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Halotolerant *Bacillus* Species as Plant Growth Promoting Rhizobacteria from Hyper – Arid Area of Algeria

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ABSTRACT
The aim of this study was to determine the diversity of aerobic halotolerant *Bacillus* plant growth promoting rhizobacteria (PGPR), their production of hydrolytic exo-enzymes and their inoculation effect on two cowpea plants. The soil dilution plate technique was performed on tryptic soy agar complemented with thermal pretreatment to select *Bacillus* strains associated with the *Phoenix dactylifera* rhizosphere growing in hypersaline and arid soil in Algeria (In Salah, Tamanrasset). The inoculation effect of these strains on cowpea plant growth was assessed based on biometric and physiological parameters. As a result, thirteen halophilic, halotolerant and non-halophilic *Bacillus* strains were isolated. Upon screening, all strains were capable of producing at least two hydrolytic enzymes under saline conditions and most of the strains (n=10/13) showed at least two plant growth promoting (PGP) traits. Strains were identified as members of *Bacillus* genera based on their phenotypic and biochemical characteristics. The inoculation of these strains in cowpeas significantly improved biometrics and physiological growth parameters of the inoculated plants. Based on their PGP effects, five strains were identified: RP 7 (*B. coagulans*), RP 8 (*B. circulans*), RP 10 (*Paenibacillus polymyxa*), RP 12 (*B. circulans*) and RP 13 (*B. cereus*). The isolation and characterization of halophilic and halotolerant *Bacillus* strains increased knowledge about the microflora in the rhizosphere associated with date palms in saline and arid soils. *Bacillus*-PGPR strains proved to be highly effective to improve cowpea plant growth and development.

Keywords: *Bacillus*, Biofertilizers, Rhizosphere, Sustainable agriculture

1. Introduction

Salinity is a natural feature of ecosystems in arid and semi-arid regions due to very low rainfall but it can also be induced by irrigation with saline water, which can be a real hindrance to plant growth. Worldwide, it is estimated that 800 million hectares of agricultural land are affected by salinity (Yasin et al. 2018). A biological approach to improving plant productivity, particularly in saline conditions, can be adopted through the use of halophilic or salt-tolerant bacteria. From this perspective, different ecological niches have been explored to isolate and characterize halophilic and halotolerant bacteria, which include the rhizospheric soil of different cultivated plants. Halophilic bacteria are a heterogeneous physiological group of microorganisms belonging to different genera and capable of developing optimally in media with a wide range of NaCl content (3-15%) (Ventosa et al. 1998). In addition, saline environments support various bacterial populations that have modified and adapted their physiological and structural characteristics under the prevailing saline conditions. These characteristics are very important in taxonomic classification.

The salinity effect is always more pronounced in the rhizosphere, where different halophilic and halotolerant bacterial species can be hosted, due to increased water absorption by plants as a result of transpiration (Ibekwe et al. 2010). Moreover, the rhizosphere is a narrow ground area adjacent to the plants (inside and around the roots) where high microbial activity is characterized by the potential to promote plant growth (Kloepper & Beauchamp 1992). The selection of non-phytopathogenic rhizospheric bacteria with halophilic or salt-tolerant character may increase plant growth in cases of stress generated by aridity, including salinity. Consequently, inoculation with these plant growth promoting rhizobacteria (PGPR) is a biological method to replace chemical fertilizers and pesticides in agricultural practice. Therefore, different mechanisms of action can be used by PGPR to improve the growth and health of the plant such as biofertilization (nitrogen fixation, phosphate and potassium

solubilization, siderophore production) and phytostimulation (phytohormone production) (Benaissa 2019). *Bacillus* species with various PGPR properties can be introduced into various root zones. Many researchers around the world use *Bacillus* PGPR to improve the growth of various plants (Kayshep et al. 2019).

Many studies have isolated PGPR from soil, but little attention has been paid to PGPR isolated from saline soil. In fact, soil salinity is a major factor in microbial selection because salt may be considered a limiting factor that reduces bacterial diversity. The adaptation of micro-organisms to salinity conditions due to salt-selective pressure makes them candidates to be salt-tolerant PGPR isolates (Jiang et al. 2018). Kushner (1978) defined the most common categories as: (i) extreme halophiles (optimal growth in salt concentrations of 2.5 to 5.2 M or 14.63-30.45% NaCl), (ii) extreme limit halophiles (optimal growth in salt concentrations of 1.5 to 4.0 M or 8.79-23.44%), (iii) moderate halophiles (optimal growth in salt concentrations of 0.5 to 2.5 M or 2.93-14.63% NaCl), and (iv) halotolerant microorganisms that do not have an absolute need for salt for growth, but tolerate many salt concentrations (1.17-30.45% NaCl) that are often very high (considered extremely halotolerant if the growth interval extends above 2.5 M of salt, and weak halophilic with optimal growth in salt concentrations of 1.17-2.93% NaCl). Non-halophilic organisms require less than 1% NaCl.

The date palm (*Phoenix dactylifera*) is considered a xerophytic and halophyte species where difficult aridity conditions do not pose a problem for cultivation. The study of the rhizobacterial community associated with plants naturally adapted to cope with extreme saline conditions could lead to several knowledge outputs: (i) understanding the plant-microbe interaction under saline conditions, (ii) defining the mechanisms underlying plant growth with promotion under saline stress, and (iii) identifying bacterial strains to design organic fertilizers for agriculture practice in arid and saline lands (Zahran 1997).

To the best of our knowledge, there are no studies conducted about the halophilic bacteria isolated from arid and saline soil in Algeria associated with the *Phoenix dactylofiera* rhizosphere. We hypothesize that the *Bacillus* group of halophilic and halotolerant rhizobacteria may have the potential to enhance plant growth and to cope with arid conditions. Therefore, the main objectives of this study were to characterize halophilic and halotolerant *Bacillus* group rhizobacteria with hydrolase activities, plant growth promoting (PGP) traits such as nitrogen fixation, hydrogen cyanide (HCN) production and phosphate solubilization and to examine their effects by inoculating two cowpea plants. The cowpea plant is particularly cultivated by peasants in small villages in southern Algeria for their own consumption.

2. Material and Methods

2.1. Sample site and collection

Soil samples were randomly collected from three palm groves located in an arid area of Algeria (In Salah) (27° 11′ 55.69″ North, 2° 26′ 45.29″ East) during December 2020 (Figure 1). The sampling technique involved inserting a sterile spatula into the soil to a depth of 15 cm glued to the roots. The sample was placed in a sterile container and transported to the laboratory in an ice box set to 4 °C. The soil texture and physicochemical analysis (Table 1) were performed as described previously (Mathieu & Pieltain 1998; Mathieu & Pieltain 2003). Soil classification (World Reference Base: WRB) was based on the Group of Arid Hapylic Solonchaks (FAO WRB 2006).

Figure 1- Geographic location of the palm grove in the In Salah region, Algeria (Google Earth, 2020) [*rhizosphere soil]

Table 1- Texture and physicochemical parameters of soil

Parameters	*Results*
pH	7.13
Electric Conductivity 1 :5 (ds/m)	2.6
Organic Matter %	0.1
Calcium Carbonate CaCO3	0%
Texture (Particle-Size)	Sandy loam

2.2. Isolation and characterization of halotolerant Bacillus strains from rhizospheric soil

Soil dilution plate technique was performed on tryptic soy agar complemented with cycloheximide (40 mg/mL) and nalidixic acid (10 mg/L) (to eliminate gram-negative bacteria and fungi in the soil, respectively) and supplemented with NaCl (0%, 15%) to allow isolation of halotolerant or/and halophilic bacteria (Kushner 1978). Surface seeding of soil dilutions was carried out on Petri dishes to select aerobic strains.

To increase the selectivity of the *Bacillus* isolation medium, thermal pretreatment (10 min at 80° C) of the soil dilution was carried out in order to select bacterial spores and eliminate all vegetative forms.

To determine whether the strains that were grown on the initial 15% NaCl medium are halophilic or halotolerant, the nutrient broth medium (NB) was used, adjusted to NaCl concentrations (0%, 3%, 20%, 25% and 30%). Halotolerant strains were classified if they grew on both media (0 and 15% NaCl), moderate halophilic strains were those grown only on the medium at 3% - 15% NaCl, while extreme halophilic strains grew on media at 15% - 30% NaCl (Kushner 1978). The Petri dishes were incubated at 27 °C for 24 to 48 hours.

The strains were phenotypically characterized using standard procedures of Gram staining and spore position, catalase and oxidase tests complemented with several biochemical tests, such as respiratory type (medium was meat-liver agar, packaged in Prévot tube, and only bacteria with surface growth were considered aerobic), Voges-Prauskeur (VP) and Methyl red (RM) (Clark and Lubs broth was seeded with a drop of bacterial suspension, after incubation for 18 h at 37 °C, the broth was divided into 2 sterile tubes. Each tube was used to reveal one of the 2 ways: RM or VP), arginine di-hydrolase (ADH), ornithine decarboxylase (ODC) (Moeller medium contains only one amino acid, glucose and purple bromocresol as an indicator of pH. Alkalization indicates the presence of these enzymes) and sugars assimilation on inclined nutrient agar containing 1% mannitol, 1% glucose, 1% fructose and 1% saccharose, respectively, with phenol red added as pH indicator. All the strains were preserved in nutrient broth with 20% glycerol added at -80 °C.

2.3. Screening for hydrolytic activity

The activity of six extracellular enzymes was investigated under non-saline (0.9% NaCl) and saline (15% NaCl) conditions. The hydrolysis test for gelatin was performed as described by Egamberdiyeva & Höflich (2004) with modifications, using a nutrient broth supplemented with 50 g/L gelatin powder as solidifying agent. Ureolytic activity was revealed on Christensen urea agar medium (peptone 1 g/L, dextrose 1 g/L, sodium chloride 5 g/L, potassium phosphate monobasic 2 g/L, urea 20 g/L, phenol red 0.012 g/L, agar 15 g/L) according to the protocol of Atlas (2010). Casein hydrolysis was tested on Mueller Hinton agar (MH) supplemented with 10% skimmed milk (Castro-Escarpulli et al. 2003) and amylolytic activity was detected on tryptic soy agar (TSA 1/10) with 1% starch added (Delarras 2014). Cellulose hydrolysis was demonstrated on agar medium with crushed pulp as a source of cellulose (Lesel et al. 1986). Lecithinase was determined on ordinary nutrient agar supplemented by an emulsion of egg yolk and distilled water (2 mL/20 mL) (Delarras 2007).

2.4. Screening for plant growth promoting activities

Molecular nitrogen fixation was tested on the Jensen medium, a free nitrogen medium. Growth on this medium after being transferred ten times in the same medium reflects the ability of bacteria to fix nitrogen. The ability of strains to produce hydrogen cyanide (HCN) was examined using the method of Lorck (1948). The qualitative solubilization activity of phosphate was tested on the National Botanical Research Institutes Phosphate (NBRIP) medium (Nautiyal 1999).

2.5. Assay of Bacillus isolates on cowpea plants growth

The seeds of two cowpea ecotypes (*Vigna unguiculata subsp. unguiculata (L.). Walp*.) from the Tamanrasset region (Bassedik et al. 2021): (accession#1 (NEA10) from Igléne (Abalessa) [4°89'E, 22°88'N], accession#2 (NEA13) from Tit (Tamanrasset) [5°14'E, 22°58'N]), were surface sterilized by brief rinsing in 95% ethanol for 30 s, then rinsed with distilled water for 5 minutes three times. The seeds were evenly distributed over the surface of sterile absorbent paper in petri dishes. The boxes were sealed with film paper and incubated in an oven set at 28 °C. The seeds with a fully widened cotyledon were selected for inoculation. Then, 24-hour cultures of *Bacillus* inoculants were first prepared by growing a colony in 10 mL of nutritious broth. All the isolated strains were tested without taking into account halophilic character so as not to neglect interesting strains that were not halotolerant.

The germinated seeds were then placed in bacterial cultures for about 30 minutes. Three inoculated seedlings were directly planted into local soil from the university of Tamanrasset, previously sterilized at 160 °C/4 h. The pots were then kept under light with 16-hour photoperiod under laboratory conditions at room temperature with regular sprinkling of 10 mL of distilled water. Plant growth was monitored and compared to controls grown without cultured bacteria. For this purpose, biometric parameters were measured and photosynthetic pigment concentrations were determined according to Lichtenthaler (1987), and the relative water content of the leaf was determined using the method described by Barrs (1968).

2.6. Statistical analysis

The data was subjected to statistical analysis using the Microsoft Excel 2010 program. All values are given as mean ± SE (standard error) of three replicates of a single sample for each experiment. The obtained data underwent unidirectional analysis of variance (ANOVA) using the Statistical Analysis System (XLSTAT) version 2016.02. The differences between individual means were considered significant at $P<0.05$. The averages were compared with the LCD test. Principal component analysis (PCA) was performed. This is a multivariate statistical method, which involves transforming interrelated variables (called "correlated" in statistics) into new variables that are not correlated with each other. These new variables are called "principal components" or main axes.

3. Results and discussion

In this study, the microflora of aerobic halotolerant-halophilic *Bacillus* strains from the rhizosphere of *Phoenix dactylifera* growing in arid and saline soil in the Algerian Sahara were studied. Thirteen strains phenotypically close to *Bacillus* and able to grow on salt-based media were selected for further analysis. They were characterized using phenotypical tests, for hydrolytic and PGP activities and inoculation potential.

3.1. Bacteria characterization

Morphological and chemotaxonomic analyses indicate that all isolates are rod-shaped, gram-positive, aerobic, sporulating, catalase positive and oxidase variable (Table 2). All isolates were characterized according to the above-mentioned phenotypic tests as members of the genera *Bacillus*. Phenotypical identification showed that 23.07% of bacterial isolates were identified as *B. circulans,* 23.07% as *B. coagulans,* 15.38% as *B. amylo-liquifaciens* and 7.69% each for *B. megaterium, B. subtilis, B. cereus, B. licheniformis* and *Paenibacillus polymyxa*. Halophilic, non-halophilic or halotolerant strains of *Bacillus* (n=13) isolated from the date palm rhizosphere represent an infinite fraction of the soil microbial community. However, the sporulation capacity of this bacterial genus promotes ubiquity on the one hand and survival in very diverse environments on the other.

Table 2- Morphological and biochemical characterization of *Bacillus* isolates associated with the *Phoenix dactylifera* rhizosphere growing in hyper-arid area of Algeria (DT: deformant terminal, TND: terminal non-deformant, CD: central deformant, CND: central non-deformant, ANAF: aero-anaerobic facultative, AS: aerobic strict)

Code / *Tests*	***RP1***	***RP2***	***RP3***	***RP4***	***RP5***	***RP6***	***RP7***
Form	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Grouping mode	chain	chain	chain	chain	chain	chain	chain
Gram	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Spore position	TD	CD	CD	CND	CND	TND	CD
Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Oxidase	+ve	+ve	+ve	-ve	+ve	+ve	+ve
Respiratory type	ANAF	ANAF	AS	ANAF	ANAF	ANAF	ANAF
Voges-Prauskeur	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Methyl red	-ve	+ve	+ve	+ve	+ve	-ve	+ve
Arginine Di-hydrolase	-ve	-ve	-ve	+ve	-ve	-ve	-ve
Ornithine Decarboxylase	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Sugar assimilation:							
-Mannitol	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Glucose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Fructose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Saccharose	-ve	-ve	+ve	+ve	+ve	+ve	+ve
Hydrolytic activities in presence of 0.9% NaCl:							
-Urease	+ve	+ve	+ve	+ve	+ve	+ve	-ve
-Gelatinase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Caseinase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Amylase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Cellulase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Lecithinase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Hydrolytic activities in presence of 15% NaCl :							
-Urease	-ve	-ve	-ve	-ve	-ve	+ve	-ve
-Gelatinase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Caseinase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Amylase	-ve	-ve	-ve	-ve	+ve	+ve	-ve
-Cellulase	-ve	-ve	-ve	-ve	-ve	-ve	-ve
-Lecithinase	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Table 2(Continue)- Morphological and biochemical characterization of *Bacillus* isolates associated with the *Phoenix dactylifera* rhizosphere growing in hyper-arid area of Algeria (DT: deformant terminal, TND: terminal non-deformant, CD: central deformant, CND: central non-deformant, ANAF: aero-anaerobic facultative, AS: aerobic strict)

Code / *Tests*	***RP8***	***RP9***	***RP10***	***RP11***	***RP12***	***RP13***
Form	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Grouping mode	chain	chain	chain	chain	chain	chain
Gram	+ve	+ve	+ve	+ve	+ve	+ve
Spore position	TD	CND	CD	TND	TD	CND
Catalase	+ve	+ve	+ve	+ve	+ve	+ve
Oxidase	-ve	-ve	+ve	+ve	-ve	+ve
Respiratory type	ANAF	ANAF	ANAF	AS	ANAF	ANAF
Voges-Prauskeur	+ve	+ve	+ve	+ve	+ve	-ve
Methyl red	+ve	+ve	+ve	+ve	+ve	+ve
Arginine Di-hydrolase	-ve	+ve	-ve	-ve	-ve	-ve
Ornithine Decarboxylase	+ve	-ve	-ve	-ve	-ve	-ve
Sugar assimilation:						
-Mannitol	+ve	+ve	+ve	+ve	+ve	+ve
-Glucose	+ve	+ve	+ve	+ve	+ve	+ve
-Fructose	+ve	+ve	+ve	+ve	+ve	+ve
-Saccharose	+ve	+ve	+ve	+ve	+ve	+ve
Hydrolytic activities in presence of 0.9% NaCl:						
-Urease	+ve	+ve	+ve	+ve	+ve	+ve
-Gelatinase	+ve	-ve	-ve	+ve	+ve	+ve
-Caseinase	+ve	+ve	+ve	+ve	+ve	+ve
-Amylase	+ve	+ve	+ve	+ve	+ve	+ve
-Cellulase	+ve	+ve	+ve	+ve	+ve	+ve
-Lecithinase	+ve	+ve	+ve	+ve	+ve	+ve
Hydrolytic activities in presence of 15% NaCl :						
-Urease	-ve	+ve	+ve	-ve	+ve	-ve
-Gelatinase	+ve	+ve	+ve	+ve	+ve	+ve
-Caseinase	+ve	+ve	+ve	+ve	+ve	+ve
-Amylase	-ve	+ve	+ve	+ve	-ve	-ve
-Cellulase	-ve	-ve	-ve	-ve	-ve	-ve
-Lecithinase	-ve	-ve	-ve	-ve	-ve	-ve

The nature of the soil impacts the diversity of the bacterial community. The rhizosphere soil of the date palm in our study is hyper-arid and saline, which are two unfavorable conditions for the growth of microorganisms. In the same way, our microbial ecology study in arid and hypersaline environments reveals that rhizobacteria isolated from saline soils can grow at different salinity levels. From the date palm's rhizosphere, 8 halotolerant *Bacillus* strains (n= 8/13) and one moderately halophilic (n=1/13) strain were isolated (Table 3).

Table 3- Types of *Bacillus* strains and their Plant Growth Promoting activities isolated from *Phoenix dactylifera* rhizosphere

Code	*Phosphate solubilization*	*HCN production*	*Nitrogen fixation*	*Type*	*Species*
RP1	+	+	+	**Halotolerant**	*B. circulans*
RP2	+	+	+	Non-halophilic	*B. coagulans*
RP3	+	-	+	**Halotolerant**	*B. coagulans*
RP4	+	+	+	Non-halophilic	*B. megaterium*
RP5	-	-	+	**Halotolerant**	*B. subtilis*
RP6	+	-	+	**Halotolerant**	*B. amylo-liquifaciens*
RP7	-	-	+	**Halotolerant**	*B. coagulans*
RP8	+	+	-	**Halotolerant**	*B. circulans*
RP9	+	+	+	**Moderately Halophilic**	*B. licheniformis*
RP10	+	-	+	Non-halophilic	*Paenibacillus polymyxa*
RP11	+	-	-	Non-halophilic	*B. amylo-liquifaciens*
RP12	-	+	+	**Halotolerant**	*B. circulans*
RP13	-	+	+	**Halotolerant**	*B. cereus*

3.2. Production of hydrolytic enzymes

Enzymes from halophilic sources are expected to have optimal activity under extreme conditions (de Lourdes Moreno et al. 2013). Our study showed that the 8 isolated halotolerant strains could produce more than three different hydrolytic enzymes, which is highly interesting. All isolates had a combination of hydrolytic activities under normal growing conditions (0.9% NaCl). It appears that almost all strains had the six hydrolytic activities tested (urease, amylase, cellulase, lipase, caseinase and lecithinase). At the same time, in the presence of salt (15% NaCl), the enzymatic activities decreased, in particular the production of urease, amylase and cellulase (Table 3). However, all strains are capable of producing at least two hydrolytic enzymes under saline conditions.

For this reason, the possibility of having a wide variety of halotolerant species producing extremozymes will provide great assistance for biotechnological applications, particularly in the agricultural field. Thus, it is very important to select enzyme-producing halotolerant-halophilic bacteria with optimal activity at different salt concentrations. However, our study showed that the enzymatic activity of our isolates decreased in the presence of 15% NaCl, which implies that salinity can pose an ecological challenge for production of certain extracellular enzymes. Indeed, some of the bacterial isolates were able to grow at 5% NaCl but failed to express hydrolytic activity at the same level. Moreover, it was recently shown that halotolerant bacteria of the genus *Bacillus* produce industrially important hydrolases and their enzymatic activities are more diverse. Enzyme-active bacilli were already isolated in saline soils (Zahran 1997) or salt marshes (Weisser & Truper 1985) with bacteria displaying significant enzymatic activity under saline stress (10% NaCl).

In this study, most of the *Bacillu*s isolates were able to produce hydrolysis enzymes. The genus *Bacillus* is well known as an enzyme producer. Many industrial processes use species belonging to this genus for the commercial production of enzymes. It is interesting to note that the combined hydrolytic activities detected in some strains could have applications for biotechnological purposes. The production of hydrolytic enzymes reflects a good adaptation of halophilic rhizobacteria to harsh environmental conditions and establish themselves in competition for the colonization of the plant's rhizosphere. For this reason, it is very important for these rhizobacteria to exhibit hydrolytic activity under saline conditions. Indeed, halophilic bacteria are a potential source of extracellular hydrolases like proteases, with a wide array of industrial applications (Shivanand & Mugeraya 2011).

3.3. Plant growth promoting traits

In terms of functional diversity, *Bacillus* strains were screened for various plant growth-promoting traits such as nitrogen fixation, HCN production and phosphate solubilization. The halotolerant *Bacillus* strains isolated in this study could for the most part (n=6/8) produce at least two PGP effects and the moderately halophilic one produced all three PGP effects studied, which are of interest in a saline environment. Most of the strains (n=10/13) had at least two plant growth-promoting activities (Table 3). Also, 11 strains (n=11/13) were able to fix nitrogen, 9 strains (n=9/13) showed phosphate solubilization activity and 7 strains (n=7/13) produced HCN.

Based on our results, halotolerant species of *Bacillus*-PGPR were identified as *B. circulans* (RP 1, RP 8 and RP 12), *B. coagulans* (RP 3), *B. amylo-liquifaciens* (RP 6), *B. licheniformis* (RP 9) and *B. cereus* (RP 13). Indeed, a large number of PGPR belonging to the genus *Bacillus* were isolated from the rhizospheres of various plants (Kashyap et al. 2019; Zafar-ul-Hye et al. 2019; Benaissa et al. 2018), including halophytes (Arora et al., 2020). Consequently, PGPRs are able to directly affect plants by promoting their development or indirectly affect them by impacting their responses to environmental constraints such as edaphic salinity (Han & Lee 2005).

3.4. Bacillus strains improves cowpea growth

A variety of biometric, physiological, and biochemical parameters were assessed for inoculated and non-inoculated plants (RP 0: control) cultivated in soil in order to explore the in vitro effects of isolates on two accessions of cowpea. Cowpea seedlings were observed on the 15th day following laboratory inoculation to determine which plants had the best growth.

Following inoculation, the isolates promoted plant development in both of the cowpea varieties studied. For instance, when compared to control plants, the two cowpea plants inoculated with RP 7 (*B. coagulans*), RP 8 (*B. circulans*), RP 10 (*Paenibacillus polymyxa*), and RP 12 (*B. circulans*) grew more effectively. Therefore, all of the aforementioned strains, aside from RP10 which is non-halophilic, were determined to be halotolerant. However, all strains studied demonstrated favorable impact on one or more physiological and biometric metrics (Table 4). As an illustration, neither strain had a favorable impact on the relative water content of the leaf in either accession.

Analysis of variance also indicated that each of the biometric and physiological parameters were highly significantly different from one strain to another for the two accessions studied (Figure 2).

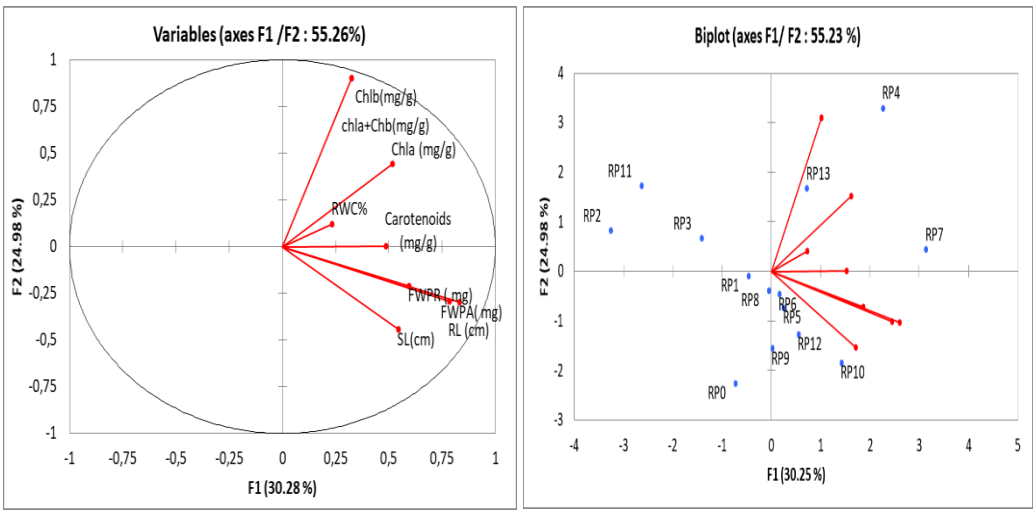
Table 4- Effect of *Bacillus* PGPRs inoculation on stem and root length, fresh weight of shoot and root, photosynthetic pigments and relative water content of cowpea plants. Standard Error (SE) shows very high significant differences (P<0.05)

Accessions	*Strains code*	*FWSP (mg)*	*FWRP (mg)*	*SL (cm)*	*RL (cm)*	*RWC%*
NEA10	RP0	209± 91cd	112.33± 28.5de	8.03±1.51abc	5.73±1.02ab	52.07±13.71ab
	RP1	327± 39b	**327± 15.94a**	8.13±1.24abc	5.23±0.64abc	53.58±18.80ab
	RP2	385.33± 29ab	257.66±15.13 b	9.36±2.53ab	**6.06±1.40abc**	50.81±15.65ab
	RP3	298± 0.57bc	124.33± 13.65cde	7.56±1.09abc	4.33±0.63abcd	48.38±10.32ab
	RP4	311± 79.26b	168.33±19.62 c	6.96±1.47bc	4.46±0.89bcd	46.98±8.21ab
	RP5	387± 1ab	174.66±28.29 c	8.1±1.47abc	4.46±0.89bcd	48.01±5.71ab
	RP6	**470± 36.59a**	131.66± 37.2cd	**10±1.04a**	3.46±0.45de	56.84±1.70ab
	RP7	**433± 3.77a**	122± 19.07cde	8.13±4.10abc	4.16±1.15cde	41.26±10.5b
	RP8	76.33± 80.99e	76.33± 17.32e	9.23±1.87ab	3.40±0.17de	51.56±12.39ab
	RP9	104.66± 67.03e	104.33± 5.85de	**10.03±0.90a**	3.2±0.26e	59.96±17.61ab
	RP10	84.33± 88.96E	84.33±4.61 E	7.63±0.41abc	3.33±0.37e	45.76±9.66ab
	RP11	107.66± 62.44e	107.66± 19.85de	8.30±1.58abc	3.96+1cde	41.35±5.27b
	RP12	123.33± 29.77de	123.33±29.36cde	5.81±0.07c	3.63±0,8de	41.35±5.27b
	RP13	84.33E± 20.55	84.33±16.25 E	7.43±1.56abc	3.5±0.6de	**67.84±27.86a**
NEA13	RP0	255±61ef	237.66±12.01bcd	10.60±3.5a	5.6±0.9bcd	84.41±5.40abc
	RP1	255±42.67ef	193.33±20.55cde	8.33±0.95abcd	4.6±0.79cde	80.76±6.61bcd
	RP2	122.33±63.7g	80±18.02f	4.3±1.47f	2.7±0.43ef	57.14±12.36d
	RP3	259.33±43	80±18.02F	4.8±0.15ef	4.83±0.15cd	87.60±3.97abc
	RP4	304±70 cdef	324.66±15.58ab	7.73±2abcde	5.66±1.4bcd	80.42±6.09bcd
	RP5	280±90.5def	**396±1a**	6.5±0.7cdef	6±0.5bc	89.04±2.72ab
	RP6	204±34.1fg	325±27.9ab	5.4±0.52def	6.96±1.61ab	66.11±7.37bcd
	RP7	**461.66±32.18a**	271.33±14.73bc	8.33±0.76abcd	**8.33±0.55a**	88.45±6.79ab
	RP8	306.66±40.41cdef	246±7.76bcd	7.33±1.15bcdef	5.4±±0.36bcd	**110.65±10.16a**
	RP9	394.33±5.85abc	241±12.50bcd	9.9±3.51ab	4.9±0.96cd	57.62±11.23d
	RP10	**423.66±4.61ab**	174.33±15.50de	8.8±2.60abc	5.9±2.68bc	62.31±8.79cd
	RP11	139.33±19.8g	137±9.29ef	4.8±2.08ef	2.53±0.45f	78.25±17.31bcd
	RP12	380.66±29.3abcd	**380±16.52a**	7.2±1.32bcdef	4.8±2.42cd	90±3.25ab
	RP13	344.33± 16bcde	181±29.53cde	8.43±2.25abcd	3.76±0.25def	89.17±1.92ab

Table 4(Continue)- Effect of *Bacillus* PGPRs inoculation on stem and root length, fresh weight of shoot and root, photosynthetic pigments and relative water content of cowpea plants. Standard Error (SE) shows very high significant differences (P <0.05)

Accessions	*Strains code*	*Chla (mg/g FPM)*	*Chlb (mg/g FPM)*	*Total Chl (mg/g FPM)*	*Carotenoids (mg/g FPM)*
NEA10	RP0	0.206±0.69b	0.244±0.28de	0.795±0.03e	0.200±0.06cde
	RP1	0.456±0.19ab	0.24±0.1de	0.687±0.18e	**0.456±0.15a**
	RP2	0.477±0.22ab	0.498±0.42cde	0.777±0.12e	0.150±0.11de
	RP3	0.456±0.13ab	0.095±0.13e	0.655±0.25e	0.184±0.01cde
	RP4	0.654±0.45a	**1.11±0.26a**	1.474±0.52abc	0.107±0.09de
	RP5	0.368±0.05ab	0.225±0.07de	0.775±0.04e	0.240±0.04bcd
	RP6	**0.7±0.18a**	0.430±0.55de	0.796±0.14e	0.328±0.09abc
	RP7	0.616±0.39ab	0.165±0,13de	0.868±0.26de	0.371±0.20ab
	RP8	0.301±0.07ab	0.381±0.10cde	1.224±0.13bcd	0.240±0.02bcd
	RP9	0.309±0.1ab	0.526±0.07cde	1.309±0.18bc	0.192±0.04cde
	RP10	0.529±0.19ab	0.668±0.21bc	1.599±0.3ab	0.192±0.01cde
	RP11	0.323±0.21ab	0.983±0.22ab	**1.758±0.05a**	0.061±0.04e
	RP12	0.498±0.14ab	0.423+0.01cde	1.262±0.09bc	0.253±0.02bcd
	RP13	0.563±0.05ab	0.550±0.10cd	1.200±0.34cd	0.121±0.13de
NEA13	RP0	0.206±0.05ab	0.264±0.07bc	0.264±0.07e	0.049±0.04b
	RP1	0.456±0.15ab	0.667±0.18bc	0.667±0.29cde	0.203±0.04b
	RP2	0.456±0.25ab	0.565±0.64bc	0.565±0.30cde	0.180±0.04b
	RP3	0.477±0.01ab	0.663bc	0.663±0.02cde	0.134±0.06b
	RP4	0.654±0.12a	**1.862±0.36a**	**1.862a**	0.330±0.04b
	RP5	0.368±0.03ab	0.570±0.07bc	0.570±0.15cde	0.143±0.34b
	RP6	**0.700±0.03a**	0.351±0.54bc	0.352±0.73e	0.228±0.04b
	RP7	0.616±0.01ab	1.094±0.03abc	1.094±0.61b	0.325±0.11&b
	RP8	0.301±0.25ab	0.66±0.03bc	0.666±0.14cde	0.157±0.09b
	RP9	0.309ab	0.528±1.01bc	0.528±0.12de	0,.133b
	RP10	0.529±0.56ab	0.213±1.01c	0.213±0.57e	**0.782±0.96b**
	RP11	0.323±0.06ab	1.014±0.03abc	1.014±0.21bcd	0.166±0.01b
	RP12	0.498±0.62ab	0.326±0.03bc	0.326±0.01e	0.156±0.23b
	RP13	0.563±0.18ab	**1.232±0.69ab**	**1.232±0.03b**	0.307± 0.12b

SL: stem length; **RL:** root length; **FWSP:** fresh weight of the shoots part; **FWRP:** fresh weight of the root part; **RWC:** relative water content; **Chl:** chlorophyll; **FPM**: fresh plant material



(a) **(b)**

Figure 2 - Principal Component Analysis (PCA) (a): Correlation circle between variables, (b): projection of PCA results on a factorial plane (F1 -F2) for the inoculation effect of PGPR-*Bacillus* strains on cowpea ecotype NEA10 growth. The first factor axis appears to oppose the strains with greatest positive effect on chlorophyll a, chlorophyll b, and chlorophyll a+b, compared to RP 4 strain. Strain RP 7 has the highest carotenoid content and appears to characterize the second factor axis. The individual projection on the factorial plane defined by axes 1 and 2 showed a fairly large distribution along the plane (Axis 1 accounted for 30.28% of the variation and was associated the characteristics on the positive side: RWC, chlorophyll a, chlorophyll b, Chlorophyll a+b and carotenoids and FWAP, FWRP, RL, SL which indicates positive correlation between the parameters of the plant, that is to say that they evolve together, with a more or less common effect).

Bacillus-PGPR strains had a positive effect on cowpea growth, which is reflected by a significant increase in biometric parameters of plant growth (Table 4). The fresh weights of shoots part (FWSP) and root part (FWRP) appear to be greater than the control, concerning strains RP 1 to RP 7 for ecotype NEA13 and strains RP 4 to RP 13 for ecotype NEA10. In the inoculated NEA10 accession (Appendix 1), fresh root weight almost tripled with the RP1 strain (327 µg) compared to the RP0 control (112 µg) and fresh weight of the aerial portion was more than double the RP0 control (209 µg) with the RP6 strain (470 µg). On the other hand, in the inoculated NEA13 accession (Appendix 2), the RP10 and RP7 strains had most positive effect on the fresh weight of the aerial part, which varied between 423 µg and 461 µg respectively, compared to the RP0 control (255 µg). Furthermore, in this same accession, the RP5 and RP12 strains were able to improve the root weight to 396 µg and 380 µg, respectively, compared to the RP0 control (237 µg).

Measurements of the lengths of the roots and stems were made both with and without inoculation. According to Table 4, the maximum stem size for inoculated seedlings was 10 cm, while the control stem sizes for NEA13 and NEA10 were 8 cm and 10 cm, respectively. Concerning the results obtained for the root length t, it is noted that the plants inoculated with most of the strains were smaller compared to control.

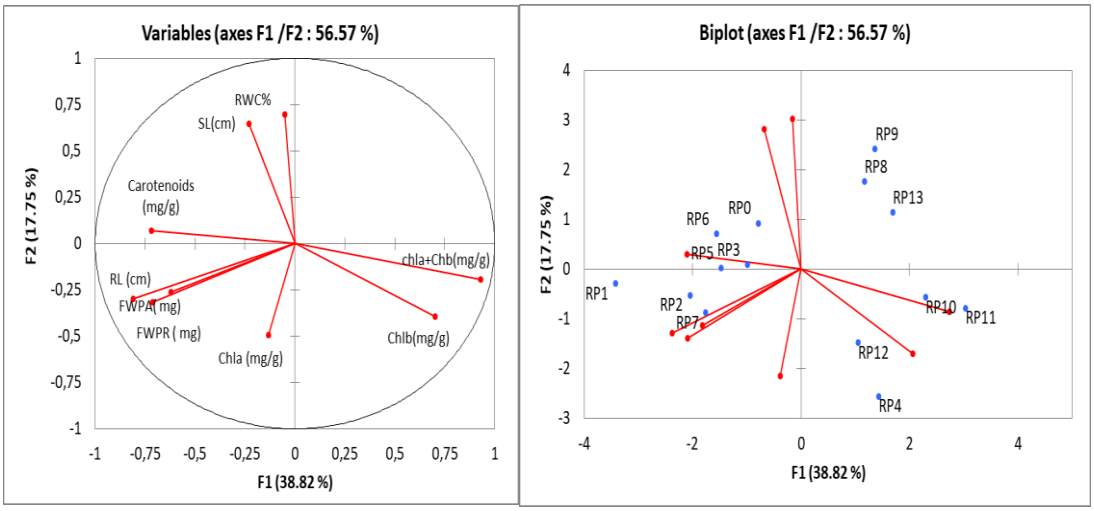
In most instances, it does not appear that inoculated plants had higher relative water content (Table 4), while the RP13 (67.84 %) and RP 8 (110.65%) strains in NEA10 and NEA13, respectively, are more interesting. The carotenoid content was higher in inoculated plants in most cases, more so with RP 5, 6, 7, 8 and RP 12 strains in NEA13 and all strains in NEA10. The total chlorophyll content was higher in plants that were inoculated with all strains, while higher values were found with strains RP11 (1.758 mg/g fresh plant material) and RP4 (1.862 mg/g fresh plant material) in accessions NEA10 and NEA13, respectively.

PCA was used to present most of the variance with a small number of principal components, represented in our case by the biometric and physiological parameters of the plant and by the strains of *Bacillus*-PGPR tested. Also, the correlation matrix (Appendix 3) shows the correlation coefficients between several variables related to plant parameters.

PCA analysis for NEA10 accession (Fig. 2) showed that Axis 1 had the following strains on the negative side: RP 6 and RP 1, RP 5, RP 2, RP 12, RP 9 and RP 10, which indicates that their effect is different on the plant. On the positive side of axis 1, the strains RP 4and RP 13 were present, this indicates that they have approximatively the same effect on the plant. Axis 2 was defined on the positive side by the strains RP 7 and RP 10. On the other hand, the negative part of the axis was defined by the strains RP 11, RP 3, and RP 2.

Concerning the effect of inoculation on NE14 accession, the correlation circle for the plane formed by the first two factorial axes is shown in Fig. 3. All the variables are well represented in this factorial plane since their correlations with the axes are relatively significant (the projections are close to the correlation circle). The first two axes alone of the PCA explain nearly 56.7%

of the variability; they were retained. This means that there is less common effect of inoculation on the plant parameters studied.



(a) **(b)**

Figure 3- Principal Component Analysis (PCA): a) Correlation circle between variables, b) projection of PCA results on a factorial plane (F1 -F2) for the inoculation effect of PGPR-*Bacillus* strains on cowpea accession NEA13 growth. The first factor axis appears to oppose strains with high stem length and water content and very low chlorophyll content, compared to RP 11 strains. The second factor axis is characterized by strains RP 11 and RP 1 with a high content of chlorophyll a, chlorophyll a +b, carotenoides and fresh weight (FWAP, FWRR), respectively. The projection of individuals on the factorial plane defined by axes 1 and 2 showed a fairly large distribution along the plane (Axis 1 isolated the following strains on the negative side: RP 12 and RP 4. On the positive side, the strains RP 13, RP 8, RP 9 and the control were isolated. Axis 2 is defined on the positive side by the RP 11 and RP 10 strains. On the other hand, the strains RP 7, RP 3, RP 1, RP 2, RP 5 and RP 6 defined the negative part of the axis).

Cowpea (*Vigna unguiculata*) is one of the most important food crops of the Fabaceae family in arid regions. The importance of this plant is linked to its seeds, which are edible and rich in protein (Aida et al. 2021). Therefore, the selection of microbial strains with beneficial activities to promote cowpea production is a great need, especially in arid regions. In our study, cowpea crop yield improvement by inoculation of *Bacillus*-PGPR strains was observed in pot experiments, which is in agreement with many studies of the species *B. megaterium*, *B. circulans, B. cereus*, *B. subtilis*, *B. amyloliquefaciens*, and *Paenibacillus polymyxa*. Thus, the use of *Bacillus* strains capable of maintaining and developing the root system has significant beneficial effects on inoculated plants, especially halophytes. In fact, PCA clearly demonstrated that the effect of inoculation was significant for all test parameters of the plant, with similar actions recorded between certain strains and specific parameters. The promotion of cowpea growth by *Bacillus*-PGPR led to a significant increase in morphological parameters such as stem size and weight and root, which were likely due to improved plant nutrition. The highest chlorophyll content was recorded with RP 4 (*B. megaterium*) and RP 11 (*B. amylo-liquifaciens)* treatment, which may be due to increased photosynthesis and nutrient intake. Some studies reported the use of PGPR-*Bacillus* as promoters of water use efficiency in *Vicia faba* (Li et al. 2016), maize and bean (Lima et al. 2019). The genus *Bacillus* is very widespread in the rhizosphere, many of them were listed as PGPR. Indeed, *Bacillus* species used as biofertilizers probably have a direct effect on plant nutrition, growth and health. In recent years, research about halophilic and halotolerant bacteria of the genus *Bacillus* has exploded in different ecological niches and several new species have been discovered, especially those with PGP effects on *Zea mays* (Mukhtar et al. 2020), *Chenopodium quinoa (*Mahdi et al. 2020) and *Triticum aestivum* (Hussain et al. 2020).

4. Conclusions

The isolation and characterization of halotolerant *Bacillus* strains has increased knowledge of rhizobacteria associated with the date palm in saline and arid soils. In general, 8 halotolerant and 1 moderately halophilic strain of the genus *Bacillus* were isolated from arid soil in the rhizosphere of the date palm. It would be interesting to study the biological properties of these microorganisms to understand how they adapt to salinity in the first place, as well as to exploit their potential applications in the second place. The results of PGP and enzymatic abilities are very interesting and show that most strains have at least two PGP effects and three hydrolytic activities even under saline conditions. Furthermore, the resistance to the physico-chemical parameters of the hyper-arid Algerian ecosystem of In Salah among the isolates is the first step to select effective PGPR capable of improving plant growth under extreme climatic conditions.

Therefore, our second investigation focused on the inoculation effects of the isolated halotolerant bacteria on cowpea plant growth. Based on the results of our study, strains RP 7 (*B. coagulans*), RP 8 (*B. circulans*), RP 10 (*Paenibacillus polymyxa*), RP 12 (*B. circulans*) and RP 13 (*B. cereus*) were identified to be highly effective strains in terms of improving cowpea plant growth

and development. Moreover, the isolated bacteria are likely to offer new opportunities for biotechnological applications in agro-ecological systems, especially in arid areas known for their saline soils. However, further research should be conducted in field trials in several arid and semi-arid locations and on several crops to provide clear evidence of their usefulness. In future studies, it would be interesting to perform molecular analysis and study the effect of our *Bacillus*-PGPR strains as bioinoculants for plants grown in saline soil.

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