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Shifts in rhizosphere and root endophyte bacterial communities under drought and salinity stress as affected by a biofertilizer consortium

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Abstract

The present research asks how plant growth-promoting bacterial (PGPB) inoculants and chemical fertilizers change rhizosphere and root endophytic bacterial communities in durum wheat, and its dependence on environmental stress. A greenhouse experiment was carried out under drought (at 40% field capacity), or salinity (150 mM NaCl) conditions to investigate the effects of a chemical fertilizer (containing nitrogen, phosphorus, potassium and zinc) or a biofertilizer (a bacterial consortium of four PGPBs). High-throughput amplicon sequencing of the 16S rRNA of rhizosphere, non-sterilized, or surface-sterilized roots, showed that *Proteobacteria, Acidobacteria*, *Bacteroidetes, Gemmatimonadetes, Thaumarchaeota, Firmicutes*, and *Verrucomicrobia* had a higher relative abundance in the rhizosphere, while *Actinobacteria* were more abundant on roots, while *Candidatus_Saccharibacteria* and *Planctomycetes* inside roots. The results indicated that the root endophytic bacterial communities were more affected by (bio-) fertilization treatments than those in the rhizosphere, particularly as affected by PGPB inoculation. This greater susceptibility of endophytes to (bio-) fertilizers was associated with increased abundance of the 16S rRNA and *acdS* genes in plant roots, especially under stress conditions. These changes in root endophytes, which coincided with an improvement in grain yield and photosynthetic capacity of plants, may be considered as one of the mechanisms by which PGPB affect plants.

Introduction

Increasing awareness of the environmental footprint of chemical fertilizers emphasizes the need to find alternative strategies that can ensure competitive crop yields, while providing environmental safety and protection thereby maintaining the long-term ecological balance in agro-ecosystems (Yaghoubi *et al.,* 2019a). Among the alternatives, plant growth-promoting bacteria (PGPB) are very promising, not only as growth-promoting agents but also to enhance plant tolerance to stressful environments (Brilli *et al.,* 2019; Szymańska *et al.,* 2019).

One of the most efficient mechanisms by which PGPB induce plant stress tolerance is through producing 1-amino cyclopropane-1-carboxylate deaminase (ACC deaminase) (Chatterjee *et al.,* 2018; Kanagendran *et al.,* 2019). Through this process, plant-associated microbes can lower ethylene level in plant roots and consequently modulate plant stress resistance by degrading ACC within roots or in exudates (Orozco-Mosqueda *et al.,* 2020). The main strategy to evaluate the possible activity of ACC deaminase in PGPB is to quantify the ACC deaminase gene (*acdS*), which is responsible for encoding ACC deaminase and is known as a suitable marker to assess complex ACC deaminase functional communities (Bouffaud *et al.,* 2018).

While the impact of chemical fertilizer application and stress such as drought and salinity on bulk soil and plant-associated microbial communities have been well documented (Kavamura *et al.,* 2018; Orozco-Mosqueda *et al.,* 2020), this is not the case for effects of PGPB bio-inoculation on microbial communities in the rhizosphere or roots. Recent insights have revealed that the endophytic and rhizosphere microbial communities are preferentially "recruited" by plant roots (Chen *et al.,* 2019), and shaped by complex multilateral interactions between the abiotic environment and its inhabitants (Rodriguez *et al.,* 2019). Hence, understanding how the

rhizosphere and root-associated microbiome respond to PGPB inoculation is essential to develop reliable applications. Therefore, the present research aims to investigate how chemical/microbiological fertilization managements shape rhizosphere and root-associated bacterial communities. We hypothesize that the rhizosphere and root endophytic bacterial community structure of durum wheat (*Triticum durum* Desf.) change under PGPB inoculation, and that chemical fertilization and stress affect the extent of change. Moreover, the size of the microbial population containing the *acdS* gene (*acdS* gene copies' number in root and soil samples) is expected to be influenced by stress and fertilization treatments.

Methods

Pot experiment

The isolation and identification of four PGPB strains, namely *Acinetobacter pittii, Acinetobacter oleivorans, Acinetobacter calcoaceticus* and *Comamonas testosterone* are fully described in Yaghoubi *et al.* (2021). These bacterial strains were isolated from a durum wheat field at Lavello (Southern Italy, Basilicata region) and showed a great potential to solubilize the insoluble forms of phosphate, potassium, and zinc and to fix N_2 gas, respectively (Yaghoubi *et al.,* 2021). The details of the pot experiment are fully explained in Yaghoubi *et al.,* (2020a). Briefly, a pot experiment was conducted in a completely randomized design on a clay loam soil (Eutri-Fulvic cambisol) that was collected from the same durum wheat field where the PGPB were isolated, for a total of seventy-two pots (4 fertilization treatments × 3 stress levels × 6 replications; details of fertilization treatments are presented in Table 1). Fertilization treatments were (i) control (no

fertilization), (ii) inoculation (seed inoculation with the selected PGPB consortium as well as adding the bacterial suspension to pots every 3 weeks), (iii) chemical fertilization (soil treated with chemical fertilizer; Table 1) and (iv) inoculation plus half dose of chemical fertilizer (Table 1). Durum wheat seeds (var. Furio Camillo) were sterilized in 1 % sodium hypochlorite solution for 10 min and washed several times with sterilized distilled water. After the inoculation treatment, ten seeds were randomly selected and planted in each pot. After the germination, six healthy seedlings were kept until sampling time. Three levels of stress included a non-stress control, "drought" at 40% of field capacity (achieved by reduced watering) and "salinity", by applying 150 mM NaCl at the booting stage (63 days after sowing (DAS)). In order to avoid osmotic shock, salinity was applied at a modest rate but repeated every three days (until 81 DAS), and the daily amount, divided in three parts, was added gradually every 2-3 hours.

Sampling and DNA extraction

Rhizosphere soil samples were collected from three replicates at 90 DAS by uprooting and shaking plants to remove soil in excess. The plant's roots were individually stored in sterile bags and moved to the laboratory at 4°C. Soil attached to the roots was collected using sterile spatulas and passed through a 0.5 mm sieve. In order to analyse endophytes in surface-sterilized roots, root tissuewas immersed in 2% (v/v) sodium hypochlorite for three min and was washed five times in sterile water, according to the method of Ling *et al.* (2020) with some modifications. DNA extraction of soil and root (non-sterilized and surface-sterilized) carried out using the DNA extraction kit for soil (MP Biomedicals™ FastDNA™ SPIN Kit, USA) and plant (Qiagen, DNeasy® Plant Pro Kit, Germany), respectively, following the manufacturer's protocols. The Fluorometer Qubit®3.0

dsDNA HS (High Sensitivity) Assay Kit (Life Technologies, Thermo Fisher Scientific Inc., USA) was used for total DNA quantification.

Amplicon sequencing and bioinformatics analyses

Aliquots of the extracted DNA were subjected to a PCR with the 515F/806R primer set targeting a ~250 bp stretch within the V4 region of the 16S rRNA (following Emsens *et al.,* 2020). The complete reagent mixture of 25 µl contained 0.2 µl of Phusion HF (High-Fidelity) DNA polymerase (1U; Thermo Fisher Scientific Inc., USA), 5 µl of HF buffer (1X), 0.5 µl of dNTP (200 µM), 0.5 µl of each primer (200 µM), 1 µl of BSA (12 mg ml^{-1}), 16.3 µl of nuclease-free water and 1 µl of template DNA (~8 ng μl^{-1}). One microliter of peptide nucleic acid (PNA) clamps (12.5 pmol μl^{-1}, pPNA, PNA Bio, Newbury Park, USA) was added to the final reaction mixture of root samples to block amplification of chloroplast 16S genes (Simmons *et al.,* 2018). There were two PCR steps involved in DNA sequencings, including tagging with the Nextera templates and tagging with sample-tags and sequencing adapters. The first PCR for the soil DNA samples consisted of an initial denaturation step at 98 °C for 60 s, followed by 27 cycles of: denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s; and an additional extension step at 72 °C for 10 min.Amplify the root DNA set to the following conditions: 94 °C for 5 min, followed by 30 cycles of denaturation at 98 °C for 45 s, PNA annealing at 67 °C for 20 s, primer annealingat 55 °C for 30 s, extension at 72 °C for 30 s; and an additional extension step at 72 °C for 10 min. The second PCR was performed after diluting (60X) the product of the first PCR and adding the dual barcoded primers with Illumina adapters. Accordingly, the reaction mixture of 25 µl was contained 11.3 µl of nuclease-free water, 5 µl of HF buffer (1X), 0.5 µl of

dNTP (200 μM), 0.2 μl of Phusion HF DNA polymerase, 3 μl of diluted PCR I product and 5 μl of mixed index primers (forward and reverse primers with barcodes). The conditions of the second PCR were set to an initial step at 98 °C for 60 s, 12 cycles at 98 °C for 10 s, 63 °C for 30 s, 72 °C for 30 s; and a final elongation step at 72 °C for 5 min. In order to confirm the success of PCR amplification and the non-amplification of negative controls (water and PCR reagents), an agarose gel electrophoresis (1.5% agarose) was run. At each PCR, there were at least five negative controls, so that in case of appearance of the band in agarose gel electrophoresis even in only one sample, the procedure was repeated.

The library was prepared by combining the PCR products for each sample, and then quantified through qPCR (KAPA Library Quantification Kit, Kapa Biosystems, USA) and sequenced on the Illumina MiSeq platform (Illumina Inc; USA) using a 2 x 250 bp V2 kit at the center of medical genetics at the University of Antwerp (Belgium). Usearch and Vsearch tools were used to perform bacterial community analysis as fully described by Emsens *et al.* (2020). Operational taxonomic units (OTUs) were clustered at 97% similarity. Singletons and non-bacterial OTUs were removed, and the OTU abundance levels rarefied based on the sample with the least number of sequences (12,319 reads, after removal of one sample with < 20 reads). Non-Metric Multidimensional Scaling (NMDS) ordination plots were computed with the Vegan package using pair-wise Bray-Curtis distance from square-root transformed OTU read numbers across sample types and fertilization treatments. The same distances were used for PERMANOVA analysis using the adonis function.

Real-time quantitative PCR analysis (qPCR)

qPCR was performed in triplicate to evaluate bacterial 16S rRNA gene copy numbers with the same primer pair as described above (515F/ 806R). The quantity of the *acdS* gene was estimated with the primer set of acdSF5 (5'-GGCAACAAGMYSCGCAAGCT-3') and acdSR8 (5'-CTGCACSAGSACGCACTTCA-3') (Bouffaud *et al.*,, 2018). qPCR amplification was performed in 20 µl containing 10 µl of KAPA SYBR FAST qPCR Master Mix (2X; KAPA™ SYBR® FAST qPCR Kit, Kapa Biosystems, USA), 0.4 µL of ROX dye (50X), 0.4 µL of each primer (10 µM), 0.6 µl of BSA, 4 µl of template DNA (final concentration < 20 ng) and 4.2 µl of nuclease-free water using a CFX96 Touch® Real-Time PCR Detection System (Bio-Rad, USA). Standard curves were obtained using a series of 10-fold dilutions of PCR products amplified from the positive control samples which were extracted from the agarose gels using the NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions and quantified by fluorometer Qubit® 3.0 dsDNA HS Assay Kit. The cycling program for all qPCR assays entailed 3 min incubation at 95 °C for enzyme activation, followed by 40 cycles of denaturation at 95 °C for 3 s, annealing at 57 °C for 16S rRNA and 67 °C for *acdS* for 20 s, and extension at 72 °C for 12 s. Amplification specificity was assessed by melting curve which was followed by ramping the temperature from 50 °C to 95 °C, with a reading every 0.5 °C.

Grain yield and photosynthetic capacity

Photosynthetic capacity of leaves was measured using the LI-COR portable photosynthesis system (LI-COR 6400/XT, USA) at 89 DAS. All six durum wheat plants from each pot were harvested at 124 DAS and then the grain yield per plant was determined, and averaged to represent the replicate

pot. All statistical analyses including a two-way analysis of variance (ANOVA), Tukey's HSD (honestly significant difference) test, ward's clustering algorithm and multiple linear regressions (backward elimination method), as well as drawing the graphs, were performed using R (ver. 3.5.1) and SigmaPlot (SigmaPlot® v11.0, Systat Software Inc., London, UK).

Results

Illumina 16S rRNA gene sequencing

NMDS ordination revealed distinct bacterial communities associated with rhizospheric soil, surface-sterilized and non-sterilized roots (Fig. 1). Although no differences were observed between bacterial communities in soil samples according to fertilization treatments (Fig. 1), these communities do separate clearly in both surface-sterilized and non-sterilized roots (Fig. 1). The relative abundance of dominant bacterial phyla in all types of samples is presented in Figure 2.

The results showed that most of the phyla were influenced by stress conditions and fertilization treatments. In this regard, PGPB inoculation and chemical fertilization significantly increased *Proteobacteria* relative abundance in all sample types as compared to the non-fertilization treatment. Although the abundance of *Actinobacteria* in soil and surface-sterilized roots was not affected by fertilization treatments, its abundance in non-sterilized roots in responses to the I, CF, and I+½CF treatments was lower than the control treatment (Fig. 2).

At the genus level, bacterial communities (relative abundance > 1%) clustered into several groups in which the distance among them varied from about 1.4 – 4.4% in the soil to 2.9 – 9.9% at the surface-sterilized root (Fig. 3). *Sphingomonas* was the dominant genus in the rhizosphere, which varied from 5.4% (control at non-stress conditions) to 10.5% (I+½CF at salinity condition). The highest proportion of reads in non-sterilized roots belonged to some bacterial genera such as *Rhizobium*, ranging from 3.6% (control under salinity conditions) to 20.4% (CF without stress), accompanied by changes in many other genera as can be seen in Figure 3.

Quantitative PCR of the 16S rRNA and acdS genes

Significant differences in the abundance of bacterial 16S rRNA gene copy numbers were observed among the different levels of stress and fertilization treatment in the surface-sterilized roots, while these did not significantly differ among any of the treatments in rhizospheric soil samples. In this regard, drought and salinity stress had a significant effect on the ribosomal gene abundance in the surface-sterilized root, which was 173.7 and 126.2% higher than that in the non-stress condition (Fig. 4). All fertilization levels increased the 16S gene abundance as compared to the unfertilized treatment. The highest abundance of the 16S gene in the surface-sterilized root was obtained from PGPB inoculations combined with the half dose of chemical fertilizer (I+½CF treatment), equal to 2.28×10^7 copies per g, which was 159.1% higher than those in non-fertilized plants, 35.7% more than PGPB-inoculated plants, and 45.2% higher than plants treated by chemical fertilizers (Fig. 4).

The results also indicated a significant increase of the *acdS* gene copy number under stress in both rhizosphere and sterilized root samples as compared to non-stress conditions, but a quite different response of the *acdS* gene to the fertilization treatments. Accordingly, in the case of rhizosphere, the *acdS* gene reached the highest abundance in the chemical fertilizer (CF) treatment (37.5%

higher than in control). However, the abundance in this treatment was not significantly higher than in the I and I+½CF treatments. Conversely, CF treatment did not show an increase in *acdS* gene abundance for the surface-sterilized root samples, while bio-inoculants (applied alone or combined with the half dose of chemical fertilizer) was able to increase this gene copy number up to 5.2 times more than the control (Fig. 4). Almost the same trends were observed for the ratio of *acdS* copy numbers to total 16S copy numbers (*acdS*/16S), which indicated that the ratio of *acdS*/16S was higher in the CF (1.3%) and I (2.1%) treatments in the soil and sterilized root samples, respectively (Fig. 4).

Grain yield and photosynthetic capacity

Grain yield and photosynthetic capacity were affected by fertilization treatments under both non-stress and stress conditions. In this regard, the grain yield reached the highest value in non-stress, drought and salinity when applied with respectively I+½CF (1.05 g/plant), I (0.46 g/plant) or I (0.61 g/plant) treatments. The maximum rates of photosynthetic capacity were observed in CF (9.21 µmol CO_2 m^{-2} s^{-1}), I+½CF (6.34 µmol CO_2 m^{-2} s^{-1}), and I (6.54 µmol CO_2 m^{-2} s^{-1}) treatments under non-stress, drought and salinity conditions, respectively, which were 16.5, 13.5 and 7% higher than control (no fertilization treatment) at the same level of stress (Fig. 5). Multiple linear regressions (backward elimination method) were used to assess which type of samples and genes could be related to the grain yield and photosynthetic capacity as affected by fertilization management. Accordingly, grain yield and photosynthetic capacity (as dependent variables) were not related to these genes' abundance in the no fertilization or chemical fertilizer treatments since no significant relationship was found between these independent and dependent variables. On the

other hand, the abundance of 16S rRNA and *acdS* genes and their ratio (*acdS*/16S) in surface-sterilized roots in responses to the PGPB inoculation has a stronger effect on grain yield (R^2 = 0.72) and photosynthesis capacity (R^2 = 0.63), than they have in the rhizosphere (Tables 2 and 3).

Discussion

The present study clearly shows the changes in rhizosphere and root endophytic bacterial communities of durum wheat in response to addition of a chemical fertilizer and a PGPB bacterial consortium. Simultaneously, shifts in bacterial communities were also found among different levels of stress treatments. When analysing each stress and fertilization treatment for each compartment (i.e. endosphere and rhizosphere) separately by cluster analysis, we found that structural shifts in the bacterial community were more pronounced in the endo- than in the rhizosphere, suggesting the former is more sensitive to these treatments. It has been reported that some environmental factors such as the availability of soil nutrients, soil pH and cation exchange capacity have a strong impact on shaping rhizosphere microbiome composition (Kusstatscher *et al.,* 2020; Lopes *et al.,* 2021). Rhizosphere bacteria may be more exposed to soil variation and stressful conditions than those inside or on roots, and therefore may already be quite stress-tolerant (Orozco-Mosqueda *et al.,* 2020; Lopes *et al.,* 2021). This may especially be true here, since these bacteria were native to Southern Italy, where the predominant calcareous soil becomes dry in the warmer seasons and pH is slightly alkaline (pH > 8) (Yaghoubi *et al.,* 2021). Alternatively, stronger endo- vs. rhizosphere effects may indicate a prominent plant-mediated mechanism: a crop's phenotypic response to fertilization management provides an altered habitat via adjusted

root architecture (Geisseler and Scow, 2014; Chen *et al.,* 2019), the effect of which could be more pronounced on- or inside roots as compared to the root-soil interface.

The difference in response of some bacterial phyla to stress and fertilization may be related to differences in their life strategy. It has already been proposed that bacterial taxa can broadly be defined in terms of response to resource availability based on their life history strategies, which are categorized from copiotrophic (fast-growing r-strategist) to oligotrophic (slow-growing K-strategist) (Fierer *et al.,* 2007). According to this idea, a greater quality and quantity of organic C and nutrient availability causes a higher relative abundance of copiotrophic taxa (Wang *et al.,*, 2019; Yaghoubi *et al.,* 2020b), and a higher number of cells or even higher average copy number of rRNA genes (Nemergut *et al.,* 2016), resulting in more 16S rRNA gene copies (Männistö *et al.,* 2016). Accordingly, our chemical fertilization and PGPB inoculation treatments revealed a trend towards higher abundance of copiotrophic phyla, such as *Proteobacteria* and *Bacteroidetes* (Fierer, *et al.,* 2007), as well as a higher copy numbers of 16S rRNA, especially in the rhizosphere. However, these changes were not significant as compared to the non-fertilization treatment. Conversely, unfertilized treatments tended to contain more oligotrophic bacteria such as *Verrucomicrobia, Acidobacteria, Planctomycetes,* and *Gemmatimonadetes,* and also lower copy numbers of 16S rRNA in the rhizospheric soil in comparison to the other levels of fertilization, but not significantly so either. Although oligotrophs have been reported to be superior to copiotrophs under environmental stress, especially where stress is due to low resource concentrations (Fierer *et al.,* 2007), here, drought and salinity had no significant effect on oligotrophs. This classification does not seem to explain the changes in microbial communities found here very well, possibly because not all members of each phylum can be generalized to be either distinctly copiotrophic or oligotrophic (Fierer *et al.,* 2007), as for instance *Actinobacteria* consist of both copiotrophic and

oligotrophic members (Ho *et al.,* 2017). Moreover, plant response should also be taken into account as it affects microbial communities both inside the roots and in the rhizosphere (Chen *et al.,* 2019).

In the present research, we used a bacterial consortium containing four beneficial strains belonging to the genera *Acinetobacter* and *Comamonas* for inoculation treatment. Interestingly, the relative abundances of sequences from these genera were very low in both rhizosphere and endosphere compartments, and undetectable in most samples even where inoculated. Accordingly, it seems that their survival rate in soil and re-colonization efficiency inside root tissue was limited, or at least that our sequencing depth did not suffice to reliably sample them. Although these results suggested that our beneficial bacteria were not able to reproduce in large enough numbers to be detected in the soil, the structure of the bacterial community was gradually affected by PGPB inoculation. One possible reason for the change in the composition of the bacterial community in the rhizosphere and endosphere may be that various bacterial taxa engage in symbiotic interactions (Faust and Raes, 2012), especially by competing with each other for resources (Gralka *et al.,* 2020) e.g. by serving the inoculant necromass as a source of nutrients (Płociniczak *et al.,* 2020). The increase in the abundance of sequences belonging to some genera, such as *Arthrobacter* (in surface-sterilized and non-sterilized root), *Actinoplanes* and *Pseudomonas* (in surface-sterilized root), could exemplify this phenomenon, since they are known for their nutritional versatility in using a variety of substrates for their oxidative metabolism such as nucleic acids (Comi and Cantoni, 2011; Płociniczak *et al.,* 2020). Furthermore, organic phosphorus in the form of bacterial DNA might be taken up by the plants' root (Paungfoo-Lonhienne *et al.,* 2010), and leave an indirect effect on associated microbial communities as previously explained.

Although these findings highlight the need for strategies such as preservatives for PGPB inoculants to increase their effectiveness and survival in the soil, the results of grain yield and photosynthetic capacity indicated a positive effect of the inoculation treatment. It seems that the possible source of phosphorus as a component of nucleic acid, as well as phosphate, potassium and zinc solubilization and N_2 fixation activity by PGPB strains during the likely short period of their survival, could have had a positive effect on grain production and photosynthetic capacity. It should also be considered the indirect effects of the changes in surrounding bacterial communities and synergistic effects among them, which has led to the stimulation of mechanisms such as increasing the expression of the *acdS* gene. In fact, inducing changes in bacterial communities in soil is also considered an important mode of action of PGPB agents (Kusstatscher *et al.,* 2020). Kang *et al.* (2013) reported similar results in increasing plant growth despite the low persistence of PGPB (*Bacillus pumilus* WP8) in soil (less than 40 days), stating that there are two types of possible mechanisms for PGPB action including high-density cell-dependent type and regulation of microbial community-dependent type. The first mechanism, as a traditional perspective, is a well-known premise regarding the necessity of establishing and maintaining a critical population density of PGPB in the soil to exert growth-promoting effects (Kang *et al.,* 2013). The latter mechanism as a regulator of soil bacterial community structure arises from competition for space or other biotic and abiotic limitations (Georgiou *et al.,* 2017).

Grain yield (significantly) and photosynthesis capacity (non-significantly) decreased in inoculated plants compared to those that were chemically fertilized under non-stress conditions, while they tended to be higher (non-significantly) under drought and salinity. Stress seems to activate some stress tolerance mechanisms in the inoculant-plant interaction, which were apparently not active without stress and/or in plants treated by chemical fertilizers. It has already been reported that

PGPB can help crop plants cope with stress through the expression of stress-related genes in plants and microbes (Nautiyal *et al.,* 2013; Etesami and Beattie, 2018), ACC deaminase activity (Kumar *et al.,* 2019), and bio-availability of soil nutrients (Yaghoubi *et al.,* 2018), which can result in enhanced photosynthesis capacity and grain yield (Yaghoubi *et al.,* 2019b; Vandana *et al.,* 2020). In this regard, it has also been shown that the expression of *acdS* genes, and the consequently increased activity of ACC deaminase, is one of the most important mechanisms inducing stress tolerance in plants by preventing excessive increases in the synthesis of ethylene under various stress conditions (Bouffaud *et al.,* 2018; Orozco-Mosqueda *et al.,* 2020). Interestingly, the gene encoding ACC deaminase, *acdS*, was present at higher abundance when the plants were exposed to PGPB inoculation and stress conditions, particularly at the surface-sterilized root. This could be the main explanation why there was a difference between the PGPB inoculation and chemical fertilizer treatments in terms of their effects on the grain production and photosynthesis capacity under stress conditions. Furthermore, as indicated by multiple linear regression analysis, increases in 16S rRNA and *acdS* genes copy number in surface-sterilized root in response to the PGPB inoculation seems to be more useful to predict the grain production and photosynthesis capacity, as compared to their abundance in the rhizosphere. Similar results were proposed by Ali *et al.* (2012) and Santoyo *et al*. (2016) who reported that PGPB endophytes had more positive effects on the plant, such as evidenced by a reduction of ethylene, compared to the rhizosphere. Also, Heydarian *et al.* (2018) stated that genes involved in photosynthesis, in particular photosystems I and II, were up-regulated in roots of plants expressing *acdS*, especially under stress conditions.

Conclusion

Using a bacterial consortium of four PGPBs as a bio-inoculant resulted in more changes in bacterial root endophyte communities compared to those in the soil rhizosphere. This was accompanied by a greater abundance of 16S rRNA and *acdS* genes, especially under stress conditions. These changes in the root endophytes may be one of the mechanisms by which PGPB's improve grain yield and photosynthetic capacity, especially compared to the control and chemical fertilization.

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Tables

Table 1. Details of the fertilization treatments including PGPB inoculation and chemical fertilizers.

Treatments	Chemical fertilizers	Seed inoculation with the bacterialsuspension	Seed inoculation with sterile KCl solution (0.9%, w/v)
Control (Co)	-	-	+
Inoculation (I)	-	+	-
Chemical fertilizers (CF)	+	-	+
I+½ CF	+	+	-

I: Seed inoculation with the selected PGPB consortium. At the first, bacterial compatibility test was performed in order to use a mixture of four PGPB strains as inoculation treatment.Then, the inoculums were prepared in flasks containing Nutrient Broth (NB) medium, starting from a NA plate 24 h bacterial culture, under agitation at 100 rpm for 24 h at 29 °C. The bacterial culture was concentrated by centrifugation at 5000 rpm for 15 min. The pellet was washed three times in a sterile KClsolution (0.9%, w/v) and then re-suspended in the same saline solution. The density of the bacterial suspension to be inoculated was brought to 10^{-6} CFU ml^{-1} corresponding to an optical density at 600 nm of 0.6–0.7. Bacterial suspension was also added to pots every three weeks.

CF: Soil treated with chemical fertilizers. Ammonium sulfate (21% N) was used as N fertilizer (290 Kg ha^{-1}), one thirds of it was added to soil before planting, while the same amount was applied at tillering and flowering stages as topdressing fertilizer. Mono ammonium phosphate (52% P_2O_5; 11% N), potassium sulfate (44% K_2O) and zinc oxide (75% Zn) were also applied equal to 115, 75 and 10 Kg ha^{-1}, respectively, as basal fertilizer before planting.

½ CF: half dose of chemical fertilizers.

Co: Control treatment (no chemical and microbiological fertilizer).

Table 2. Multiple linear regressions **(**backward method**)** between grain yield (dependent variable) and gene copy numbers (independent variables) influenced by fertilization treatments (N=9).

	Independent variables	Sig. level					
		Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
Contr o1 (no fertilization)	(Constant)	0.88	0.87	0.09	0.02	0.00	0.00
	16S gene in soil	0.92	-	-	-	-	-
	16S gene in root	0.77	0.72	0.50	0.48	0.40	-

	AcdS gene in soil	0.84	0.72	0.76	-	-	-
	AcdS gene in root	0.75	0.69	0.34	0.27	0.19	0.12
	acdS/16S in soil	0.84	0.72	0.76	0.91	-	-
	acdS/16S in root	0.83	0.81	-	-	-	-
	R Square	0.42	0.42	0.40	0.39	0.39	0.30
PGPB inoculation	(Constant)	0.51	0.01	0.00	0.00		
	16S gene in soil	0.63	-	-	-		
	16S gene in root	0.44	0.33	0.26	**0.02** *		
	*acdS*gene in soil	0.61	0.81	-	-		
	*acdS*gene in root	0.51	0.45	0.30	**0.04** *		
	acdS/16S in soil	0.49	0.45	0.39	-		
	acdS/16S in root	0.43	0.34	0.27	**0.03** *		
	R Square	0.81	0.78	0.78	0.72		
Chemical Fertilizers	(Constant)	0.65	0.48	0.14	0.10	0.00	0.00
	16S gene in soil	0.82	0.68	-	-	-	-
	16S gene in root	0.66	0.61	0.69	0.50	-	-
	*acdS*gene in soil	0.64	0.51	0.47	0.45	0.16	-
	*acdS*gene in root	0.84	-	-	-	-	-
	acdS/16S in soil	0.60	0.47	0.44	0.66	0.10	0.26
	acdS/16S in root	0.65	0.59	0.67	-	-	-
	R Square	0.52	0.51	0.47	0.45	0.43	0.17

Constant: y-intercept, the value at which the regression line crosses the y-axis.
*: Significant at $P< 0.05$ level

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Table 3. Multiple linear regressions **(**backward method**)** between photosynthetic capacity (dependent variable) and gene copy numbers (independent variables) influenced by fertilization treatments (N=9).

	Independent variables	Sig. level					
		Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
Control (no fertilization)	(Constant)	0.39	0.10	0.05	0.01	0.00	0.00
	16S gene in soil	0.52	0.49	0.42	0.63	-	-
	16S gene in root	0.72	0.89	-	-	-	-
	*acdS*gene in soil	0.57	0.56	0.48	-	-	-
	*acdS*gene in root	0.69	0.64	0.17	0.16	0.12	0.10
	acdS/16S in soil	0.49	0.45	0.36	0.23	0.22	-
	acdS/16S in root	0.73	-	-	-	-	-
	R Square	0.78	0.76	0.76	0.72	0.70	0.58
PGPB inoculation	(Constant)	0.78	0.15	0.01	0.00		
	16S gene in soil	0.61	0.52	0.50	-		
	16S gene in root	0.58	0.51	0.29	**0.04** *		
	*acdS*gene in soil	0.69	-	-	-		
	*acdS*gene in root	0.58	0.54	0.35	**0.02** *		
	acdS/16S in soil	0.67	0.74	-	-		
	acdS/16S in root	0.50	0.47	0.26	**0.04** *		
	R Square	0.85	0.83	0.82	0.63		
Chemical Fertilizers	(Constant)	0.64	0.09	0.07	0.04	0.00	0.00
	16S gene in soil	0.91	-	-	-	-	-
	16S gene in root	0.46	0.35	0.48	0.38	-	-
	*acdS*gene in soil	0.68	0.44	0.84	-	-	-
	*acdS*gene in root	0.50	0.34	0.37	0.32	0.60	-
	acdS/16S in soil	0.66	0.45	-	-	-	-
	acdS/16S in root	0.42	0.31	0.23	0.18	0.15	0.14
	R Square	0.74	0.74	0.66	0.65	0.56	0.53

Constant: y-intercept, the value at which the regression line crosses the y-axis.
*: Significant at $P< 0.05$ level

Figures

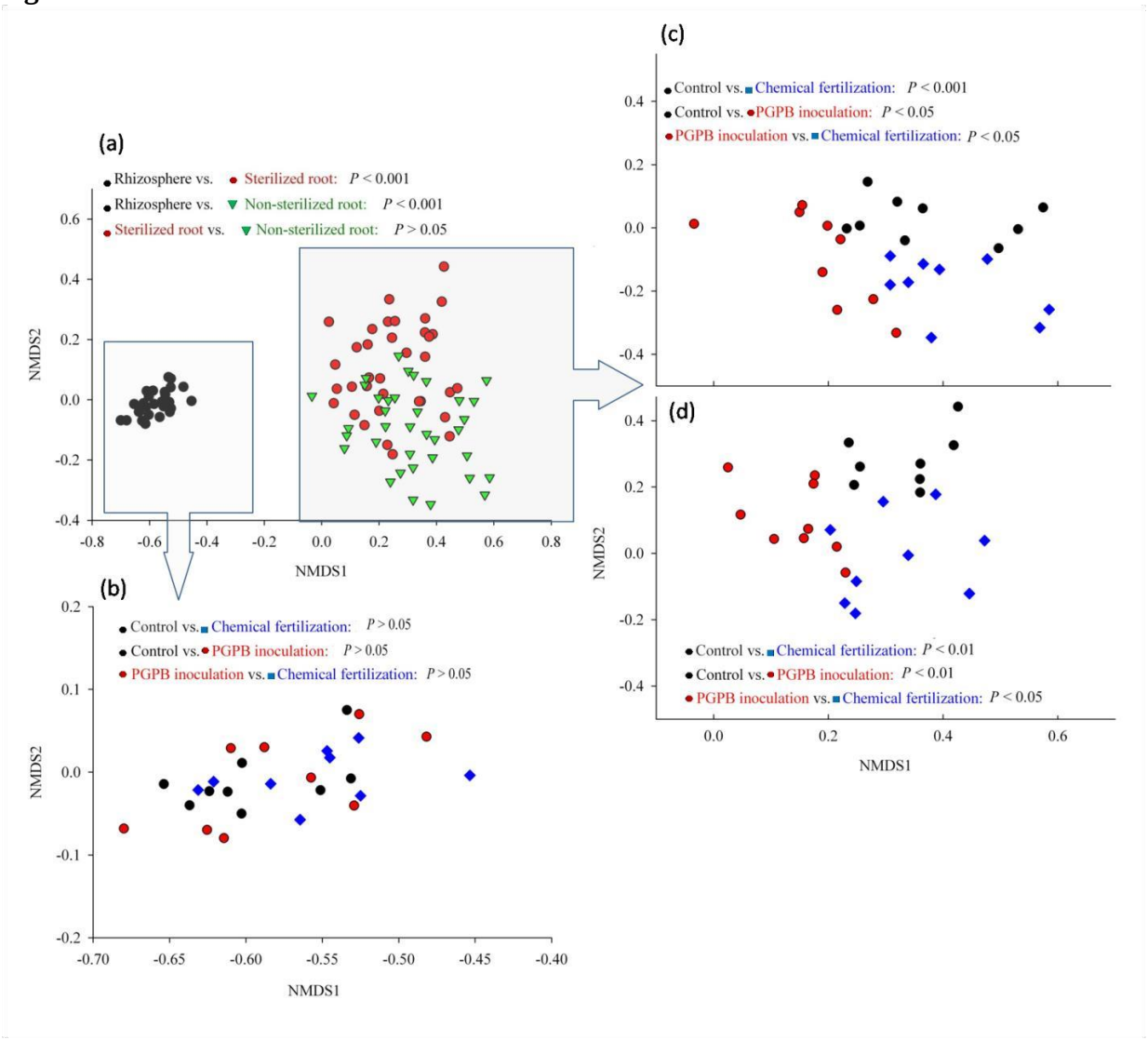

Fig. 1. Non-metric multidimensional scaling (NMDS) of bacterial community composition in three types of samples including rhizospheric soil, surface-sterilized and non-sterilized root using the Bray–Curtis dissimilarity matrix. NMDS of bacterial OTU compositions in soil and roots (a); rhizosphere soil (b), non-sterilized root (c), and surface-sterilized root (d) samples under different fertilization managements. NMDS stress = 0.089.

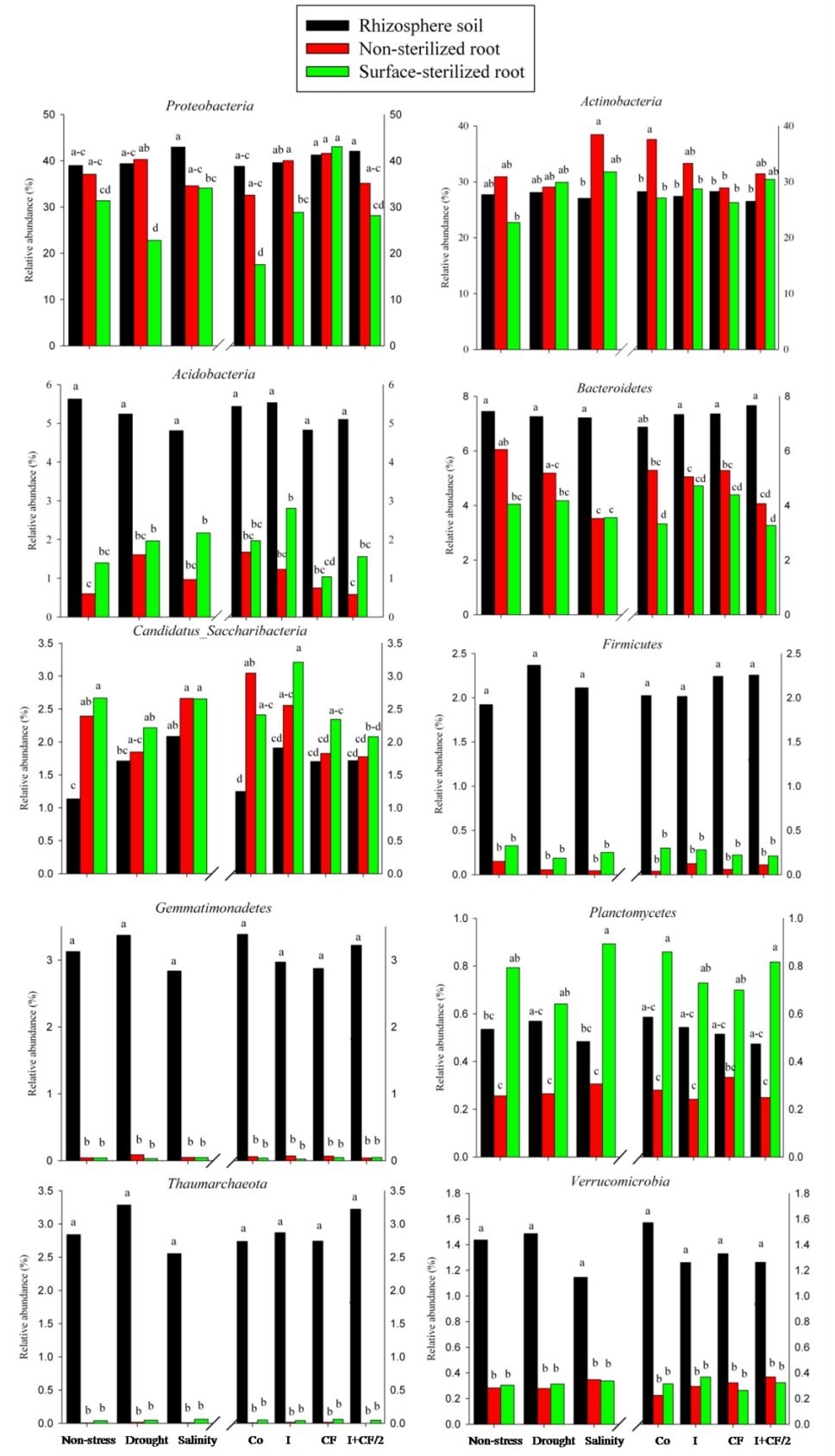

Fig. 2. Relative abundance of bacterial *phyla (*relative abundance > 1%*)* for bacterial communities in rhizosphere soil, non-sterilized and surface-sterilized roots under fertilization treatments and stress conditions. Means in each phylum and factor followed by similar letter(s) are not significantly different at 5% probability level (Tukey's HSD test).

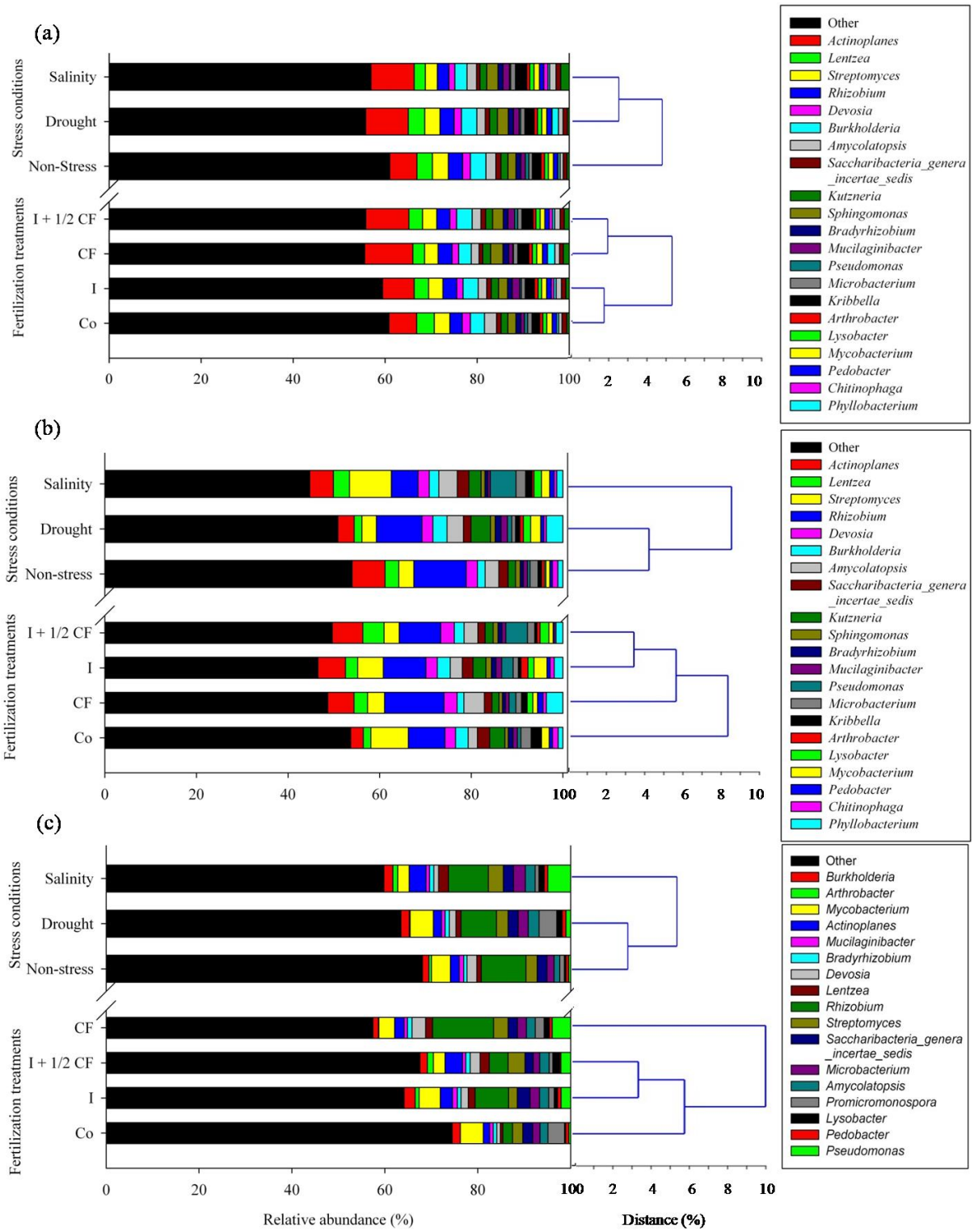

Fig. 3. Cluster analyses (ward's method) of the 16S rRNA composition of rhizospheric soil (a), non-sterilized (b), and surface-sterilized root (c) bacterial communities at the genus level as affected by stress conditions and fertilization treatments.

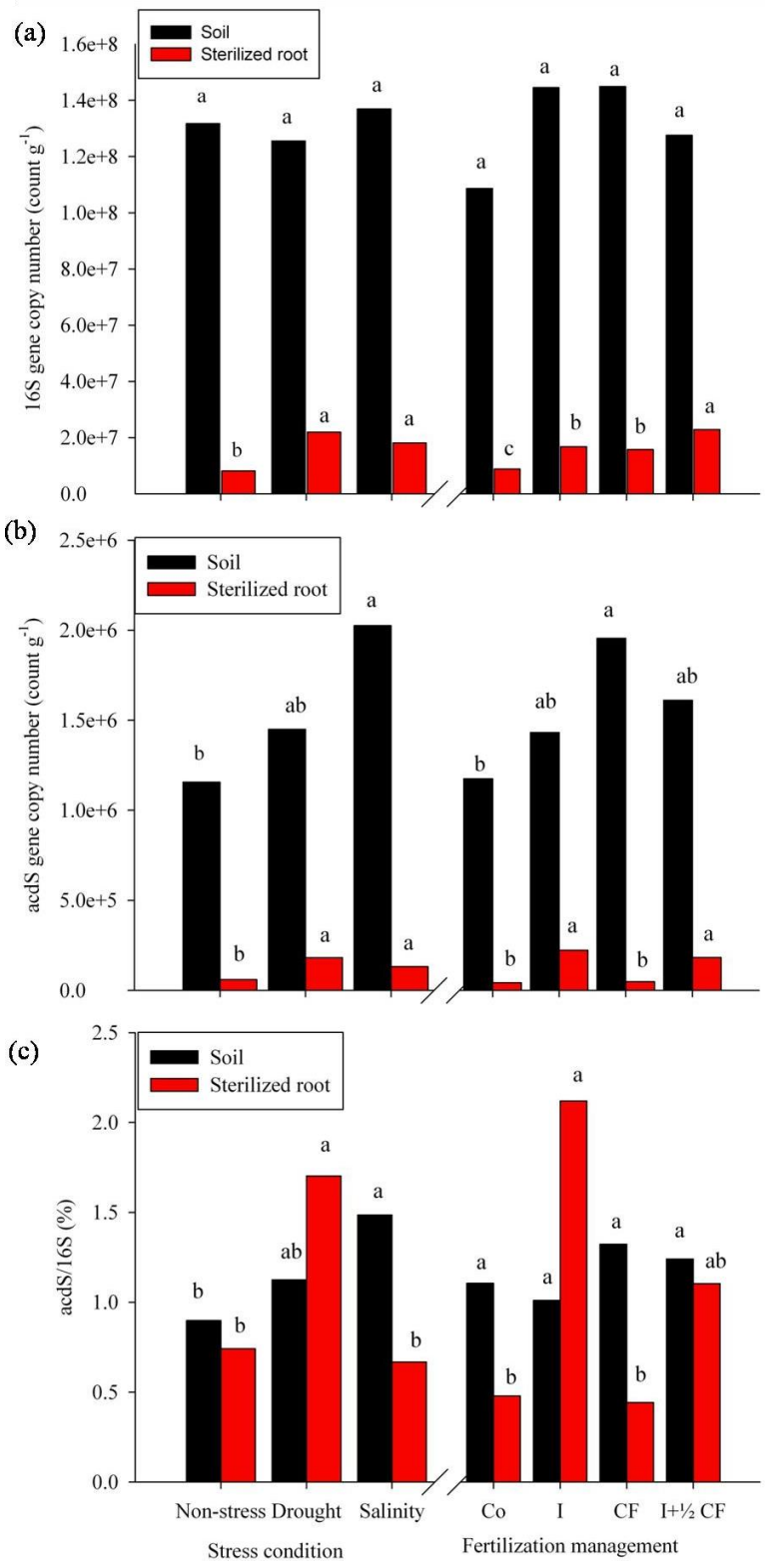

Fig. 4. 16S (a) and *acdS* (b) gene copy numbers and the ratio of *acdS* to 16S abundance (c) under different stress conditions and fertilization managements. Means in each type of sample and factor followed by similar letter(s) are not significantly different at 5% probability level (Tukey's HSD test).

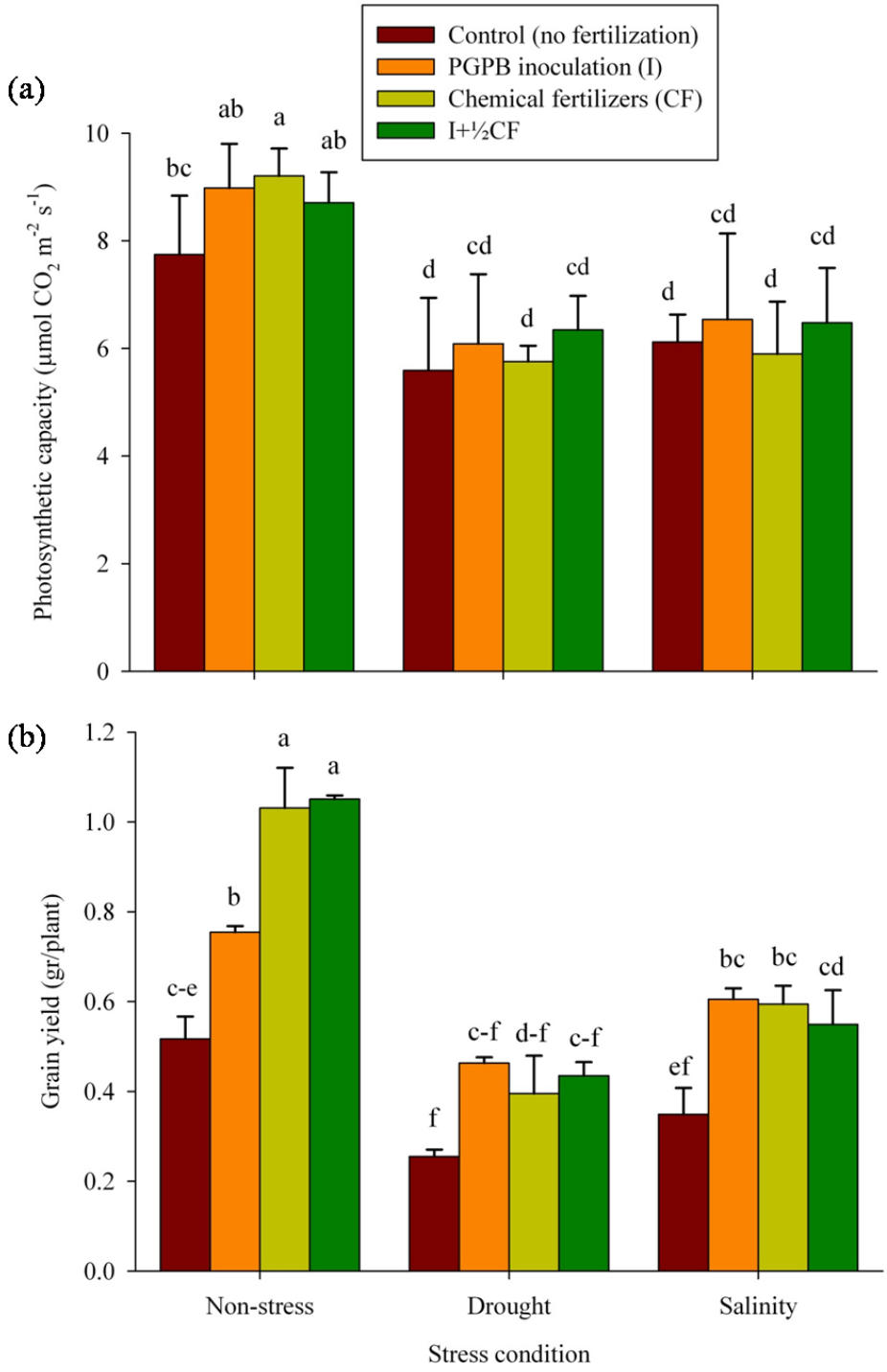

Fig. 5. Photosynthesis capacity (a) and grain yield (b) in response to the fertilization treatments under stress conditions. Means followed by similar letter(s) are not significantly different at 5% probability level (Tukey's HSD test).