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## Biopriming of durum wheat seeds with newly halotolerant PGPB bacterial isolates for improving their potential of plant growth under stressful conditions

*Adel Hadj Brahim<sup>a\*</sup>, Asmahen Akremi<sup>a</sup>, Mouna Jlidi<sup>a</sup>, Manel Ben Ali<sup>a,b</sup>, Brabra Wided<sup>a</sup>,  
Naser Aliye Feto<sup>c</sup>, Mamdouh Ben Ali<sup>a,b\*</sup>*

*\*E-mail addresses:*

*<sup>a</sup> Laboratory of Microbial Biotechnology, Enzymatic, and Biomolecules (LMBEB), Centre of Biotechnology of Sfax (CBS), University of Sfax, P.O. Box 1177, Sfax 3018, Tunisia*

*<sup>b</sup> Astrum Biotech, Business incubator, Center of Biotechnology of Sfax (CBS), University of Sfax, Road of Sidi Mansour km 6, PO Box 1177 Sfax 3018, Tunisia.*

*<sup>c</sup> OMICS Research Group, Department of Biotechnology, Vaal University of Technology, Johannesburg-Vanderbijlpark, South Africa*

**Abstract.**

Worldwide salinity is one of the most severe abiotic stresses limiting crop growth and productivity. Further salt-affected area in Tunisia is fast escalating due to intrusion of saline water on arable land and use of chemicals compounds. To overcome these agricultural problems, the aim of this study is the selection of bacterial strains from saline soil sites for their capacity of promoting growth plants under stressful conditions after a seed biopriming approach.

Differents biochemical parameters of plant growth were analyzed such as bacterial growth under 100 and 150 mM, phosphate solubilization, ACC metabolic pathway, AIA secretion, siderophores, HCN, antimicrobials compounds,...

Four strains within fifty were selected for their high ability to produce many growth factors under stressful conditions (150mM) and promote seed germination of Tunisian var. durum wheat. Moreover, seed biopriming with strain MA13 had the highest ability of seed germination after 2 days of incubation at room temperature under obscurity light.

**Keywords:** seed biopriming; halotolerant bacteria, durum wheat, salinity.

## 1. Introduction

Salinization is recognized as the main threats to environmental resources and human health in many countries, affecting almost 1 billion ha worldwide/globally representing about 7% of earth's continental extent. Plants face various biotic and abiotic stresses in adverse environmental conditions. Abiotic stress indeed is a complex process, which informs cells to adapt themselves at the molecular, biochemical, and physiological levels [1].

Salt stressed soils are known to suppress the growth of plants [2]. Plants in their natural environment are colonized both by endocellular and intracellular microorganisms [3]. For the enhancement of sustainable agricultural production under stressful conditions, use of bacterial inoculants or PGPB (Plant Growth Promoting Bacteria) is becoming a more widely accepted practice in intensive agriculture in many parts of the world. Rhizosphere microorganisms, particularly beneficial bacteria and fungi, can improve plant performance under stress environments and, consequently, enhance yield both directly and indirectly [4]. Some plant growth-promoting rhizobacteria (PGPR) may exert a direct stimulation on plant growth and development by providing plants with fixed nitrogen, phytohormones, iron that has been sequestered by bacterial siderophores, and soluble phosphate [5, 6].

Besides developing mechanisms for stress tolerance, microorganisms can also impart some degree of tolerance to plants towards abiotic stresses like drought, chilling injury, salinity, metal toxicity and high temperature. In the last decade, bacteria belonging to different genera including *Rhizobium*, *Bacillus*, *Pseudomonas*, *Pantoea*, *Paenibacillus*, *Burkholderia*, *Achromobacter*, *Azospirillum*, *Microbacterium*, *Methylobacterium*, *Variovorax*, *Enterobacter* etc. have been reported to provide tolerance to host plants under different abiotic stress environments [7, 8]. Use of these microorganisms per se can alleviate stresses in agriculture thus opening a new and emerging application of microorganisms [9]. Microbial elicited stress tolerance in plants may be due to a variety of mechanisms proposed from time to time based on studies done. Therefore, the present study aims to (i) isolation of bacteria from rhizosphere of halophyte grown in saline sodic soils, (ii) identification of

plant growth promoting (PGP) attributes, and (iii) analyze the seed germination ability through seed coating with potent PGPB.

## **2. Materials and Methods**

### **2.1. Materials**

Roots of *salicornia brachiata* and soil samples were collected from sabkha site in Tunisia (34°46'16''N10°48'24''E). Many laboratories materials were used such as rotary spectrophotometer, checker, Marie bath, Bacterial temperature Incubator, Erlenmeyer flasks,...

### **2.2. Methods**

#### **2.2.1. Sampling and isolation of diazotrophs bacteria**

Ten grams of soil collected from a native site of sabkha in Tunisia (34°46'16'' N10°48'24'' E) were added into 90 mL of sterile saline (NaCl, 0.85%) in Erlenmeyer flasks, which were shaken for 30 min. Then, a 10-fold serial dilution was prepared, and 0.1 mL aliquots were spread in Petri plates in duplicate over the Burk's N-free medium. The plates were kept at 30 °C for 7 days.

The macerat of washed roots were inoculated in a culture flask containing Burk's N-free medium and incubated for 7days at 30°C. Then, a 6-fold serial dilution was prepared, and 0.1 mL aliquots were spread in Petri plates in duplicate over the Burk's N-free medium. The plates were incubated for 7 days at 30°C and morphologically different colonies appearing on the medium were isolated and subcultured for further analysis.

#### **Diazotrophic potential of the isolates**

Bacterial isolates were examined for their nitrogen-fixation (diazotrophic) potential first by testing their growth on the liquid and solid mineral nitrogen free medium [10] ( medium removing NaNO<sub>3</sub>) with oil as a sole source of carbon and energy. Isolates were then tested for nitrogenase, using the method of Quantification of N fixing capacity.

#### **Quantification of N fixing capacity**

The isolates which showed positive and predominant growth during the first 3 to 4 days were selected for quantification using the Kjeldahl N digestion and distillation system [11] (Kelplus system, Classic

Dx[VA]). The selected isolates were incubated in 10 ml of Jensen's broth in a rotary shaker at 150 rpm for 5 days at 28° C. The amount of N fixed in the microbial tissues contained in the broth was determined by the method described by [12]. From the quantification result, one potent N fixing isolates having the highest N fixing ability was selected for further characterization.

### **Effect of physiological conditions on the growth of potent N fixing Bacteria**

Effect of various growth conditions such as temperature, salt tolerance and pH on the growth of the most potent N fixers were checked in nutrient broth. For studying the effect of temperature, potent bacteria were incubated at temperatures viz., 25°C, 30°C, 35°C, and 40°C for 24 h at 150 rpm. Nutrient broth supplemented with different concentrations of NaCl (ranging from 50-200mM) was used for salt tolerance studies and the hydrogen ion concentration in the range of 5-8 was selected for pH studies. The flasks were incubated at 30°C for 24 h in a rotary shaker at 150 rpm. The growth and activity of the potent N fixing bacteria in the given growth conditions were observed by taking the optical density of the medium.

### **Antifungal assay**

The agar well diffusion method as adopted earlier [13] was used with minor modification. The bacterial isolates tested for their antifungal activity were fully grown in LB medium.

Wells of 8mm diameter of test fungus were punched into in the Potato dextrose agar (PDA) slants and filled with 200 ml ( $2 \times 10^7$  CFU/ml) of bacterial culture. Potato dextrose broth was taken as negative control. The plates were incubated for 5–6 days at 28°C. The antifungal activity was evaluated by measuring the growth inhibition zone against test fungi.

### **Screening of phosphate solubilizing Bacteria**

Modified Pikovskaya agar plates were prepared and test isolates were streaked on plates, then the plates were the incubated at 37°C and observed for 2-7 days [14]. The strains forming zone of clearance were maintained by streaking on nutrient agar slants and stored at 4°C.

### **IAA production**

Indole acetic acid (IAA) production was detected as described by Patten et al, (2002) [15]. Bacterial cultures were grown for 7 days in halophilic medium containing supplement of 20g NaCl at 37°C. Fully grown cultures were centrifuged at 3,000 rpm for 30 min. The supernatant (2 mL) was mixed with 2 drops of orthophosphoric acid and 4 mL of Salkowski reagent (50 mL, 35% of perchloric acid and 1 mL 0.5 M FeCl<sub>3</sub> solutions). Development of pink color indicated IAA production.

### **Siderophore production**

Production of siderophore was detected by standard method [16] using chrome azurol S (CAS) as indicator. The isolates were spot inoculated at the center of the plate and incubated for 7 days. The change in the colour of the medium around the bacterial spot was an indication of siderophore production.

### **Hydrogen cyanide (HCN) production**

HCN production was determined by color change of filter paper [17]. Loopfull of bacterial suspension was inoculated on nutrient agar medium (Merck, Germany) containing 4.4 g L<sup>-1</sup> glycine. Filter papers were soaked in a reagent solution (sodium carbonate 2% and picric acid 0.5%) and placed in the upper lid of Petri dishes. To prevent volatilization, the plates were sealed with parafilm and incubated at 37°C for 7 days. One plate without inoculation of bacterium was considered as control. If HCN was produced, yellow filter papers changed to cream, light brown, dark brown and eventually turn into reddish-brown.

### **Production of Ammonia**

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 7 days at 37°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production [18].

### **Exopolysaccharide production**

The qualitative determination of exopolysaccharide production was performed according to Paulo et al. (2012) [19]. Each strain was inoculated onto 5-mm diameter paper discs disposed in a medium (2%

yeast extract; 1.5% K<sub>2</sub>HPO<sub>4</sub>; 0.02% MgSO<sub>4</sub>; 0.0015% MnSO<sub>4</sub>; 0.0015% FeSO<sub>4</sub>; 0.003% CaCl<sub>2</sub>; 0.0015% NaCl; 1.5% agar) modified by the addition of 10% of saccharose, pH value of 7.5. The production was characterized by the size of the halo produced and its slime appearance.

#### **Preparation of inoculum and seed coating:**

Seeds of wheat variety “aouija” were obtained from Agriculture Research Station in Tunisia. Bacterial strains were grown overnight in LB broth at 28±2°C with constant shaking. Cells were harvested by centrifugation and re-suspended in normal saline to get an optimum growth (OD 10<sup>8</sup> cells per mL at λ600). Seeds were constantly shaken along-with the bacterial suspension with continuous addition of the sterile carrier material until the seeds become coated with a thin film of bacterial suspension and carrier material. Coated seeds were air-dried before sowing.

#### **Seed germination test**

After seed coating with potent PGPB strains, uniform seeds were sowed in pots and germination of seeds was observed after 5<sup>th</sup> day.

### **3. Results and Discussion**

To date, soil salinity becomes a huge obstacle for food production worldwide since salt stress is one of the major factors limiting agricultural productivity. To embark upon this harsh situation, numerous strategies such as plant breeding, plant genetic engineering, and a large variety of agricultural practices including the applications of plant growth-promoting rhizobacteria (PGPR) and seed biopriming technique have been developed to improve plant defense system against salt stress, resulting in higher crop yields to meet human’s increasing food demand in the future [20]. Biopriming of durum wheat seeds with the potent PGPB is the main approach studied for improving the germination rate in this mini-scientific research. Four bacteria were selected from 22 halotolerant strains isolated on minimal medium devoid of any nitrogen source. These bacteria were subjected to an ecophysiological study *in vitro* in order to determine their resistance and ability to survive under different physiological stressors such as tolerance to osmotic pressure (salt), temperature and growth pH.

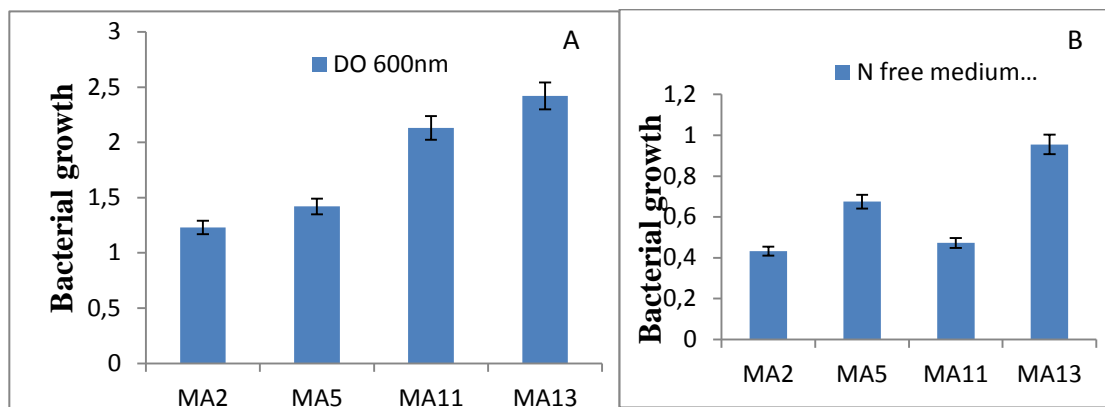
The growth study shows that these bacteria have the ability to survive to different degrees under all used salt concentrations and the same for growth at different temperatures and pH (Table 1). The MA11 bacterium shows a good ability to grow under all the conditions tested. While MA2 is resistant to osmotic pressure, sensitive to temperature greater than 30°C and a pH less than or greater than 7. All bacteria have an identical growth optimum of 100 mM, 30°C, and 7 respectively for salt concentration, temperature and pH except for MA13 of 150mM (Table 1). The ameliorative functions of PGPR consist of three aspects, namely, the ability to protect themselves against hyperosmotic conditions and abnormal NaCl concentrations, the capacity to aid plant tolerate better to elevated salinity, and to improve soil quality [21]. Regarding the alleviating roles of PGPR in promoting plant salinity tolerance, PGPR exhibit beneficial traits in mitigating the toxic effects of high salt concentrations on morphological, physiological, and biochemical processes in plants, resulting in the significant rescue of yield loss.

**Table 1:** Bacterial growth of selected isolates under different temperature, pH, and salt concentrations.

Isolate code	Salt tolerance (mM)				Temperature tolerance(°C)				pH tolerance			
	50	100	150	200	25	30	35	40	5	6	7	8
MA2	++	+++	++	++	+	+++	+	+	+	+	+++	+
MA5	+	+++	++	+	++	+++	+	+	+	++	+++	+
MA11	++	++++	+++	++	+	++++	+++	++	+	++	++++	++
MA13	+	+++	++++	++	+	+++	+++	++	+	+++	++++	+

The bacterial growth of the isolates was put under their optimum salt at 30°C and pH 7 under two media: one rich LB and the other poor lacking a nitrogen source to enhance the growth capacity of the strains to support stressful and non-stressful conditions. Growth on LB as well on N-free medium shows that MA13 exhibits the best growth ability. While MA5 shows good growth on N-free medium than MA11 unlike their growth on LB medium (Fig. 1 A; B).

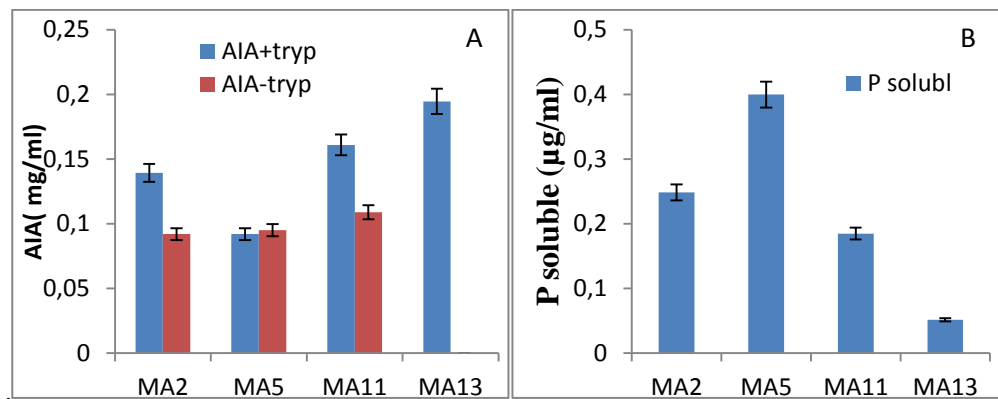




**Figure 1:** Bacterial growth in LB and N free medium after 24h under optimum osmotic pressure (mM NaCl) at 30°C and pH 7.

Furthermore, PGPR can also mitigate salt stress symptoms by producing  $\text{Na}^+$  binding exopolysaccharides (EPS), improving ion homeostasis, decreasing ethylene levels through enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and synthesizing phytohormones [22-24]. The most reported mechanism predominantly used to explain the positive PGPB effects on plant growth is their ability to produce auxin. The production of indol-acetic acid of the strains was studied on a medium with and without tryptophan (Fig.2). All strains tested produced IAA at concentrations ranging from 0.092 to 0.194 mg / ml. Strain MA13 is a strain dependent on tryptophan to produce growth hormone IAA, whereas the strains produce IAA even in the absence of tryptophan. We note that these three strains produce IAA at the same non-variable concentration. And the production of IAA in the presence of tryptophan is 0.139; 0.092 and 0.161 respectively for MA2, MA5, and MA11 (Fig. 2). Auxins function in geotropism and phototropism, vascular tissue differentiation, apical dominance, root initiation (lateral and adventitious), cell division, stem and root elongation [25]. Bacteria that solubilize phosphorus are referred to as phosphate solubilizing bacteria [26]. They supply phosphate in a more acceptable way to the plants and are not deleterious to the environment. They convert insoluble organic and inorganic phosphate to a form which can be readily accessible to plants. Environmental conditions, plant and soil conditions, and bacterial strains all affect the actions of

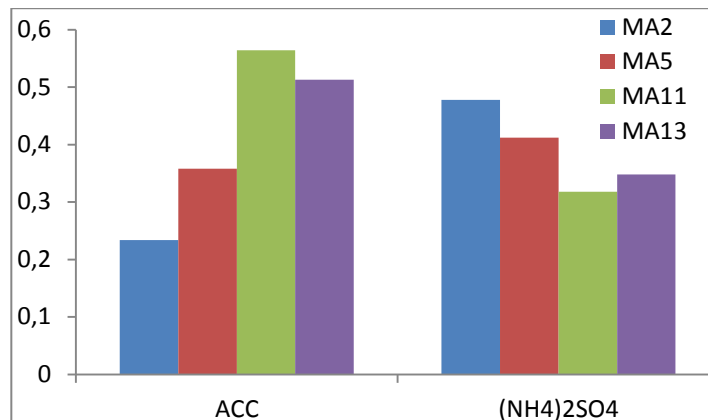
phosphate solubilizers [27, 28]. The principal mechanism of inorganic phosphate solubilization is the use of mineral-dissolving compounds like hydroxyl ions, organic acids, protons, siderophores, and carbon dioxide (CO<sub>2</sub>) [29]. The phosphate solubilization pathway is significantly activated in MA5 by the solubilization of 0.4 µg/ml, whereas this pathway has minimal activity in MA13 by the solubilization of 0.0515 µg/ml. While MA2 and MA11 solubilize 0.2485 and 0.18475 µg/ml respectively (Fig.3).



**Figure 2:** (A) IAA production, (B) P-solubilization of selected strains in the presence of 2% NaCl. Error bars show the standard deviation of the mean values of three replicates ( $P < 0.05$ ).

Ethylene synthesis in a particular plant is affected by the presence and concentration of other plant hormones, temperature, gravity, light, nutrition, and the presence of various degrees of biotic/abiotic stress which the plant may be subjected [30]. Its production more than its threshold level by the action of ACC oxidase enzyme in plant tissues causes “stress ethylene” which affects the root and shoot development in plants. Colonization of “stress ethylene” plant rhizosphere by ACC deaminase producing PGPB help to alleviate this situation and restores normal plant development. In order to test the ability of strain to assimilate 1-aminocyclopropane-1-carboxylate (ACC), precursor of ethylene responsible of senescence and stress hormone in plants, bacterial growth is measured in the presence of ACC and another source of inorganic nitrogen ( $(\text{NH}_4)_2\text{SO}_4$ ) in the minimum medium. Our results show that the growth of MA2, using both sources, is minimal for ACC and maximal for  $(\text{NH}_4)_2\text{SO}_4$  and contradictory in MA11 whose activity is maximal for ACC and minimal for  $(\text{NH}_4)_2\text{SO}_4$ . Whereas in MA5 the use of  $(\text{NH}_4)_2\text{SO}_4$  is better than ACC and MA13 assimilates ACC more than  $(\text{NH}_4)_2\text{SO}_4$ . The strain which able to assimilate

In this regard, bacteria that express ACC deaminase, by lowering plant ACC levels (and subsequently plant ethylene levels) can decrease the detrimental effect on plants from different stresses [31]. The ACC is being converted by ACC deaminase in the PGPB to  $\alpha$ -Ketobutyrate and ammonia



**Figure 3:** ACC deaminase activity expressed in bacterial growth of selected bacteria on N-free medium (NFMM) supplemented with ACC (1-aminocyclopropane-1- carboxylate) and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as nitrogen source.

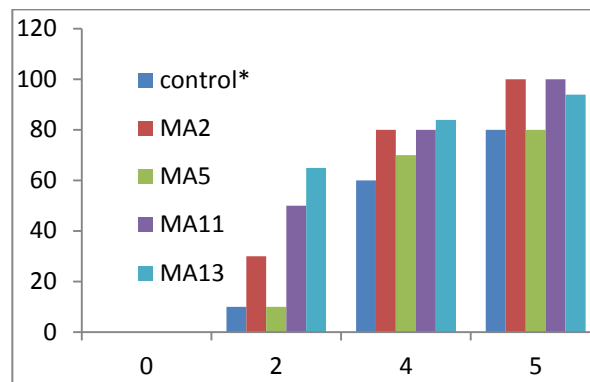
On average, various plant diseases reduce plant yields by around 10%/year in more developed countries and by about 20% /year in less developed countries of the world (<http://www.fao.org/home/en/>). In an effort to decrease the widespread use of chemicals as a means of preventing phytopathogen damage to plants, scientists have been developing the use of certain environmentally friendly PGPB as biocontrol agents. The antifungal activity of all strains was checked against *fusarium solani*, *fusarium oxysporum*, *fusarium graminearum* and *rhizoctonia solani* using PDA medium (Table2). The antifungal activity of the tested strains varied according to PGPB and phytopathogenic fungal strain. MA11 and MA13 were the most effective against all fungal strains. It should be noted that only with MA11 and MA13, this activity was efficient against *fusarium oxysporum*. Moreover, no antifungal activity was noticed with MA5 for *fusarium solani* and *fusarium oxysporum* and with MA2 for *fusarium oxysporum* and *rhizoctonia solani*. However, with MA11 and MA13, the antifungal activity was highly effective against *rhizoctonia solani* and *fusarium graminearum* respectively, but less effective against *fusarium solani* and *fusarium oxysporum*. PGPB as

biocontrol agents with different pathways: antibiotics production, cell wall degrading enzymes, competition, hydrogen cyanide, induced systemic resistance (ISR), quorum quenching, and bacteriophages.

**Table 2:** Bioprotection ability of PGPB strains against wheat fungal wilt caused by *Fusarium* species.

Nb.	Strain	<i>Fusarium oxysporum</i>	<i>Fusarium solani</i>	<i>Rhizoctonia solani</i>	<i>Fusarium graminearum</i>
1	MA2	-	+	-	++
2	MA5	-	-	+	+
5	MA11	+	+	+++	++
6	MA13	+	+	++	+++

According to the FAO (<http://faostat3.fao.org>), after sugarcane the next three first crops in terms of production (million tons) in the world are the cereal maize (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticum aestivum L. subseq. durum*). Wheat represents a major renewable resource for food, feed, and industrial raw material and it is the most widely grown worldwide crop. For this, it is interesting to develop new techniques, such as seed biopriming, for improving and protecting crops against limiting factors. Seed biopriming proved the germination rate of wheat seeds after five days of incubation at 25°C. Compared with a control treated with salt without biopriming, germination is more favored after seed treatment with the strains. After 5 days, the germination of the seeds is improved in MA2 and MA11 by 100%. While, the germination rate after seed biopriming with MA5 and MA13 is 80 and 94% respectively. Hadj Brahim et al (2019) [32] were reported that biopriming durum wheat seeds with halotolerant and diazotrophic bacteria enhanced germination rate of seeds under stressful and unstressful conditions.



**Figure 4:** Effect of seed biopriming with PGPB strains on germination rate of durum wheat under salt stress conditions. The seeds were incubated in a suspension of  $10^8$  bacteria on 150 mM NaCl at room temperature for 30 min.

#### 4. Conclusion

*In vivo* studies showed that MA2, MA5, MA11 and MA13 strains are PGPBs seen that all these tested strains can promote wheat germination through some important biochemical traits. All these strains are able to grow under different growth parameters, to produce auxin with different levels, to solubilize phosphoric matter, to assimilate ACC, and to secrete antimicrobial compounds against phytopathogenic species. The germination test after seed biopriming demonstrated that, after five days of incubation, MA2 and MA11 can germinate all tested seeds than other tested strains. It is interesting to check the potential of these strains to contribute in plant production in the arid and semi-arid regions, especially the salt affected sites, besides its potential role in improving healthy plants production under biotic and abiotic stresses.

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