MOL2NET, International Conference Series on Multidisciplinary Sciences
MODEC-05: Nat. Prod. & Agro-Indust. Proc. in Amazon, UEA, Puyo, Ecuador, 2020

Seed biopriming of durum wheat with purified and combined bacterial culture improved seed germination under salt stress

Adel Hadj Brahim[a], Lobna Daoud[a] , Manel Ben Ali[a,b] , Asmahen Akremi[a], Mouna Jlidi[a] , Houda Hmani[a] ,Naser Aliye Feto[c], Mamdouh Ben Ali[a,b]*

**E-mail addresses*: *adelhadjibrahim@gmail.com*

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

[b] *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.*

[c] *OMICS Research Group, Department of Biotechnology, Vaal University of Technology, Johannesburg-Vanderbijlpark, South Africa*

Abstract:

Background and aim: Improved crop productivity under stressful conditions is a major asset of global agriculture. Salinity is one of the most severe abiotic stresses limiting crop yield. Further, a salt-affected area in Tunisia is fast escalating due to intrusion of saline water on arable land and the use of chemical fertilizers and pesticides. Moreover, climate change scenarios showed the increased risk of salinization at different latitudes. Therefore, a great effort is required for maintaining crop production under limiting factors. The present study was conducted to isolate and identify PGPB associated with the halophyte *Salicornia brachiata* from the coastal saline sites and evaluation their bacterization effect of durum wheat seeds with "Biopriming" technology.

Methods: The selection parameters of PGPB strains were based on the ability to promote the growth of plants under stressful conditions. On the other hand, the effect of selected isolates on germination of durum wheat (*Triticum durum*) was assessed *in vivo* conditions. The treatment with bacteria was applied on purified and combined strain under 25 and 125 mM NaCl.

Results: A total of 22 isolates were selected *in-vitro* for studying their plant growth-promoting (PGPB) ability including, tolerance with salt concentration, ACC deaminase activity, N_2 fixation, phosphorus solubilization, and indole-3-acetic acid (IAA), out of which, three strains (MA9, MA32, and SA62) were selected. Our data revealed that experiments using treated with NaCl and bioprimed seed permitted us to identify the most efficient isolates in a combined culture which offered the best rate of germination and the highly vegetable growth of explants (roots and shoots). In fact, the benefic effect of seed biopriming was more pronounced in samples added with NaCl than that of untreated samples.

Conclusion: Seed biopriming with efficient PGPB strains induced salinity tolerance of wheat and therefore enhanced their rate of germination and growth of explants under salinity.

Keywords: Co-inoculation; PGPB; halotolerant bacteria; seed biopriming; durum wheat; salinity.

1. Introduction

To date, soil salinity becomes a huge obstacle for food production worldwide since salt stress is one of the major factors limiting agricultural productivity. It is estimated that a significant loss of crops (20–50%) would be due to drought and salinity. Salinity not only hampers crop productivity, but also threatens the sustainability of agro-ecosystems worldwide. The osmotic stress caused by high salinity (100–200 mM) is originated from the reduction in solute potential of soil solution [1]. Further, a salt-affected area in Tunisia is fast escalating due to intrusion of saline water on arable land and the use of chemical fertilizers and pesticides [2]. Moreover, climate change scenarios showed the increased risk of salinization at different latitudes. Therefore, a great effort is required for maintaining crop production under limiting factors.

Due to the rising severity of salinity on global food production, numerous strategies have been offered to cope with the increasing challenging soil conditions. Along with plant breeding [3], plant genetic engineering [4], and genetic transformation [5], agricultural practices have dramatically contributed to the improvement of plant tolerance to salinity stress. Among these agricultural approaches, the bacterization of plant crops with PGPB and the implementation of these useful rhizobacteria in seed biopriming have demonstrated their beneficial properties in enhancing plant growth and development, and in augmenting plant salt stress tolerance through different mechanisms [6-9].

Therefore, the present study was conducted to isolate and identify PGPB associated with the halophyte *salicornia brachiata* from the coastal saline sites and evaluation their bacterization effect on germination rate and explants growth of durum wheat seeds with “Biopriming” approach.

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, shecker, 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 macerate 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.

2.2.2. 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 NaNO3) 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.

2.2.3. 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 50-400mM) 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 corresponded temperature 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.

2.2.3. Plant growth parameters

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 [11]. 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) [12]. 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 $FeCl_3$solution). Development of pink color indicated IAA production.

Siderophore production

Production of siderophore was detected by standard method Schwyn and Neiland (1987) [13] 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 color 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 [14]. Loopful of bacterial suspension was inoculated on nutrient agar medium (Merck, Germany) containing 4.4 g L-1 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 [15].

Exopolysaccharide production

The qualitative determination of exopolysaccharide production was performed according to Paulo et al. (2012) [16]. Each strain was inoculated onto 5-mm diameter paper discs disposed in a medium (2% yeast extract; 1.5% K_2HPO_4; 0.02% $MgSO_4$; 0.0015% $MnSO_4$; 0.0015% $FeSO_4$; 0.003% $CaCl_2$; 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.

Antifungal assay

The agar well diffusion method as adopted earlier by Mehmood et al., (1999) [17] 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. 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.

2.2.4. Preparation of inoculum and seed coating:

Seeds of wheat variety "aouija" were obtained from Agricultural Research Institute INGREF Tunis-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 10^8 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.

2.2.5. Seed germination test

After seed coating with potent PGPB strains, uniform and en seeds were sowed in pots and germination of seeds was observed after 3th day. The seed germination was observed

3. Results and Discussion

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	150	200	400	25	30	35	40	5	6	7	8
MA9	+	++	+	+	++	+++	+	+	+	++	+++	+
MA32	++	++++	+++	++	++	+++	++	++	++	+++	++++	++

Accordingly, these strains (MA9, and MA32) were found to be able to metabolize sucrose, mannitol, glucose, starch, citrate, and nitrate with API 20E (data not shown). The isolates were also evaluated for their tolerance to different concentration of salt and growth at different temperature and pH values. We found that almost all bacterial strains were able to grow under all of the NaCl concentrations tested (50 to 400 mM) (Table 1), with an optimal growth at 30^{o}C temperature and a pH of 6.5-7 and 150 mM of NaCl for respectively, MA9, and MA32.

Table 2: Plant growth-promoting properties of selected strains isolated from coastal saline soil site in Tunisia.

Isolate code	PGP properties						Mycelial growth inhibition			
	HCN	Ammonium	Siderophores	Diazotrophic potentiel	ACC deaminase	EPS	*Rhizoctonia solani*	*Fusarium solani*	*Fusarium oxysporum*	*Fusarium graminarum*
MA9	-	+++	+	+	+++	++++	+++	+	-	+
MA32	-	++++	+++	+++	+++++	++++	+++	++++	++++	++++

In addition, the strains were positive for phosphate solubilisation, production of antimicrobial compounds, production of auxin and siderophores, as well as for important plant growth promoting (PGP) properties including dinitrogen fixation ability (Table 2), and hydrolytic potential of 1-aminocyclopropane-1-carboxylate (ACC deaminase) (Table 2; Fig. 1).

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 (Table 2). The antifungal activity of the tested strains varied according to PGPB and phytopathogenic fungal strain whose MA32 as the most effective against all fungal strains. It should be noted that only with MA32 this activity was efficient against *fusarium oxysporum*. Moreover, no antifungal activity was noticed with MA9 for *fusarium oxysporum*, whereas the strain MA9 had the same activity of MA32 under *rhizoctonia solani*. PGPB as biocontrol agents with differents pathways: antibiotics production, cell wall degrading enzymes, competition, hydrogen cyanide, induced systemic resistance (ISR), quorum quenching, and bacteriophages.

The biodegradation of ACC, precursor of ethylene which had a negative impact on bacterial growth under stress and therefore, limits the plant growth and productivity, was assessed with another laboratory assay for better understand the bacterial biological pathways of ACC. The results shown in Fig. 2 affirmed that the strain MA32 was a more activated biological pathway of ACC assimilation than MA9

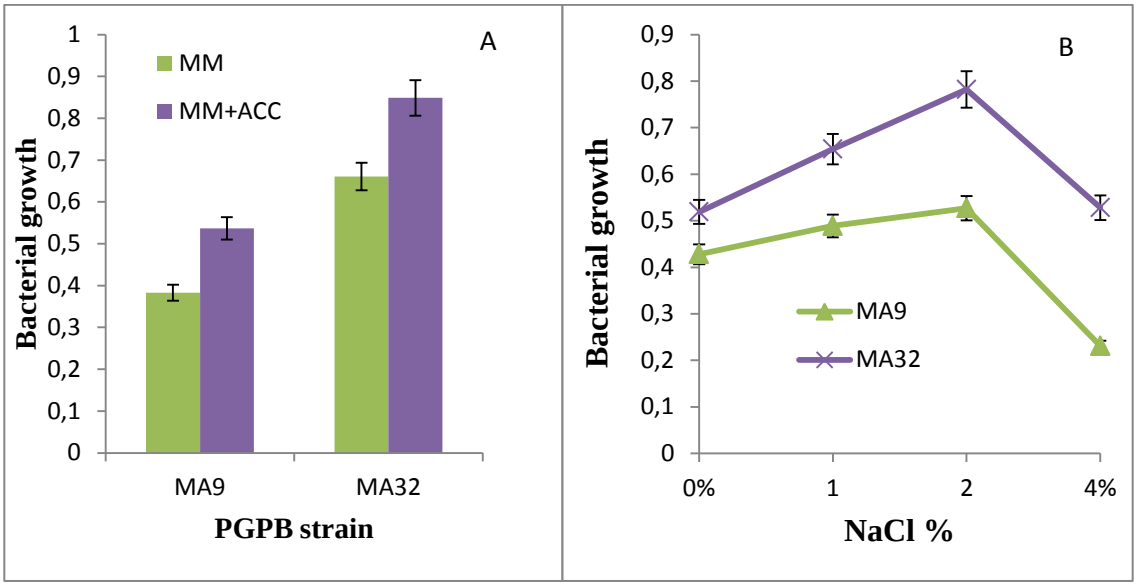


Figure 1: Bacterial growth in N free medium (A) and LB (B) under optimum of osmotic presure (mM NaCl) à 30°C et pH 7.

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 (Fig 1B) as well on N-free medium (Fig. 1A) shows that MA32 exhibits the best growth ability. Therefore, MA32 had higher growth in a ACC medium than MA9.

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 [18]. 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 ($(NH_4)_2SO_4$) in the minimum medium. Our results show that the growth of MA9, using both sources, is minimal for ACC and maximal for $(NH_4)_2SO_4$ and contradictory in MA32 whose activity is maximal for ACC and minimal for $(NH_4)_2SO_4$ (Fig.2). Whereas, the strain, which able to assimilate ACC, have active pathway of secretion of hydrolytic enzyme of ACC named ACC deaminase. This biological pathway is active in other tested strain MA9, but not in the same rate of secretion.

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 [19]. The ACC is being converted by ACC deaminase in the PGPB to α-Ketobutyrate and ammonia.

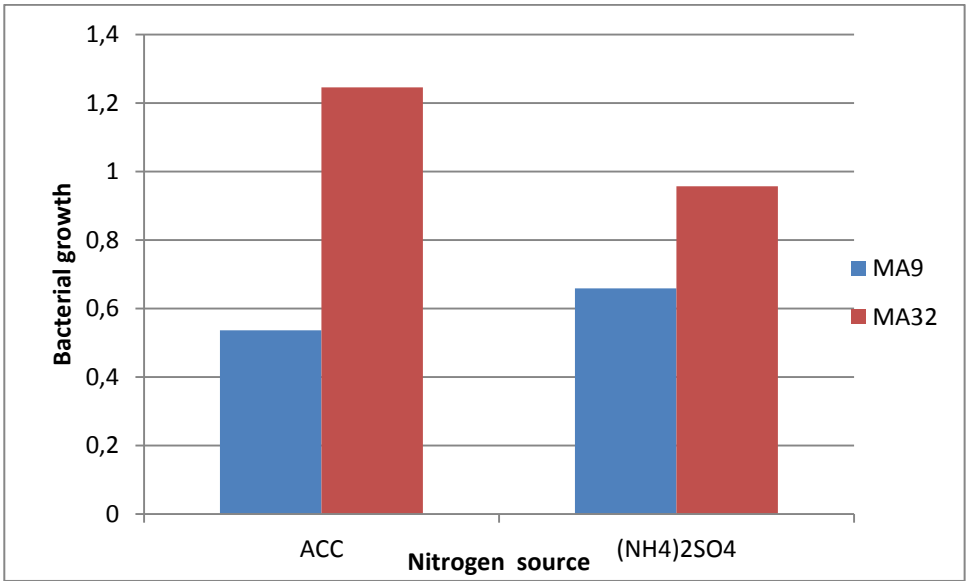


Figure 2: ACC deaminase activity expressed in bacterial growth of selected bacteria on N-free medium (NFMM) supplemented with ACC (1-aminocyclopropane-1- carboxylate) and $(NH_4)_2SO_4$ as nitrogen source.

The tested strains were found to produce variable amounts of auxins (Fig. 3A) ranging from 82 to 209 µg/ml in a free-tryptophan medium. The two strains MA9 and MA32 produced relatively more auxin with tryptophan at 202 and 376 µg/ml, respectively.

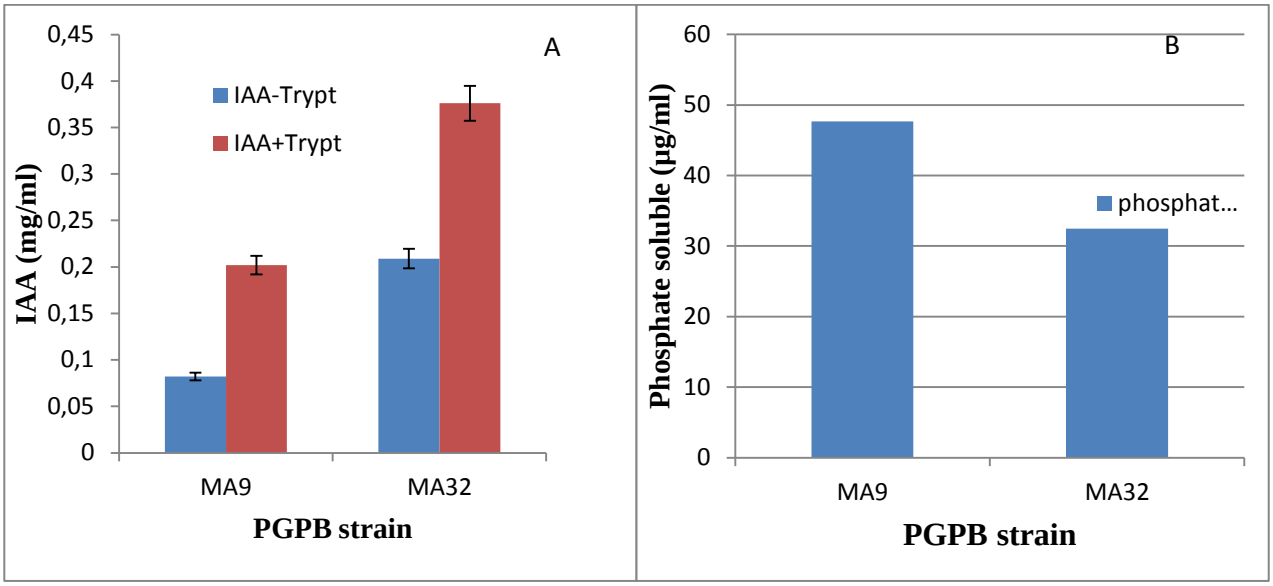
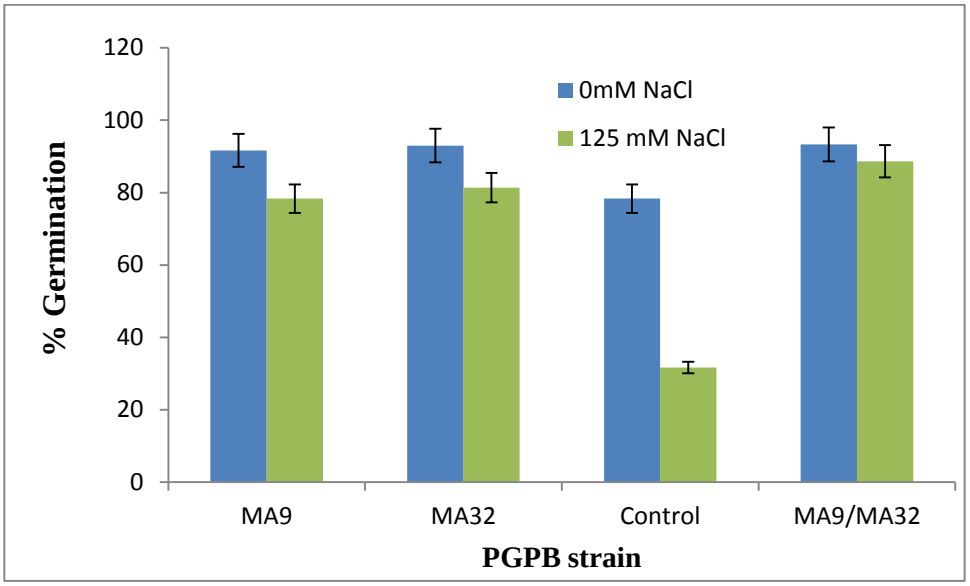


Figure 3: (A) IAA production, (B) P-solubilization of selected strains in the presence of 2% NaCl. Error bars show the standard deviation of the mean values of three replicates (P<0.05).

The idea of eliminating the use of all chemicals inputs in agriculture which are sometimes environmentally unsafe is slowly becoming a reality because of the emergence of microorganisms that can serve the same purpose or even do better [20]. Depletion of soil nutrients through leaching into the waterways and causing contamination are some of the negative effects of these chemical inputs that prompted the need for suitable alternatives. This brings us to the idea of using microbes that can be developed for use as biological inputs (biofertilizers; biostimulators; bioinductors of defense system;…) [20]. They are environmentally friendly as they are natural living organisms. They increase crop yield and production and, in addition, in developing countries, they are less expensive compared to chemical fertilizers. These biofertilizers are typically called plant growth-promoting bacteria (PGPB).



Wheat (*Triticum aestivum L. subseq. durum*) 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 obscurity incubation at 25°C. Compared with unbioprimed seeds treated with salt, the germination rate is more pronounced in untreated and unbioprimed seeds. Therefore, germination is more favored after seed treatment with the strains. Under control conditions, after five days, the germination is improved in bioprimed seeds with MA9 and MA32 by 91 and 93% respectively. While, under treated seeds with 125 mM salt, the germination rate is 78 and 81% respectively seed biopriming with MA9 and MA32.

As it was mentioned that the two strains exhibit all the biochemical characteristics of plant growth promotion under 2% NaCl, it is interesting to exploit the combined effect of these two complementary strains to better have a detailed idea on the formulation of efficient bio-inoculants in agriculture. This combined effect is tested to germinate durum wheat seeds under stress to ascertain the effect of stress on growth and on the germination rate of seeds.

Our results reveal that the biopriming of the seeds with a combination of two relevant strains, markedly improves the germination rate that it was 93 and 88% respectively with MA9 and MA32 in untreated and treated seeds.

4. Conclusions

The strains MA9 and MA32 were found to have PGPB characteristics as they produced indole-3-acetic acid, siderophores, and lytic enzymes, fixed free atmospheric nitrogen, and solubilized inorganic phosphate, *in vitro*. Yet, biopriming with combined bacterial formulation MA9/MA32 offered the

highest germination promotion and salinity tolerance. Hence, it would be worth to test this combination strains under field conditions as a step towards its commercial production. Moreover, these strains could be further assessed for its potential role in bioprotection and growth promotion of other crop plants.

5. References

1. Ha-Tran, D.M., et al., *Roles of Plant Growth-Promoting Rhizobacteria (PGPR) in Stimulating Salinity Stress Defense in Plants: A Review.* International Journal of Molecular Sciences, 2021. **22**(6): p. 3154.
2. Bai, Y.-C., et al., *Soil chemical and microbiological properties are changed by long-term chemical fertilizers that limit ecosystem functioning.* Microorganisms, 2020. **8**(5): p. 694.
3. Fita, A., et al., *Breeding and domesticating crops adapted to drought and salinity: a new paradigm for increasing food production.* Frontiers in Plant Science, 2015. **6**: p. 978.
4. Wang, W., B. Vinocur, and A. Altman, *Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance.* Planta, 2003. **218**(1): p. 1-14.
5. Jha, S., *Transgenic approaches for enhancement of salinity stress tolerance in plants*, in *Molecular Approaches in Plant Biology and Environmental Challenges*. 2019, Springer. p. 265-322.
6. Moeinzadeh, A., et al., *Biopriming of Sunflower ('Helianthus annuus' L.) Seed with'Pseudomonas fluorescens' for Improvement of Seed Invigoration and Seedling Growth.* Australian Journal of Crop Science, 2010. **4**(7): p. 564.
7. Ansari, T., et al., *Biopriming of wheat seeds with rhizobacteria containing ACC-deaminase and phosphorate solubilizing activities increases wheat growth and yield under phosphorus deficiency.* Pakistan Journal of Agriculture, Agricultural Engineering and Veterinary Sciences, 2015. **31**(1): p. 24-32.
8. Meena, S.K., et al., *Effect of nitrogen levels and seed bio-priming on root infection, growth and yield attributes of wheat in varied soil type.* Biocatalysis and Agricultural Biotechnology, 2017. **12**: p. 172-178.
9. Brahim, A.H., et al., *Seed-Biopriming of Durum Wheat with Diazotrophic Plant Growth Promoting Bacteria (PGPB) Enhanced Tolerance to Fusarium Head Blight (FHB) and Salinity Stress.* 2019.
10. Sorkhoh, N., et al., *Crude oil and hydrocarbon-degrading strains of Rhodococcus rhodochrous isolated from soil and marine environments in Kuwait.* Environmental Pollution, 1990. **65**(1): p. 1-17.
11. Mehta, S. and C.S. Nautiyal, *An efficient method for qualitative screening of phosphate-solubilizing bacteria.* Current microbiology, 2001. **43**(1): p. 51-56.
12. Patten, C.L. and B.R. Glick, *Role of Pseudomonas putida indoleacetic acid in development of the host plant root system.* Appl. Environ. Microbiol., 2002. **68**(8): p. 3795-3801.
13. Schwyn, B. and J. Neilands, *Universal chemical assay for the detection and determination of siderophores.* Analytical biochemistry, 1987. **160**(1): p. 47-56.
14. Alström, S. and R.G. Burns, *Cyanide production by rhizobacteria as a possible mechanism of plant growth inhibition.* Biology and Fertility of Soils, 1989. **7**(3): p. 232-238.
15. Cappuccino, J. and N. Sherman, *Biochemical activities of microorganisms.* Microbiology, A Laboratory Manual. The Benjamin/Cummings Publishing Co. California, USA, 1992: p. 188-247.
16. Paulo, E.M., et al., *An alternative method for screening lactic acid bacteria for the production of exopolysaccharides with rapid confirmation.* Food Science and Technology, 2012. **32**(4): p. 710-714.

17. Mehmood, Z., et al., *Indian medicinal plants: a potential source for anticandidal drugs.* Pharmaceutical Biology, 1999. **37**(3): p. 237-242.
18. Gamalero, E. and B.R. Glick, *Bacterial modulation of plant ethylene levels.* Plant Physiology, 2015. **169**(1): p. 13-22.
19. Glick, B.R., *Bacteria with ACC deaminase can promote plant growth and help to feed the world.* Microbiological research, 2014. **169**(1): p. 30-39.
20. Olanrewaju, O.S., B.R. Glick, and O.O. Babalola, *Mechanisms of action of plant growth promoting bacteria.* World Journal of Microbiology and Biotechnology, 2017. **33**(11): p. 1-16.