca* convey significant osmoprotection to microorganisms under salt stress [11]. These algal extracts contain high amounts of different betaines, amino acids, proteins and dimethylsulphoniopropionate [11] which can be used as osmoprotective substances by organisms.

The effects of salinity on plant growth have been extensively studied [12–14]. However, no data are yet available on the combined effects of osmotolerant *A. brasilense* strains and different osmoprotectants on plant growth under saline stress in the field. Here, the authors describe phenotypic and 16S rDNA-based phylogenetic analyses of a new osmotolerant bacterial isolate from a salt-affected soil (Bejaïa, Algeria), and report its effect on wheat (*Triticum durum* var. *waha*) growth under saline conditions and the influence of *U. lactuca* extracts.

2 Materials and Methods

Soil Sampling and Bacterial Isolation

Soil samples were collected from the top 30 cm of a red soil field (Bejaïa, Algeria) with 1 % salinity (10 g/kg). Ten g of each sample was suspended in 100 mL sterile saline solution (0.85 % NaCl, Tween 80) [15]. After preparing appropriate dilutions, 1 mL of each sample was used to inoculate the N-free semisolid medium (NFB) [g/L] [16, 17]: K_2HPO_4 , 6.0; KH_2PO_4 , 4.0; $MgSO_4 \times 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.02; $FeCl_3$, 0.01; NaMoO, 0.002, and malic acid 5.0. After adjusting the pH to 6.8 with NaOH (0.2 mol/L), 5 mL 0.5 % alcoholic solution of bromothymol blue (BBT), yeast extract (Bacto) (0.05 g/L), and agar (Bacto) (1.75 g/L) were added and autoclaved. The incubation conditions were 35 °C for 7 days.

Tubes with subsurface pellicle growth were used to inoculate NFB agar plates (agar 20 g/L) including 0.05 g/L of yeast extract. Plates were incubated at 35 °C for 7 days. Potato agar plates were prepared from 200 g of potatoes (boiled with the skin in one liter of H_2O), filtered, and 0.25 % potassium malate and 0.25 % sucrose were added. The microscopic observations of the cell morphology were performed with a light microscope (Carl Zeiss, Jena, Germany) using an immersion oil objective ($\times 100$).

Utilization of Different Carbon Sources

The utilization of sugars as the only source of carbon was studied in NFB medium without BBT [16]. The liquid medium was augmented with 2.5 g/L of NH_4Cl and the pH was adjusted to 7.1. Malic acid was replaced in each case by the following sugars: glucose, cellobiose, raffinose, starch, xylose, glycerol, melibiose, maltose, arabinose, saccharose, mannitol, sorbose, mannose, lactose, salicin, rhamnose, galactose, trehalose, levulose, fructose, dulcitol, sorbitol, ribose, N-acetyl glu-

cosamine, glycerol contained in sterile bulbs; they were added to a final concentration of 1 %. Each tube was inoculated with 0.1 mL bacterial solution and then incubated at 37 °C for 72 h. The growth was monitored by measurements of the absorbance at 560 nm (A_{560nm}).

Production of Acid from Glucose

Glucose peptone broth (5 mL) containing the BBT as a pH indicator was inoculated with 0.1 mL 48-hour-old bacterial culture, and incubated for 96 h at 32 °C. The acidification of glucose is indicated by the change of color to yellow [18].

Biotin Requirement

Semisolid NFB (1.75 g/L of agar) was supplied with 100 μ g/L biotin. The same medium was prepared without biotin and used as a control. The two media were inoculated with 0.1 mL bacterial solution and were incubated at 37 °C for 48 h [18]. Growth was determined by the appearance of a subsurface pellicle in the semisolid medium and a color change of the medium to brilliant blue.

Production of Acid from Glucose and Fructose under Anaerobic Conditions

Glucose peptone broth and fructose peptone broth were used as growth media. The tubes were inoculated with a 48-h-old culture, paraffin oil was added on top and incubated at 37 °C for 72 h. The acidification of glucose or fructose is indicated by the color change to yellow [18].

Acidification of Mannitol, Ribose, and Sorbitol under Aerobic Conditions

Malic acid contained in semisolid medium NFB (1.75 g/L of agar) with the BBT was replaced by each sugar which was added to a final concentration of 1 %. Incubation was carried out at 37 °C for 96 h.

Utilization of Organic Acids as a Carbon Source

NFB medium without BBT was used by adding to each tube the following organic acids (5 g/L) after their sterilization using sterile filtration (0.22 μ m): lactate, malate, propionate, pyruvate, citrate and succinate. The medium was inoculated with 0.1 mL 48-h-old bacterial culture and then incubated at 37 °C for 48 h [16]. The effect of pH and temperature were monitored by measurement of OD at 560 nm on liquid NFB medium without BBT [18]. To assess the effect of NaCl on the isolates, NFB medium without BBT was prepared with concentrations of NaCl ranging from 100 to 1000 mmol/L. It should be noted that the basic medium was taken as a control

(1.72 mmol/L NaCl). The pH was adjusted using KOH to 6.8. The media were inoculated with 0.1 mL bacterial suspension (48-h-culture) and incubated at 32 °C for 24 h. The growth was monitored by measurement of absorbance at 560 nm ($A_{560\text{nm}}$).

Tests for Enzymes and Growth in 1 % Bile

Tests for the enzymes catalase, oxidase, phosphatase, urease, esculine hydrolase, starch hydrolysis and gelatinase and growth in 1 % bile were carried out according to Bergey's manual of bacterial systematics [17].

Nitrate Reduction Assay

The ability of colonies to reduce nitrate to nitrite was determined using the nitrate reductase assay. The isolate was grown on NFB solid medium plates for 2 days at 32 °C. 100 μL of sodium nitrate (1 %) were added to the colony on the Petri plate. After 10 min of incubation at room temperature, 100 μL of the nitrate detection reagent (containing 2 parts of 4 % [w/v] sulphanilamide in 20 % of concentrated HCl and 1 part of 0.08 % [w/v] naphthyl ethylene diamine in 1 % of concentrated HCl) were added and the occurrence of reddish pink color indicated the existence of nitrite and therefore the aerobic reduction of nitrate to nitrite [15].

Nitrite Assay

The isolate was grown in defined liquid medium with different nitrogen sources (either 10 mmol/L of NO_2^- or NO_3^- or NH_4Cl) by shaking at 150 rpm at 32 °C for 12–16 h. Cells were harvested by centrifugation at 6000 rpm for 3 min and 1 mL of supernatant was transferred into 3 mL cuvettes. Then, 0.02 mL of nitrite determination reagent was added to the same cuvettes left at room temperature for 20 min and the color was measured spectrophotometrically at 540 nm [15].

Production of Indole Acetic Acid

The assay was performed according to Bric et al. [19]. The bacteria were grown in liquid NFB medium supplied with NH_4Cl (0.1 g/L) and tryptophan (0.5 mg/L) under shaking conditions (100 rpm) at 30 °C to the late stationary phase. Culture supernatant was obtained by centrifugation at 10,000 rpm for 15 min. An aliquot of 2 mL was added to 0.1 mL of 10 mM orthophosphoric acid and 4 mL of reagent (1 mL of 0.5 M FeCl_3 in 50 mL of 35 % HClO_4) and mixed. After an incubation of 25 min at room temperature, the absorbance of pink color was measured at 530 nm. Pure IAA (Sigma-Aldrich, Steinheim, Germany) was used to prepare a standard curve.

16S rDNA-Based Strain Characterization

Total DNA was isolated, PCR-amplified and sequenced following Xia et al. [20]. The 16S rDNA partial sequence (1050 bp) was compared to databases using Blast Software [21]. Sequence alignment and phylogenetic reconstruction were inferred using Clustalw 0.82 Software [22] and Phylip 3.6b Package, respectively [23].

Preparation of Algal Extract

Ulva lactuca was harvested from the Gulf of Bejaïa on March 2005 and 2006. At room temperature, 50 g algal fresh material was homogenized in 70 % [v/v] ethanol, filtered through an absorbent pad and evaporated to dryness. The material was dissolved in 10 mL distilled water and these extracts were used at a 10⁻² final dilution to the growth medium [11].

Determination of NaCl-Tolerance

The salt content of NFB-liquid medium was increased by the addition of NaCl concentrations varying from 250 to 650 mmol/L. 10 % of algal extract [v/v] were added to the NFB medium and autoclaved at 110 °C and 1.21 atm for 30 min. The same experiment was carried out using 1 mmol/L of glycine betaine (GB) in place of algal extract. Growth experiments were performed using 2 mL of medium at 32 °C for 48 h with shaking at 100 rpm. Bacterial growth was recorded by measuring OD 560 nm. Controls without NaCl, GB, and algal extract were used.

Wheat Growth Experiments in Pots

Two pot experiments were performed simultaneously to test the effect of the *A. brasilense* inoculation and application of osmoprotectants on wheat (*Triticum durum* var. *waha*) growth under different salinity conditions in the greenhouse. The soil used for pot experiments was from the field experimental site (Ihaddadhen, Bejaïa, Algeria) located at 500 m altitude and oriented north towards the sea. Chemical characteristics of the soil were: organic matter, 31 %; total N, 19.48 %; mineral N, 3.77 %; P, 5.2 %; K, 13 %; water capacity, 25 %; pH, 6.4; salinity, 0.85 %.

Pot experiments 1 and 2 were performed at an initial NaCl concentration of 100 and 150 mmol/L, respectively. Each pot experiment consisted of 10 treatments (see Tab. 1) with 50 plants respective pots each (finally 1 plant per pot). 1.5 L pots were filled with autoclaved soil (110 °C for 30 min). For surface disinfection of seeds, immersion in 1 % NaOCl solution for 3 min was performed followed by thorough washing. Algal extracts (100 mL, 0.5 g/mL) and 10 mL of bacterial suspension (10⁶ CFU/mL) were added to the appropriate seeds immediately after sowing. No fertilizers were applied throughout the entire experiment. Five surface-disinfected seeds were

sown per pot and then reduced to one after emergence of the first leaf to make one plant per pot. The 1000 pots used for the two pot experiments were completely randomized and, to avoid cross-contamination, pots were placed 30 cm apart and the soil surface was covered with sterile vermiculite. Plants were irrigated once a week with 500 mL of water from a nearby well. In the first three weeks, 500 mL of 100 or 150 mmol/L NaCl solution was used to irrigate the appropriate plants instead of water. The conductivity was measured using a conductivity meter (Condi 3031). All potted plants were isolated from precipitation water using a greenhouse. The experiment was conducted under a natural light/dark regime from March to June 2006.

Field Experiment

The field experiment was carried out at Ihaddadhen, Bejaia, Algeria, located at 500 m altitude and oriented north towards the sea. The soil characteristics are given above and no further fertilization was performed. An initial NaCl concentration of 150 mmol/L was applied. Wheat seeds (*Triticum durum* var. *waha*) were surface disinfected before use as described above. Inoculation and treatment of algal extract was performed as in the pot experiment. Ten treatments with 30 plants each were analyzed (see Tab. 1). The 30 plants of each treatment were sown in a single line with 80 cm between the plants and 75 cm between the lines. This made a total of 300 plants cultivated on an overall surface area of 225 m².

Plants were irrigated once a week with 500 mL of water from a nearby well. In the first three weeks, 500 mL of 150 mmol/L NaCl solution was used to irrigate the appropriate plants instead of water. The conductivity was measured

using a conductimeter (Condi 3031). The temperatures ranged from 25–32 °C during the experimentation period. The field experiment was performed from March to June 2006.

Measured Plant Parameters

Stem heights and spike lengths (hairs not included) were measured nine weeks after sowing. For statistical comparison, Fisher's Least Significant Difference test was applied.

3 Results

3.1 Taxonomic and Phylogenetic Characteristics

The isolate NH formed a subsurface growth pellicle 2–5 mm deep in semisolid NFB medium containing bromothymol blue. After one week of incubation at 35 °C, the medium color turned from yellowish green to brilliant blue. On solid NFB medium plates containing BBT and yeast extract (0.05 g/L), white and small dense colonies appeared on the surface. Characteristic colonies, more or less pinkish, usually wrinkled and partially growing into the agar, were observed on infusion potato agar plates. The cells were Gram-negative, vibroid with very high motility (typical spiral movement) and contain poly-β-hydroxybutyrate granules, visible under the oil immersion microscope. The isolate NH used the same sugars, organic acids and amino acids as carbon sources as reported for *Azospirillum brasilense* [18]. It showed neither acidification of glucose on glucose peptone broth nor any fermentative metabolism at both aerobic and anaerobic conditions. The hydrolysis tests were positive for phosphatase, urease, catalase, oxidase and esculine and negative for gelatine and starch. The isolate NH tolerated the presence of 1% bile and reduced nitrate to nitrite and then to gaseous N₂. It did not show biotin requirement. Its optimal pH, temperature and NaCl concentrations for growth were 6–7, 30–37 °C and 200 mmol/L NaCl, respectively.

Table 1. Comparison of growth of *Triticum durum* var. *waha* under different salt stress conditions. 1: Soil (without NaCl stress, control); 2: Soil (control) + *Ulva lactuca*; 3: Soil + NaCl; 4: Soil + NaCl + *U. lactuca*; 5: Soil + NaCl + *Azospirillum brasilense* NH; 6: Soil + NaCl + *A. brasilense* Sp7; 7: Soil + NaCl + NH + *U. lactuca*; 8: Soil + NaCl + Sp7 + *U. lactuca*; 9: Soil (control) + Sp7; 10: Soil (control) + NH.

The numbers represent arithmetic means ± SD of stem heights and spike lengths (cm); A–H and a–c express the statistic evaluation and compare the 10 treatments within each experiment (columns) and the three experiments within the same treatment (lines), respectively. Values accompanied by the same letter are not significantly different according to Fisher's Least Significant Difference test ($P < 0.05$).

Treatments	Pot experiment 1 [*]		Pot experiment 2 [†]		Field experiment [†]	
	Stem height	Spike length	Stem height	Spike length	Stem height	Spike length
¹ Control	60.1 ± 0.4 ^{Ea}	6.4 ± 0.1 ^{Ea}	60.1 ± 0.4 ^{Ea}	6.4 ± 0.1 ^{Da}	67.9 ± 0.4 ^{Fb}	6.9 ± 0.1 ^{Fb}
² U	65.1 ± 2.4 ^{Ha}	6.7 ± 0.1 ^{Fa}	65.1 ± 2.4 ^{Ga}	6.7 ± 0.5 ^{Da}	72.1 ± 0.4 ^{Hb}	7.3 ± 0.1 ^{Hb}
³ NaCl	37.6 ± 0.5 ^{Ab}	3.0 ± 0.1 ^{Ab}	No growth ^{Aa}	No growth ^{Aa}	No growth ^{Aa}	No growth ^{Aa}
⁴ NaCl+U	46.9 ± 0.7 ^{Bc}	3.9 ± 0.4 ^{Bc}	30.5 ± 4.8 ^{Ba}	3.7 ± 0.5 ^{Cb}	41.5 ± 1.9 ^{Gb}	3.5 ± 0.2 ^{Ca}
⁵ NaCl+NH	51.7 ± 1.7 ^{Dc}	4.4 ± 0.2 ^{Dc}	38.6 ± 0.6 ^{Ca}	3.0 ± 0.1 ^{Bca}	45.4 ± 2.6 ^{Db}	3.7 ± 0.3 ^{Db}
⁶ NaCl+Sp7	38.0 ± 2.3 ^{Ab}	3.0 ± 0.5 ^{Ab}	No growth ^{Aa}	No growth ^{Aa}	No growth ^{Aa}	No growth ^{Aa}
⁷ NaCl+NH+U	61.7 ± 0.5 ^{Fc}	6.5 ± 0.1 ^{Ec}	45.9 ± 0.4 ^{Da}	3.5 ± 0.1 ^{Ca}	47.2 ± 2.0 ^{Eb}	4.1 ± 0.2 ^{Eb}
⁸ NaCl+Sp7+U	48.7 ± 1.0 ^{Cc}	4.0 ± 0.1 ^{Cc}	30.1 ± 0.4 ^{Ba}	1.8 ± 0.5 ^{Ba}	38.1 ± 4.0 ^{Fb}	3.3 ± 0.2 ^{Bb}
⁹ Sp7	61.2 ± 0.8 ^{Fa}	6.5 ± 0.1 ^{Ea}	61.2 ± 0.8 ^{Fa}	6.5 ± 0.1 ^{Da}	69.8 ± 0.5 ^{Gb}	7.2 ± 0.2 ^{Gb}
¹⁰ NH	62.1 ± 0.5 ^{Ga}	6.5 ± 0.1 ^{Ea}	62.1 ± 0.5 ^{Fa}	6.5 ± 0.1 ^{Da}	69.3 ± 0.4 ^{Gb}	7.0 ± 0.1 ^{Gb}

The pot and field experiments were performed at 150^{*} and 200[†] mmol/L NaCl concentrations, respectively.

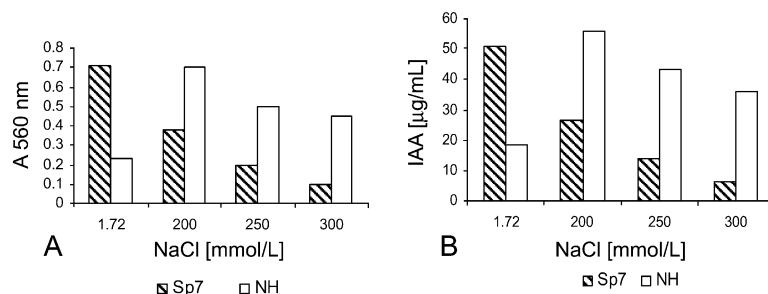


Figure 1. Comparison between the indole acetic acid (IAA) production of *A. brasilense* Sp7 and *A. brasilense* NH at different NaCl concentrations. (A) Growth (A_{560nm}); (B) IAA production ($\mu\text{g/mL}$).

to the type strains *A. brasilense* Sp7 (ATTC 29145) (GenBank accession number: AY324110.1).

3.2 Halotolerance Properties

The *A. brasilense* strain NH showed a halophilic phenotype, since growth was much retarded at 1.7 mmol/L NaCl while it was optimal at 200 mmol/L NaCl (see Fig. 2A). Figs. 2B–D summarizes the osmotolerance characteristics of *A. brasilense* NH. Growth gradually and significantly decreased as NaCl concentrations increased. When no osmoprotectant was added (see Figs. 2A and D), bacterial growth was completely inhibited at 400 mmol/L NaCl. In the presence of glycine betaine (see Fig. 2B) this limit was increased to 450 mmol/L NaCl, and with algal extract to 650 mmol/L (see Fig. 2C). Interestingly, in the presence of algal extracts bacterial growth was significantly higher at 250 and 300 mmol/L NaCl than at 200 mmol/L NaCl (see Figs. 2C and D).

The *A. brasilense* strain NH was resistant to 60 $\mu\text{g/L}$ 3,4 dehydroproline (DHP), an antimetabolite of the osmoprotectant amino acid proline. This resembles the connection of DHP-resistance and NaCl-tolerance found in spontaneous mutants of *A. brasilense* Sp7 [10].

3.3 Impact on Wheat Growth

Both *A. brasilense* strains Sp7 and NH increased the growth of wheat plants in pot and field experiments in the absence of salt stress (see Tab. 1, treatments 1 and 9–10), which confirms the plant growth promoting activity of *A. brasilense* strains. In the presence of NaCl, wheat growth was partially (at 150 mmol/L NaCl) or completely (at 200 mmol/L NaCl) inhibited. *U. lactuca* extracts revealed to be an efficient osmoprotectant for wheat plants (see Tab. 1, treatments 3–4). Clearly, *A. brasilense* NH showed more osmoprotection of wheat plants than Sp7 (see Tab. 1, treatments 1, 3, 5–6), especially at higher osmolarities. Obviously, there is a cooperation between the strains NH or Sp7 and algal extracts in conferring higher osmotolerance to wheat plants (see Tab. 1, treatments 3–8), as

could be measured with the stem heights or spike lengths of wheat plants in pot and field experiments under salinity stress.

4 Discussion

The phenotypic and physiological properties of the isolate NH completely matched the properties described for *A. brasilense* [17, 18]. Its 16S rDNA sequence showed very high similarity (up to 98.3%) with an *A. brasilense* Sp7 type strain. This characterization of a new osmotolerant *A. brasilense* strain NH confirms earlier findings that *A. brasilense* can be isolated from salt-affected soil and rhizosphere, showing an increased salt tolerance [24]. The different degree of halophilicity and halotolerance as well as many other taxonomic differences clearly separates the isolate NH from *A. halopraeferans* [25].

Algal extract was a far more efficient osmoprotectant for *A. brasilense* NH as compared to, e.g., glycine betaine (GB) in laboratory culture experiments (see Fig. 2). *U. lactuca* extract has been reported to be rich in various betaines, amino-acids and dimethylsulphoniopropionate [11]. According to [9] and literature cited therein, amino acids and betaines help bacterial cells as osmoprotectants and compatible solutes to face osmotic stress and to preserve enzymatic functions. GB is better as an osmoprotectant than amino acids (e.g., proline) and choline when used separately [24]. The superiority of *U. lactuca* extracts as osmoprotectants suggests that their constituents may act in synergy or may contain some unidentified strongly osmoprotective substances.

Since *A. brasilense* NH showed maximum growth at 200 mmol/L NaCl, in the absence of osmoprotectants, while growth and IAA-production was retarded at 1.7 mmol/L NaCl, it can be characterized as a halophilic and moderately halotolerant bacterium. It appears to be more osmotolerant than *A. brasilense* Sp7 (type strain) with optimal growth at 100–150 mmol/L NaCl and optimal growth at very low NaCl concentrations (see Figs. 1 and 2). For *A. brasilense* NH, the addition of algal extract had a better osmoprotective effect than glycine betaine (GB), since it could tolerate NaCl stress up to 400 and 600 mmol/L NaCl, respectively.

A. brasilense NH was resistant towards 60 $\mu\text{g/mL}$ 3,4 dehydroproline (DHP) concentrations. This is quite remarkable, because DHP-resistant spontaneous mutants of *A. brasilense* Sp7, showing a superior osmotolerance than the wild type strain, had been obtained at a rather high frequency under laboratory conditions [10]. In addition, the selection of spontaneous mutants of *A. brasilense* Sp7 with improved osmotic tolerance at NaCl stress at 400 mmol/L also yielded DHP-resistant cells [10]. This observation suggests that *A. brasilense* NH may eventually be a product of natural selection or evolution under salt stress in arid Algerian soil.

Inoculation with *A. brasilense* NH alone and especially in combination with algal extract had a remarkable plant growth stimulatory effect which was clearly superior to the effect observed with *A. brasilense* Sp7. This could be in part based on a direct osmoprotective effect of algal extracts on the develop-

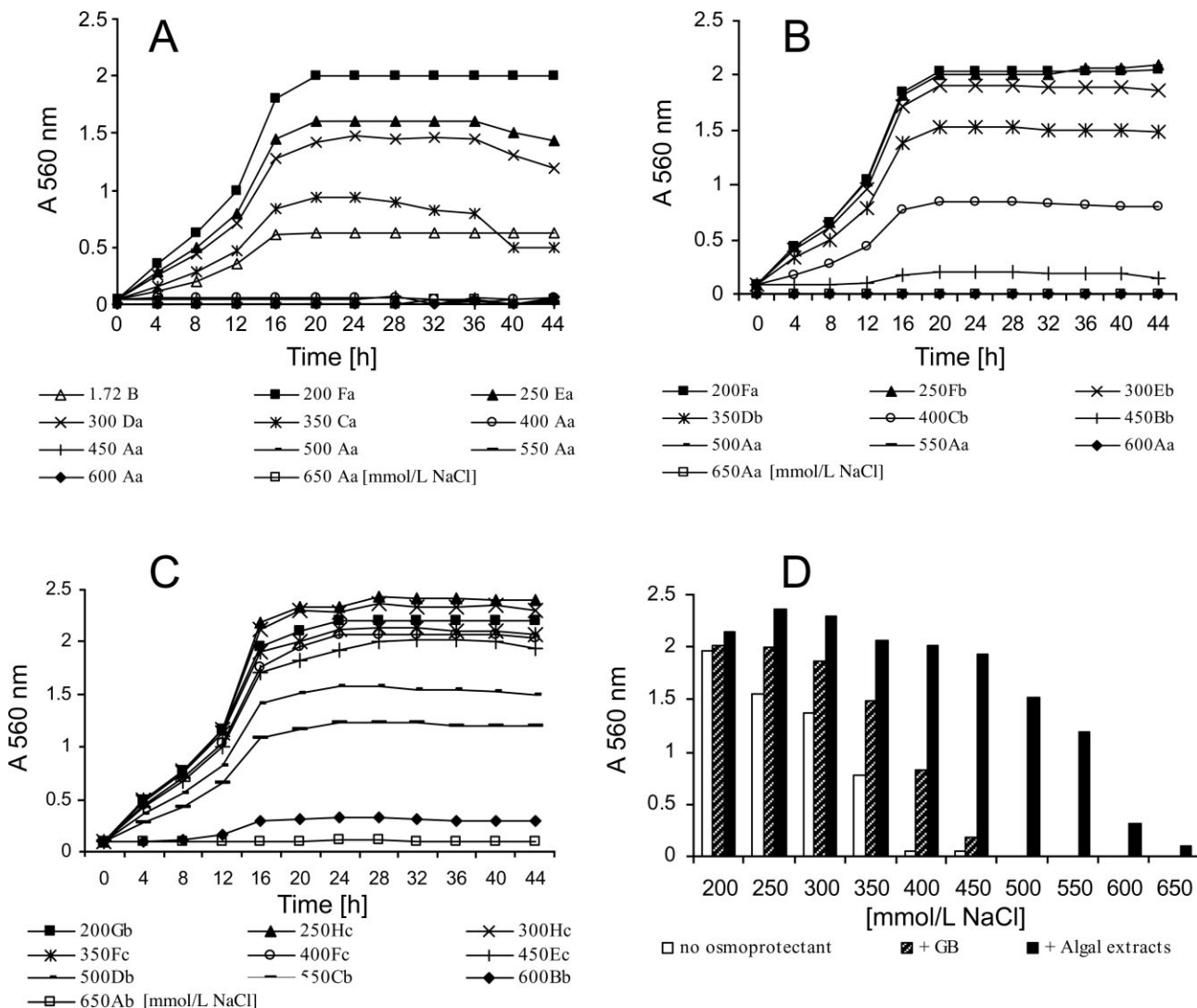


Figure 2. Growth of *Azospirillum brasilense* NH in the presence or absence of glycine betaine (1 mmol/L) and *Ulva lactuca* extract (10% [v/v]) at increasing NaCl concentrations. A: No osmoprotectant added; B: with glycine betaine added; C: with algal extract added; D: comparison of stationary growth phases in the experiments A, B and C. A–H and a–b express the statistic evaluation of the values for bacterial growth (A_{560nm}) at different NaCl concentrations within and between the experiments A, B, and C, respectively. Values accompanied by the same letter are not significantly different according to Fisher’s Least Significant Difference test ($P < 0.05$).

ment of the wheat plants under salinity stress, which may directly use the osmoprotective compounds in the algal extract. It has been shown that osmotically stressed plants use similar osmolytes than bacteria [26]. In addition, due to a better performance of the inoculants in the presence of algal extracts, their plant promoting effect may have been supported. Plant growth was better in the field experiment as compared to the pot experiment 2 (see Tab. 1). This could be explained by the limiting conditions and different microflora in the pot experiment, where the soil had been autoclaved. There may be a cooperation of several soil microorganisms to result in better nutrient uptake and osmotolerance under natural conditions.

In conclusion, these results justify the continuation of selecting *A. brasilense*-wheat couples with improved osmotolerance for the sake of a more efficient agriculture in salt-affected soils. The combination of *A. brasilense* NH and *Ulva lactuca* extract may successfully improve agriculture in salt-affected soils.

Acknowledgements

The authors would like to thank Prof. Yaacov Okon (Department of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Rehovot, Israel) for his helpful advices

and Mr. Mokhtar Harrat (Cybercafé Harrat, Targa Ouzemour, Bejaïa, Algeria) and Mrs. Angelika Schulz (GSF) for their valuable technical assistance.

References

- [1] Y. Okon, C. A. Labandera-Gonzalez, Agronomic application of *Azospirillum*: An evaluation of 20 years worldwide field inoculation, *Soil. Biol. Biochem.* **1994**, *26*, 1551–1601.
- [2] S. Doebbelaere, A. Croonenborghs, A. Thys, D. Ptacek, J. Vanderleyden, P. Dutto et al., Responses of agronomically important crops to inoculation with *Azospirillum*, *Austr. J. Plant Physiol.* **2001**, *28*, 871–879.
- [3] Y. Bashan, G. Holguin, *Azospirillum*-plant relationships, environmental and physiological advances (1990–1996), *Can J. Microbiol.* **1997**, *43*, 103–121.
- [4] A. Hartmann, J. I. Baldani, The Genus *Azospirillum*, in *The Prokaryotes: Proteobacteria: Alpha and Beta Subclasses* (Eds: M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, E. Stackebrandt), Vol. 5, Springer-Verlag, New York (USA), **2006**, 114–140.
- [5] S. Dobbelaere, J. Vanderleyden, Y. Okon, Plant growth-promoting effects of diazotrophs in the rhizosphere, *CRC Crit. Rev. Plant Sci.* **2003**, *22*, 107–149.
- [6] A. Hartmann, H. Fu, R. H. Burris, Influence of amino acids on nitrogen fixation ability and growth of *Azospirillum* sp., *Appl. Environ. Microbiol.* **1988**, *54*, 87–93.
- [7] N. Riou, D. Le Rudulier, Osmoregulation in *Azospirillum brasilense*: glycine betaine transport enhances growth and nitrogen fixation under salt stress, *J. Gen. Microbiol.* **1990**, *136*, 1455–1461.
- [8] A. Hartmann, S. R. Prabhu, E. A. Galinski, Osmotolerance of diazotrophic rhizosphere bacteria, *Plant Soil* **1991**, *137*, 105–109.
- [9] A. Hartmann, Osmoregulatory properties of *Azospirillum* spp., in *Azospirillum IV: Genetics, Physiology, and Ecology* (Ed: W. Klingmüller), Springer-Verlag, Berlin (Germany) **1988**, 122–130.
- [10] A. Hartmann, C. Guendisch, W. Bode, *Azospirillum* mutants improved in iron acquisition and osmotolerance as tools for the investigation of environmental fitness traits, *Symbiosis* **1992**, *13*, 271–279.
- [11] M. Ghoul, J. Minet, T. Bernard, E. Dupray, M. Cornier, Marine macroalgae as a source for osmoprotection for *Escherichia coli*, *Microb. Ecol.* **1995**, *30*, 171–181.
- [12] M. Saqib, J. Akhtar, R. H. Qureshi, Pot study on wheat growth in saline and waterlogged compacted soil: I. Grain yield and yield components, *Soil Tillage Res.* **2004**, *77*, 169–177.
- [13] S. E. El-Hendawi, Y. Hu, G. M. Yakout, A. M. Awad, S. E. Hafiz, U. Schmidhalter, Evaluating salt tolerance of wheat genotypes using multiple parameters, *Eur. J. Agron.* **2005**, *22*, 243–253.
- [14] A. R. Sepaskhah, A. R. Bazrafshan-Jahromi, Z. Shirmohammadi-Aliakbarkhani, Development and evaluation of a model for yield production of wheat, maize and sugar beet under water and salt stresses, *Biosyst. Eng.* **2006**, *93*, 139–152.
- [15] E. Çelen, A. K. Mehmet, Isolation and characterization of aerobic denitrifiers from agriculture soil, *Turk J. Biol.* **2004**, *28*, 9–14.
- [16] J. Döbereiner, I. E. Marriel, M. Nery, Ecological distribution of *Spirillum lipoferum* Beijerinck, *Can. J. Microbiol.* **1976**, *22*, 1464–1473.
- [17] J. I. Baldani, N. R. Krieg, V. L. D. Baldani, A. Hartmann, J. Döbereiner, Genus *Azospirillum* Tarrand, Krieg and Döbereiner 1979, in *Bergey's Manual of Systematic Bacteriology*, Vol. 2: The Proteobacteria, Part C. The Alpha-, Beta-, Delta-, and Epsilonproteobacteria (Eds: D. J. Brenner, N. R. Krieg, J. T. Staley), 2nd ed., Springer-Verlag, New York (USA) **2005**, 7–26.
- [18] J. J. Tarrand, N. R. Krieg, J. Döbereiner, A taxonomic study of the *Spirillum lipoferum* group with description of a new genus *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. sp. nov. and *Azospirillum brasiliense* sp. nov., *Can. J. Microbiol.* **1978**, *24*, 967–980.
- [19] J. M. Bric, R. M. Bostock, S. E. Silverstone, Rapid in situ assay for indole acetic acid production by bacteria immobilization on a nitrocellulose membrane. *Appl. Environ. Microbiol.* **1991**, *57*, 535–538.
- [20] Y. Xia, T. M. Embley, A. G. O'Donnell, Phylogenetic analysis of *Azospirillum* by direct sequencing of PCR-amplified 16S rDNA, *Syst. Appl. Microbiol.* **1994**, *17*, 197–201.
- [21] S. F. Altschul, L. T. Madden, A. S. Alejandror, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman, "Gapped BLAST and PSI-BLAST: A new generation of protein database search programs", *Nucleic Acids Res.* **1997**, *25*, 3389–3402.
- [22] J. D. Thompson, D. G. Higgins, T. J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res.* **1994**, *22*, 4673–4680.
- [23] J. Felsenstein, *PHYLIP Phylogeny Inference Package*, Version 3.6b (beta release), Department of Genome Sciences University of Washington. Washington (USA) **2000**.
- [24] B. Reinhold, T. Hurek, I. Baldani, J. Döbereiner, Temperature and salt tolerance of *Azospirillum* spp. from salt-affected soils in Brazil, in *Azospirillum IV: Genetics, Physiology, and Ecology* (Ed: W. Klingmüller), Springer-Verlag, Berlin (Germany) **1988**, 234–241.
- [25] B. Reinhold, T. Hurek, I. Fendrik, B. Pot, M. Gillis, K. Kertters et al., *Azospirillum halopraeferans* sp. nov., a nitrogen fixing organism associated with roots of kallar grass [*Leptochloa fusca* (L.) Kunth], *Int. J. Syst. Bacteriol.* **1987**, *37*, 43–51.
- [26] R. G. Wyn Jones, R. Storey, Betaines, in *Physiology and Biochemistry of Drought Resistance in Plants* (Ed: L. G. Paleg, D. Aspinall) Academic Press, Sydney (Australia), **1981**, 171–204.