Seed-Biopriming of Durum Wheat with Diazotrophic Plant Growth Promoting Bacteria (PGPB) Enhanced Tolerance to Fusarium Head Blight (FHB) and Salinity Stress

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Abstract

Background There is growing interest in the use of bioinoculants based on plant growth promoting bacteria (PGPB) to promote plant growth under biotic and abiotic stresses. To our knowledge much work has not been, thus far, done on seedbiopriming of durum wheat for tolerance to biotic and abiotic stresses. In the present work, we report detailed account of the effectiveness a potent bacterial strain with proven plant growth-promoting ability and antimicrobial activity. The isolate was selected following screening of several bacterial strains isolated from halophytes that grow in a coastal saline soil in Tunisia for their role in enhancing durum wheat tolerance to both salinity stress and head blight disease. **Results** Accordingly, *Bacillus* strains MA9, MA14, MA17 and MA19 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. The in vivo study that involved in planta inoculation assays under control (25 mM NaCl) and stress (125 mM NaCl) conditions indicated that all PGPB strains significantly ($P < 0.05$) increased the total plant length, dry weight, root area, seed weight, nitrogen, protein and total mineral content. On the other hand, strain MA17 reduced *Fusarium* Head Blight (FHB) disease incidence in wheat explants by 64.5%, showing that the strain has antifungal activity as was also displayed by in vitro inhibition study. **Conclusions** Both in vitro and in vivo studies showed that MA9, MA14 MA9, MA14, MA17 and MA19 strains were able to play the PGPB role. Yet, biopriming with *Bacillus* strain MA17 offered the highest bioprotection against FHB, plant growth promotion, and salinity tolerance. Hence, the MA17 strain should further be evaluated under field condition and formulated for commercial production. Besides, the strain could further be evaluated for its potential role in bioprotection and growth promotion of other crop plants. We believe, the strain has potential to significantly contribute to wheat production in the arid and semi-arid region, especially the salt affected Middle Eastern Region, besides its potential role in improving wheat production under biotic and abiotic stresses in other parts of the world.

Background

Biotic and abiotic stresses have been reported to severely limit crop growth and yield [1]. Salinity is one of the most important abiotic stress factors affecting crop production. Several studies reported that climate change could potentially increase risk of salinization at different latitudes. In Tunisia, salt-affected areas are fast escalating (Fig. S1[2]) due to intensive exploitation by drainage and irrigation [2], intrusion of saline water into arable lands and the use of chemical fertilizers and pesticides.

To reduce the toxic effects of salt (NaCl) stress on plants, different tools have been developed such as plant genetic engineering [3], osmopriming with different chemical factors such as aerated solution of ascorbate, salicylic acid, kinetin and CaCl_2 [4] and the use of plant growth-promoting bacteria (PGPB) [5].

Plant Growth Promoting Bacteria (PGPB) are known to stimulate plant growth via different mechanisms [6]. These PGPB strains can be applied using different methods to improve crop productivity. The first one as direct inoculation of pure [7] or mixed [8] PGPB strains or co-application of PGPB and chemicals like N

fertilizers [9] and the second as seed biopriming before the germination phase [10]. The latter one is a method used to enhance seed germination either under optimal or stress conditions [11]. Interestingly, the positive impact of priming was found to be more pronounced under stress than normal conditions [12], a finding that yet needs further investigation to understand the underlying mechanism. Relatively, seed biopriming is considered as a simple, low cost and low risk technique as compared to other techniques that render glycophytes grow under salt-stress conditions [13-15]. This technique was successfully used in many crops including hot pepper [16, 17], lettuce [18], maize [19], okra [20], tomato [21], pea [22], pepper [23], milk thistle [24] and soybean [25]. The application of PGPB strains via seed biopriming through soaking grains for a specified period of time in a bacterial suspension initiated the physiological processes related to seed germination, while radicle and plumule do not emerge [26]. Seed biopriming reported to improve tolerance of crop plants to salt-stress. To the effect different PGPB strains have so far been used, while strains belonging to *Bacillus* species have largely been used. For instance, *Bacillus* species have successfully been used as priming agents in crops like potato [27], radish [28], rice, mung bean and chickpea [29].

The halophytes are able to grow under salt stress through different physiological and biochemical mechanisms besides their interactions with the associated PGPB. However, there are no published works on the mechanisms behind the effect of the endophytic or rhizospheric bacteria associated to the halophytes in natural saline conditions. Hence, in the current study we report (1) isolation and identification of different strains with PGPB traits, (2) evaluation of the potential bioprotection effect of the strains against *Fusarium* Head Blight in durum wheat, and (3) study of role of PGPB on promoting plant growth under salt stress condition.

Results

Biochemical characterization of PGPB strains

Accordingly, our strains (MA9, MA14, MA17 and MA19) were found to be able to metabolise sucrose, mannitol, glucose, starch, citrate, and nitrate. The isolates were also evaluated for their tolerance to different concentration of salt and growth at different temperature and pH. As a result it was observed that almost all of them were able to grow under NaCl concentrations ranging from 0 to 7%, with optimal growth at of 30 °C and pH 6.5 (Fig. S2).

In addition, the strains were positive for β -glucanase, protease, chitinase as well as for important plant growth promoting (PGP) properties including production of siderophores (Fig. 1), antimicrobial compounds and hydrolytic potential of 1-aminocyclopropane-1-carboxylate (ACC deaminase +) (Table 1). The majority of the tested strains were found to produce variable amounts of auxins (Fig. 2) ranging from 35 to 376 $\mu\text{g/mL}$ except for MA17 and MA19, which produced relatively less auxin with and without tryptophan at 56/109 and 35/125 $\mu\text{g/mL}$, respectively. Particularly, MA14 was found to produce indole acetic acid (IAA) more than the rest of tested strains and even higher yield than values reported in literature [30]. The isolates also showed phosphate-solubilizing activity as evidenced by the formation of

clear zones on NBRIP agar plates. In the liquid NBRIP medium, bacterial strains solubilized variable amounts of phosphates ranging from 78 to 476 µg/ml (Fig. 2).

Antifungal activity for the different isolates was evidenced by the formation of inhibition zones between the bacterial isolates and the fungal isolates (Table 1). Among which, MA17 showed the highest antifungal activities against *F. oxysporum* (Table 1). All of the tested diazotrophic bacteria, except MA14, showed antagonistic effects to both *R. solani*, and *F. graminearum*.

Molecular profiling of the PGPB strains

The analysis of the 16S rRNA gene-based sequences for the different PGPB strains was done using BLASTn of the sequences against the NCBI's database. We, accordingly, found that the sequences of isolates MA9 and MA17 showed 99% similarity with *Bacillus pumilus* Bp02 (DQ910855.2) and *Bacillus pumilus* ML270 (KC692158.1), respectively. While isolate MA19 showed 98% similarity and MA14 showed 84% similarity with *Bacillus tequilensis* BPR061 (KU161291.1) and *Virgibacillus* sp. SKW19 (KU132375.1), respectively.

Diazotrophic potential of the isolates

The results in Table 2 confirms the diazotrophic activity of the PGPB strains. Most of the tested strains were able to grow in the mineral medium without any nitrogen source as 1-Aminocyclopropane-1-carboxylate (ACC) and glucose as sole carbon source (Table 2; Fig. S2). The MA14 and MA17 strains were the most efficient ones in terms of biofilm formation after growing in a NFMM medium without nitrogen source, but MA14 exhibited the lowest capacity of acetylene reduction. Both strains (MA14 and MA17) were also found to produce ethylene after 72 h of incubation (data not shown) based on the acetylene reduction test and the CPG analysis of gases. These strains were shown to have nitrogenase activities ranging from 0.452 to 3.125 µmol C₂H₄ mg protein/h based on the acetylene reduction assay (ARA). In addition, MA17 isolate showed maximum ARA and Kjeldhal levels of nitrogen but this strain had the least potential of growth on an N-free medium. The ARA test was verified by measuring N₂ by the Kjeldhal method of bacterial culture in NFMM medium. Thus, the results imply that the strains had functional nitrogenase activity. The growth test revealed that the three strains MA9, MA14 and MA17 grew very well in the ACC medium, while, MA19 grew equally on NFMM and NFMM+ACC media (Fig. S2).

Evaluation of mechanisms regulating plant homeostasis

ACC deaminase activity assays *in vitro* in a chemically defined medium were carried out to determine whether different strains might play a role in regulating plant stress homeostasis.

In fact, bacterial culture in NFMM medium with 5 µg/mL of 1-aminocyclopropane-1-carboxylate (ACC) suggested active metabolic pathways of ACC degradation for the PGPB strains (MA9, MA14, MA17 and MA19) (Fig. 3). As known, the ACC metabolic pathway promotes plant growth under stress conditions by reducing the ethylene concentration in plant cells. In this context, numerous PGPB strains that are able to produce AcdS were found to promote plant growth under various stress factors [31-33].

Biocontrol effect of PGPB strains against *Fusarium graminearum* head blight in wheat

Biocontrol experiments were conducted to evaluate the bioprotection effect of two selected PGPB strains (MA17 and MA14) against *Fusarium graminearum* in wheat. The data obtained revealed that head blight severity was reduced in samples inoculated by the two strains compared to the untreated control. It is worth mentioning that strain MA14 did not exhibit an *in vitro* antifungal activity against *F. graminearum* despite the bioprotection it rendered to the plant, which means indirect mechanisms might have been involved (induced systemic resistance, ISR) or niche exclusion in its *in planta* activity. MA17 strain provided a more efficient bioprotection against the disease in the soil without fertilizer compared to MA14 with the disease index of 36 and 76%, respectively. Moreover, MA17 was found to render the plant growth promotion as well as bioprotection against fusarium head blight (Table 4, 5). The biocontrol efficiency was also reflected by enhanced morphological parameters of the explant such as root length, shoot length, total length, total dry weight, total survival plants after treatment, and disease index. Thus, seed biopriming with both MA14 and MA17 provided a significant resistance to head blight in wheat explants, nevertheless, MA17 was more efficient than MA14 in terms of disease reduction efficiency (Fig.S3 - S8).

Effect of seed bacterization by PGPB on wheat growth under salt stress

PGPB seed bacterization enhanced germination rate

Tolerance level of selected PGPB strains to different salt concentrations was measured after seven days of culture. The results indicated that both MA17 and MA19 strains were halotolerant as they showed greater tolerance to salt stress compared to both MA9 and MA14.

The sensitivity of durum wheat cv. *Aouija* was assessed at different NaCl concentrations in order to determine the effective salt concentration on limiting wheat plant growth to be used in the entire experiments. Accordingly, plant growth was affected at 50 mM NaCl and severely retarded at 120 mM NaCl. Thus, the later concentration (120 mM NaCl) was chosen for the further assays.

Regarding germination assays, we found that all PGPB strains enhanced wheat seed germination under salt stress compared to the negative control (non-bacterized but at 120 mM NaCl) (Fig. 4). The MA9,

MA14, MA17 and MA19 significantly improved the germination rate by 47, 50, 57 and 40% under 120 mM NaCl treatment, respectively. Besides, most of PGPB strains significantly improved the germination rate of wheat seeds under normal conditions (positive control or no salt stress) (Fig. 4).

Biomass promotion of wheat plants by PGPB seed biopriming

The treatment of durum wheat seeds with halotolerant strains significantly improved root length under stress (125 mM NaCl) as well as under normal growth conditions except for MA19 strain, which reduced root length by 71% in the absence of stress. The growth promotion under stressful condition was 40, 131 and 70% for MA9 MA14 and MA17, respectively. While under normal growth condition the growth promotion was 48, 459, 27 and 70% for MA9, MA14, MA17, and MA19, respectively. However, the plant biomass was markedly increased after seed biopriming with MA17 and MA19 strains. The latter showed a significant plant development under 25 and 120 mM, as opposed to biopriming by MA9 and MA14 whose biopriming improved the length of the roots. However, it had no remarkable effect on the plant biomass which was reduced by 10% with MA9 under normal and by 7% with MA14 under stress conditions. The development of root length can be explained by an aggressive nutrient deficiency of the plant after inoculation by these two strains which have an inefficient biological activity spectrum under stress. The highest growth promotion effect on under stress was observed in MA14 strain (459%) in the length of roots and MA17 in the vegetative mass (98.94%) (Table 4) (Fig. S9).

Therefore, biopriming of seed with diazotrophic strains (MA9, MA14, MA17 and MA19) increased the dry weight of durum wheat var "*Aouija*". This induction effect was more pronounced under salinity conditions than under control conditions (Table 5) (Fig. S10). In fact, the percentage of improvement under (stress and non-stress), for MA9, MA14, MA17 and MA19 was (145, 51), (162, 82), (637, 200) and (166,114), respectively. The highest plant growth promotion percentage was observed in MA17 bioprimed-seeds under stress with a plant dry weight and 100-seed mass of 63.7 and 77%, respectively (Fig. S12).

In addition, seed bacterization with MA14, MA17 and MA19 strains induced an increase in root length by 48, 45.9, 27, and 70%, respectively, and in shoot length by 15, 7, 99 and 39%, respectively as compared to controls (untreated seeds) subjected to NaCl. However, treatment with MA19 decreased root length by 71% and enhanced shoot length by 48% in comparison with the controls (untreated seeds). In contrast, the strain MA14, displayed no significant impact on wheat plant growth parameters under non-saline conditions. Moreover, seed biopriming with PGPB strains, except MA9, offered greater growth promotion in comparison to control plants, whereas under natural and saline treatments, they showed a promotion in shoot length and total dry weight parameters (Table 5, 6). In fact, plant dry weight was increased at 25 and 120 mM NaCl for all the PGPB strains. However, at 120 mM NaCl, except of MA14, inoculated plants showed significant increase in root length. At 25 mM NaCl, the root length of inoculated plants with MA17 was markedly increased compared to uninoculated and other PGPB-inoculated plants (Fig. S9 - S13). These results indicated and the usefulness of MA19 to enhance wheat plant growth under salt stress.

Effect of PGPB treatments on nitrogen (N) and protein contents in wheat seeds

The N and protein contents in wheat seeds in response to PGPB isolates under both stress and control conditions are shown in Fig. 5. Both N and protein contents in wheat seeds were significantly ($P < 0.05$) affected in response to stress treatments. In fact, the total nitrogen content in untreated seeds was 1.06 and 1.71 g.100g⁻¹, while, it ranged from 1.796 to 1.93 g.100g⁻¹ and from 1.799 to 1.85 g.100g⁻¹ for bioprime-plants under stress and natural conditions, respectively. Among the different amendments, seed bioprime with the different isolates before germination provided an enhancement of these parameters compared to both untreated seeds under stress and natural conditions. The highest total N content of 1.93 g.100g⁻¹ was recorded in plants bioprime with MA17 under stressful conditions (125 mM NaCl) followed by those with MA19, MA14 and MA9 with 1.826, 1.807 and 1.796 g.100g⁻¹, respectively. The comparative upsurge in total nitrogen content in plants bioprime with MA9, MA19, MA14 and MA17 compared to the negative control (salinized soil) was 69, 72, 70 and 82%, respectively, and 4.6, 8.2, 7 and 8.2%, respectively compared to the control (soil without NaCl). A such increase in N content by PGPB relied on by their capacity to colonize the soil colonization and to grow stress environments. Nitrogenase activity was recorded in all PGPB treatments (Table2), indicating the interplay between nitrogen and protein contents in plant and N₂-fixation (Fig. 5). Application of different PGPBs under stressful condition significantly ($P < 0.05$) increased plant protein contents in the range of 10.25 and 11.07 g.100 g⁻¹ literally doubling the content as compared to the non-treated control. The highest protein content of 11.07 g.100 g⁻¹ was recorded for MA17 under salt stress followed by that for MA17 under normal conditions (10.61 g.100 g⁻¹). In the controls (without bioprime and without NaCl), the total protein content was 10.02 g.100 g⁻¹. Moreover, there was a relative increase in protein content in plants treated with PGPB strains MA9, MA14, MA17 and MA19 compared to the control by 2.29, 3.4, 10.47 and 3.6 %, respectively.

Seed bioprime enhanced the total mineral content

The total mineral content in positive control plants (no PGPB, no NaCl) decreased by 72 % compared to those subjected to NaCl treatment. In addition, a reduction by 40% in dry weight was noted in negative control plants (with NaCl, no PGPB) when compared to controls (Fig. 6). The plants inoculated with selected PGPB showed an increase in their total mineral content either in the presence or absence of NaCl treatment. The plants inoculated with MA9 showed an increase in their mineral content by 14.6 to 441 %, in salted and unsalted soils compared to negative controls (Table 5). While plants inoculated with MA17 (*B. pumilus*) showed 24 and 56% higher total mineral contents compared to those of the control plants under primary and secondary salinity, respectively. The total mineral content in MA14-treated plant was greater than those measured in MA9-treated ones.

Discussion

The study was conducted to isolate bacterial strains from saline soil and screening for potential PGPB traits *in vitro* with the eventual aim of employing the strains for durum wheat seed-bioprimering to promote tolerance to salt stress. To our knowledge, the present study is the first in evaluating halotolerant diazotrophic PGPB from saline soil. *Bacillus* spp. are known for their ability to grow under various biotic and abiotic stresses. Thus, it is considered in numerous plant studies as the dominant isolate [34], and is therefore recognized as a aerobic or facultative anaerobic [35].

Soil application requires large amounts of inoculants, which is economically demanding to the commercial farms. Solid inoculants are easy to apply, while liquid ones need careful handling during the transportation and after field inoculation. In terms of mechanisms of action of the PGPBs, it was reported that PGPB might release lytic enzymes, siderophores [36] and/or antibiotics [37] leading to pathogens growth restriction. In our work, we found that the strain MA17 was the most efficient in terms of both plant growth promotion under stress conditions and bioprotection against head blight resistance. This was accompanied by the production of diverse biologically active compounds by the strain including siderophores, lytic enzymes, growth hormones and a stable ACC deaminase activity. It is known that plant growth promoting rhizobacteria (PGPR) with 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity has the potential to promote plant growth and development under adverse environmental conditions [33]. Several approaches were described to promote plant growth under stressful conditions. Among which, the treatment of the seeds (priming) either by physical or chemical agents [4] during storage or just before the sowing. Interestingly, appropriate conditions have been standardized for an efficient colonization of the seed by the bacterial inoculum during the seed priming process [38] to allow multiplication of PGPB in the seed as well as in the spermosphere even before sowing [39]. Thereafter, PGPB can invade the inner seed tissue, and trigger important biological activities in the seed. In this manner, germination might occur rapidly and plant growth can be promoted [40]. In our study, we found that most of the tested PGPB had different PGPB traits, of which MA17 is the only one that exhibited all of such traits.

Moreover, most of the PGPB used offered significant ($P < 0.05$) improvement of plant growth ability under salinity stress as demonstrated by observed agro-morphological traits of the bioprimered wheat plants. Our results are in accordance with previous report describing the ability of different PGPB strains, like those belonging to the *Bacillus* genus, to provide abiotic-stress tolerance in various plant species including potato [27], radish [28] rice, mung bean and chickpea [29].

In addition, it was reported that associating bio-osmopriming with bacterial coating greatly improved uniformity of the germination and plant growth traits [41]. Also, combining *Azotobacter chroococcum*, with *Azospirillum lipoferum* and 80 kg ha⁻¹ urea and 60 kg ha⁻¹ P₂O₅ as seed priming significantly improved barley yield parameters such as grain weight, dry matter accumulation, biological yield, grain yield and harvest index [42]. Moreover, priming with *Azotobacter* and *Azospirillum* strains promoted growth of wheat plants as revealed by the increase in total length of root, vegetative parts, dry mass of

plants and grains, root surface and nitrogen, proteins and mineral content. On the same trend, biopriming of safflower with *Pseudomonas* strain 186 increased grain number per head, heads per plant, the number of branches, diameter of head, grains per plant, grain yield of the plants and 100 grain weight [43].

On the other hand, our data of biopriming in durum wheat revealed an increase in N and other essential elements, which are sufficiently transferred to plants by the tested diazotrophic PGPB strains. Clearly, such an increase of these elements in plant organs is related to N₂ fixation[44] and P solubilization capacity of the selected PGPB strains via their ability to solubilize insoluble phosphates[45], allowing plant uptake via several processes such as acidification, chelation, and ion-exchanger actions [46]. The promotion in plant growth and N/protein levels in bioprimed-plants clearly reflects the ability of the bacterial isolates to offer a higher nutrient flux to the plant resulting in a higher plant biomass. In a related study, it was demonstrated that the N-content in wheat shoot increased by 1.7 – 2.43 % following pretreatment with PGPB strains as compared to untreated control [47].

Moreover, the increase in total mineral content in plant tissues might be due to efficient biochemical activity of the selected PGPB (MA17, and MA19) strains. Some additional important traits detected in our tested PGPB strains were production of hydrolytic enzymes, (β -glucanase, protease, amylase), EPS and ACC deaminase activity, which are known to stimulate plant growth under stress conditions directly. On the other hand, it was reported that PGPB can indirectly improve plant growth under stress conditions through production of siderophores, antimicrobial compounds and chitinase known for their role in bioprotecting host plants from pathogens [48, 49].

Conclusion

Both *in vitro* and *in vivo* studies showed that MA9, MA14, MA17 and MA19 strains were able to play significant PGPB role. Yet, biopriming with *Bacillus* strain MA17 offered the highest bioprotection against Fusarium Head Blight (FHB), plant growth promotion, and salinity tolerance. Hence, the MA17 strain should further be evaluated under field condition and formulated for commercial production. Besides, the strain could further be evaluated for its potential role in bioprotection and growth promotion of other crop plants. We believe, the strain has a potential to significantly contribute to wheat production in the arid and semi-arid region, especially the salt affected Middle Eastern Region, besides its potential role in improving wheat production under biotic and abiotic stresses in other parts of the world.

Methods

Isolation of diazotrophic bacteria

Prior to isolation, root samples were washed to eliminate soil traces and then successively washed by sterile deionized water in order to selectively isolate diazotrophic bacteria. The macerate of clean roots was inoculated in a culture flask containing Burk's N-free medium and incubated for 7 days at 30°C. Then 0.1 mL aliquots of a 6-fold serial dilution of root macerate was spread on Burk's N-free medium

supplemented with yeast extract at 0.5%. After an incubation for 7 days at 30°C, then the morphologically-different colonies were isolated and subcultured for further analysis.

Moreover, root samples were repeatedly washed and treated by a chlorinated solution for 2 min and alcohol solution for 45 s and repeatedly rinsed with sterile deionized.

Assessment of the nitrogen-fixation (diazotrophic) potential of the isolates

In order to understand nitrogen-fixing potential of the strains, we grew the isolates on two mineral-nitrogen-free media (NFMM and NFb). Then in order to investigate the nitrogenase activity and quantify N-fixing capacity of the strains we performed the ARA test. The isolates that showed positive and predominant growth during the first 3 to 4 days were selected for quantification based on the acetylene reduction method in nitrogen-free semisolid NFb medium [50] and the Kjeldahl N-digestion and distillation system. The selected isolates were incubated in 10 mL of NFb broth in a rotary agitator for 5 days at 150 rpm and 28°C. The N content in the microbial cells was determined according to Kizilkaya [51].

ACC deaminase activity assay

The assessment of the ACC deaminase activity was carried out by determining the content of α -ketobutyric acid released after ACC cleavage as previously reported by Penrose and Glick [52]. In brief, the bacterial cells were grown in minimal medium containing ACC as the sole nitrogen source in a 15 mL TSB medium up to log phase to elicit their ACC deaminase activity. The production of α -ketobutyrate was monitored at 540 nm absorbance wave length. The standard curve of α -ketobutyrate ranging from 0.1 to 1.0 μ mol was used to calculate the ACC deaminase activity that was expressed as the amount of α -ketobutyrate generated per mg of protein per hour.

Production of lytic enzymes

B-glucanase, chitinase, protease and phytase activities were assessed by using B-glucan, chitin, milk and phytic acid, respectively as substrate in a modified LB agar medium [1.0% Enzyme substrate, 1% NaCl, 0.5% levure extract, 0.5% Peptone, 0.5% $(\text{NH}_4)_2\text{HPO}_4$, 0.02% KCl, 0.3% $\text{MgSO}_4/7\text{H}_2\text{O}$]. The isolates were spotted in plates, incubated for 3 days at 30°C and the halo zone was checked.

***In Vitro* plant growth promoting and biocontrol potential of bacterial isolates**

The isolated bacteria were investigated for various plant growth promoting (PGP) as well as biocontrol activities. The PGP parameters included HCN production [53], IAA production in the presence or absence of 0.5% tryptophan [54, 55], siderophore production in agar plates using chromeazurol S (CAS) [56], phosphate solubilization on pikovskya's medium [57, 58], and organic acid production on NFMM medium using bromophenol blue as pH indicator [59, 60].

Biocontrol activity against wheat fungal attack in greenhouse

To investigate the ability of various bacterial strains to suppress head blight disease in wheat, wheat seeds were disinfected by immersion in NaOCl (2%) for 3 min followed by an incubation in ethanol (75%) for 2 min before rinsing 3 times in sterile distilled water. On the other hand, our selected bacterial strains were grown in LB medium and harvested by centrifugation for 10 min at 5000 x g and washed twice with phosphate-buffered saline (PBS, pH 7.0) .

The bioprimered seeds in a 1×10^8 bacterial suspension for 1h, were placed on 9-cm plates covered with sterile wet filter paper and incubated for 72 h at 25 °C for germination. Thereafter, the obtained plantlets were placed in the sterile soil in a growth chamber at 25 °C under 16/8h photoperiod and irrigated with a 1/2 hoagland solution. One week later, wheat seedlings were inoculated by a 2 ml of *F. graminearum* suspension (10^7 cfu/ml).

In order to determine the biocontrol activity of the two selected PGPB strains (MA14 and MA17) we used pots filled with sterilized agricultural soil in a controlled growth chamber. The treatments were: control 1: not bioprimered; control 2: only inoculated with *F. graminearum*; treatment: seed bioprimered with MA14 and inoculated with *F. graminearum*; treatment 2: seed bioprimered with MA17 and inoculated with *F. graminearum*. Data for the percentage of disease incidence and severity was collected on 30th day after sowing. Each treatment had seven replicates. The control efficiency (CE) was determined according to the formula: $[CE (\%) = (DI \text{ control-2} - DI \text{ T}) / DI \text{ control-2} \times 100\%]$, where, DI: disease index, T: one of the treatments.

Wheat inoculation assays

Inoculum preparation and seed coating

Wheat seeds of "Aouija" variety was provided by the Agriculture Research Station at the Centre of Biotechnology of Sfax. The PGPB strains were incubated in LB broth overnight with constant shaking at 28 ± 2 °C. Cells were, then, harvested by centrifugation and resuspended in normal saline to an optimum concentration ($OD = 10^8$ cells per ml at $\lambda = 600$ nm). Seeds of "Aouija" were surface-sterilized with HgCl₂ (0.1%) for 2 min and rinsed twice with sterile distilled water. Then, the bacterial suspension was added to

seeds and subjected to a constant shaking along with a sterile carrier material till a thin film is formed as a proof of seeds being coated. Finally, the coated seeds were air-dried before sowing.

Pot trials

Natural soil without sterilization was used in the pot assay. The used soil was iso-humic (Ustifluvent) with pH 7.05, EC 0.21 mS m⁻¹, 4.40 g kg⁻¹ organic carbon, 89 kg ha⁻¹ available N (alkaline permanganate extractable). After germination of the coated seeds in 7 days, plantlets were placed in a glasshouse for five months in a completely randomized design. **Trial 1:** In this experiment was conducted by seed without PGPB in small pots (9 cm diameter) containing natural soil salinized by 150 mM NaCl as a negative control. The plants grown without PGPB and sand salinization were used as positive control. **Trial 2:** In this experiment, seed biopriming with PGPB in small pots containing natural soil (1Kg/pot) salinized by 150 mM without any bacterial treatment.

Trial 3: The experiment was conducted by seed biopriming with PGPB in small pots containing natural sand (1Kg/pot) .

Statistical Analysis

The data obtained from biochemical characterization, seed germination and plant growth parameters were subjected to the Duncan's Multiple Range Test (DMRT) using the Statistica software (ver. 10.1). For seed biopriming, wheat growth test, and for antagonism test the data were analyzed by the Duncan's Multiple Range Test (DMRT) using the IBM SPSS software (ver. 21.1, SPSS Inc. www.spss.com). The significance level for all analyses was P = 0.05.

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and in supplementary materials.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Experimental design and write up by AHB, MJ, LD, AA, MBA, HH, MBA & NAF. MBA, AA and HH carried out the statistical analysis. NAF final editing & correspondence. All authors approved the final version.

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Tables

Table 1: Plant growth-promoting and other properties of selected isolates from coastal saline soil in Tunisia.

PGPB strain	Hydrolytic enzyme properties				PGP properties		Antifungal activity			
	B-Glucanase	Protease	Chitinase	Siderophore	ACC deaminase*	EPS	<i>Rhizoctonia solani</i>	<i>Fusarium solani</i>	<i>Fusarium oxysporum</i>	<i>Fusarium graminearum</i>
MA9	++	+++	+	+	+++	++++	+++	+	-	+
MA14	+++	++++	++	++++	++	+	-	-	-	-
MA17	+++	++++	+++	++	+	++++	+++	+++	++	+++
MA19	+++	+++	++++	+++	+++	+	+++	+	-	+++

*ACC deaminase activity was measured after 7 days of bacterial growth. Data showing only four identified isolates, all experiments were done in duplicate. (–) absence of activity, (+) low, (++) medium, (+++) high, very high (++++).

Table 2: Plant growth promoting attributes of salt tolerant rhizobacteria at 2% NaCl concentration.

Strain	Pellicle formation	N-free growth medium	Blue bromothymol color change	Dinitrogen fixation (mg N ₂ fixed 50 ml ⁻¹ culture media 72h ⁻¹)	ARA(nmol of C ₂ H ₄ formed ml ⁻¹ culture media 72 h ⁻¹)
MA9	+	++	Green blue	24.548b	2.348b
MA14	++	+++	Yellow	09.458b	0.452a
MA17	++	++	Blue	51.023b	3.125b
MA19	+	+++	Yellow	37.149b	2.428b
MTCC2460*	+	++	Blue	5.03a	1.64a

Data showing only of four identified isolates, all biochemical analysis are in triplicate. Means in the columns followed by same superscript letters indicate no significant difference (P = 0.05) by Duncan's Multiple Range Test (DMRT). * *Azotobacter*

vinelandii.

Table 3: Bioprotection efficiency of PGPB strains against wheat fungal wilt caused by *Fusarium graminearum*.

Treatment	Disease index (%)	Bioprotection efficiency (%)
Control-1	0 ± 0	-
Control-2	89.58 ± 0.38a	-
Treatment-1	76 ± 0.18b	24.5
Treatment-2	36 ± 0.89c	64.5

Control1 (not inoculated with any microbe), Control-2 (only inoculated with *F.graminearum*), Treatment1 (inoculated with MA14 and *F.graminearum*) and Treatment-2 (inoculated with MA17 and *F.graminearum*). Values with the different letter within the same column are significantly different at P < 0.05 according to Duncan Multiple Range Test (DMRT). Numbers followed by the “±” are standard errors.

Table 4: Influence of seed biopriming by PGPB on growth parameters (root and shoot length) of wheat plants, under non-saline (control) and saline (*in planta*) conditions.

Bacterized seed with	NaCl mol.l ⁻¹	Root length (cm)	%Increase	Shoot length (cm)	%Increase
Control*	0.025	5ab	-	42.57ab	-
MA9	0.025	7cd	40	38.57ab	0
MA14	0.025	16.2e	131.42	61bc	14.33
MA17	0.025	8.5d	70	63.64d	49.49
MA19	0.025	5ab	0	63.14 cd	48.32
Control*	0.125	3.7a	-	37.87a	-
MA9	0.125	5.5abc	48.64	43.45bc	14.73
MA14	0.125	20.2f	459.46	35.14a	0
MA17	0.125	4.7ab	27	75.34d	99.2
MA19	0.125	6.3bcd	70.27	52.72bc	39.21

* Seed without bacterial treatment.

Table 5: Influence of seed biopriming by PGPB on growth parameters (total dry weight and 100 grain weight) of wheat plants, under non-saline (control) and saline (*in planta*) conditions.

Bacterized seed with	NaCl mol.l ⁻¹	Total Dry Weight (g/plant)	Dry Mass Increase (%)	Grain Weight (g)*	Grain Weight Increase (%)
Control**	0.025	1.68a	-	4.182b	-
MA9	0.025	2.55b	51.78	4.181b	0
MA14	0.025	3.09b	83.92	4.48cd	07.17
<u>MA17</u>	0.025	<u>5.04b</u>	<u>200</u>	<u>5.44e</u>	<u>30.14</u>
MA19	0.025	3.61b	114.88	4.67 d	11.72
Control**	0.125	1.01a	-	3.38a	-
MA9	0.125	2.48b	145.54	4.16b	23
MA14	0.125	2.65b	162.37	4.31bc	27.51
<u>MA17</u>	0.125	<u>7.45b</u>	<u>637.62</u>	<u>6f</u>	<u>77.51</u>
MA19	0.125	2.69b	166.33	4.55d	34.61

*Weight of 100 grains

**Seed without bacterial strain

Additional Figure Legends

Fig. S1: Distribution cartography of soil with different degree of salinity in Tunisia.

Fig. S2: (A) Salinity tolerance assays with selected strains on LB medium after 48h (B) Bacterial growth assay after 7 days of selected bacteria on N free medium (NFMM) and (C) Bacterial growth assay after 7days of selected bacteria on NFMM+ACC as the only available nitrogen source. Error bars show the standard deviation of the mean values of three replicates (P < 0.05).

Fig. S3: Effect of seed-biopriming with PGPB strains on *Fusarium* wilt growth as expressed in root length (cm) following inoculation of in seedling explants of durum wheat with *Fusarium graminearum* under controlled growth chamber.

Control1 (not inoculated with any microbe), Control 2 (only inoculated with *F. graminearum*), Treatment1 (inoculated with MA14 and *F. graminearum*) and Treatment 2 (inoculated with MA17 and *F. graminearum*). Values with the different letter within the same column are significantly different at P < 0.05 according to Duncan's test.

Fig. S4: Effect of Seed bioprimering with PGPB strains on *Fusarium* wilt suppression expressed in shoot length (cm) following inoculation with *Fusarium graminearum* in seedling explants of durum wheat under controlled growth chamber.

Control 1 (not inoculated with any microbe), Control 2 (only inoculated with *F. graminearum*), Treatment1 (inoculated with MA14 and *F. graminearum*) and Treatment2 (inoculated with MA17 and *F. graminearum*). Values with different letter within the same column are significantly different at $P < 0.05$ according to Duncan's test.

Fig. S5: Effect of seed-bioprimering with PGPB strains on *Fusarium* wilt suppression expressed in total length (cm) following inoculation with *Fusarium graminearum* in seedling explants of durum wheat under controlled culture chamber.

Control 1 (not inoculated with any microbe), Control 2 (only inoculated with *F. graminearum*), Treatment1 (inoculated with MA14 and *F. graminearum*) and Treatment 2 (inoculated with MA17 and *F. graminearum*). Values with the different letter within the same column are significantly different at $P < 0.05$ according to Duncan's test.

Fig. S6: Effect of seed-bioprimering with PGPB strains on *Fusarium* wilt suppression expressed in total dry weight (mg/explant) following inoculation with *Fusarium graminearum* in seedling explants of durum wheat under controlled growth chamber.

Control 1 (not inoculated with any microbe), Control 2 (only inoculated with *F. graminearum*), Treatment 1 (inoculated with MA14 and *F. graminearum*) and Treatment 2 (inoculated with MA17 and *F. graminearum*). Values with the different letter within the same column are significantly different at $P < 0.05$ according to Duncan's test.

Fig. S7: Effect of seed-bioprimering with PGPB strains on *Fusarium* wilt suppression expressed in survival explants following inoculation with *Fusarium graminearum* in seedling explants of durum wheat under controlled growth chamber.

Control 1 (not inoculated with any microbe), Control 2 (only inoculated with *F. graminearum*), Treatment 1 (inoculated with MA14 and *F. graminearum*) and Treatment 2 (inoculated with MA17 and *F. graminearum*). Values with the different letter within the same column are significantly different at $P < 0.05$ according to Duncan's test.

Fig. S8: Effect of seed-bioprimering with PGPB strains on *Fusarium wilt* suppression expressed in disease index (DI) following inoculation with *Fusarium graminearum* in seedling explants of durum wheat under controlled growth chamber.

Control 1 (not inoculated with any microbe), Control 2 (only inoculated with *F. graminearum*), Treatment 1 (inoculated with MA14 and *F. graminearum*) and Treatment 2 (inoculated with MA17 and *F. graminearum*).

Values with different letter within the same column are significantly different at $P < 0.05$, according to Duncan's test.

Fig. S9: Effect of seed biopriming with PGPB strains on the total length (cm) of durum wheat plants under different salinity regimes.

Bars represent standard error of mean (SEM). Different letters above the error bars indicate significant difference at $P = 0.05$. Control = no bacterial inoculation under normal conditions; NaCl = no bacterial inoculation under salinized soil; T1 = biopriming seed; 9 = *Bacillus pumilus*; 14 = *Virgibacillus* sp.; 17 = *Bacillus pumilus*; 19 = *Bacillus tequilensis*.

Fig. S10: Effect of seed-biopriming with PGPB strains on the total dry weight (g/plant) of durum wheat plants under different salinity regimes.

Bars represent standard error of mean (SEM). Different letters above the error bars indicate significant difference at $P = 0.05$. Control = no bacterial inoculation under normal conditions; NaCl = no bacterial inoculation under salinized soil; T1 = biopriming seed; 9 = *Bacillus pumilus*; 14 = *Virgibacillus* sp.; 17 = *Bacillus pumilus*; 19 = *Bacillus tequilensis*.

Fig. S11: Effect of Seed biopriming with PGPB strains on the root area (cm²) of durum wheat plants under different salinity regimes.

Bars represent standard error of mean (SEM). Different letters above the error bars indicate significant difference at $P = 0.05$. Control = no bacterial inoculation under normal conditions; NaCl = no bacterial inoculation under salinized soil; T1 = biopriming seed; 9 = *Bacillus pumilus*; 14 = *Virgibacillus* sp.; 17 = *Bacillus pumilus*; 19 = *Bacillus tequilensis*.

Fig. S12: Effect of seed biopriming with PGPB strains on seed mass (g/100 seed) of durum wheat plants under different salinity regimes.

Bars represent standard error of mean (SEM). Different letters above the error bars indicate significant difference at $P = 0.05$. Control = no bacterial inoculation under normal conditions; NaCl = no bacterial inoculation under salinized soil; T1 = biopriming seed; 9 = *Bacillus pumilus*; 14 = *Virgibacillus* sp.; 17 = *Bacillus pumilus*; 19 = *Bacillus tequilensis*.

Figures

Figure 1

Production of β -Glucanase (A), proteases (B) and siderophores (C) in vitro by bacterial isolate: 1: MA9, 2: MA14, 3: MA17 and 4: MA19 after 7 days of growth at 28 ± 2 oC. The formation of halo zone around the colonies shows the positive activity.

Figure 2

(A) AIA production, (B) P-solubilization of selected strains on 2% NaCl. Error bars show the standard deviation of the mean values of three replicates ($P < 0.05$).

Figure 3

ACC deaminase activity expressed in $\mu\text{mol ketobutyrate mg}^{-1} \text{ h}^{-1}$ (A) of selected bacteria on N-free medium (NFMM) added by ACC (1-aminocyclopropane -1- carboxylate) as nitrogen source. Error bars show the standard deviation of the mean values of three replicates ($P < 0.05$).

Figure 4

Effect of seed bio-priming by PGPB of germination of durum wheat under salt stress and natural conditions. The seeds were incubated in a suspension of 108 bacteria on 0 and 150 mM NaCl at room temperature for 30 min. Error bars show the standard deviation of the mean values of three replicates ($P < 0.05$).

Figure 5

Effect of seed biopriming with PGPB strains on the wheat N-content (g/100g wheat) (A) and the total protein content (g/100g wheat) (B) of durum wheat plants under different salinity regime. Bars represent standard error of mean (SEM). Different letters above the error bars indicate significant difference at $P = 0.05$. Control = no bacterial inoculation under normal conditions; NaCl = no bacterial inoculation under salinized soil; T1 = biopriming seed; 9 = *Bacillus pumilus*; 14 = *Virgibacillus* sp.; 17 = *Bacillus pumilus*; 19 = *Bacillus tequilensis*.

Figure 6

Effect of seed biopriming with PGPB's strains on the total mineral content (g/100g wheat) of durum wheat plants under different salinity regime. Bars represent standard error of mean (SEM). Different letters above the error bars indicate significant difference at $P \leq 0.05$. Control = no bacterial inoculation under normal conditions; NaCl = no bacterial inoculation under salinized soil; T1 = biopriming seed; 9 = *Bacillus pumilus*; 14 = *Virgibacillus* sp.; 17 = *Bacillus pumilus*; 19 = *Bacillus tequilensis*.

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