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Abstract

We investigated the effect of plant growth-promoting bacterial strains (PGPB) as biofertilizer on grain metabolic composition of durum wheat (*Triticum durum* Desf.). To this aim, we conducted a greenhouse experiment where we grew durum wheat plants supplied with a biofertilizer consortium of four PGPB and/or chemical fertilizer (containing nitrogen, phosphorus, potassium, and zinc), under non-stress, drought (at 40% field capacity), or salinity (150 mM NaCl) conditions. Nutrient accumulations in the grain were increased in plants treated with biofertilizer consortium, alone or along with a half dose of chemical fertilizers, compared to those in no fertilization treatment. A clear benefit of biofertilizer application in the improvement of protein, soluble sugar, starch and lipid contents in the grains was observed in comparison with untreated controls, especially under stress conditions. The most striking observation was the absence of significant differences between biofertilizer and chemical fertilizer treatments for most parameters.

Moreover, the overall response to the biofertilizer consortium was accompanied by the greater changes in amino acids, organic acids, and fatty acid profiles. In conclusion, PGPB improved metabolic and nutrient status of durum wheat grains to a similar extent as chemical fertilizers, particularly under stress conditions, demonstrating the value of PGPB as a sustainable fertilization treatment.

Keywords: Biofertilizer; Durum wheat; Metabolite profiling; Plant growth-promoting bacteria; Stress

1. Introduction

Durum wheat (*Triticum durum* Desf.) is one of the most widespread crops in the Mediterranean basin, where it often suffers from climate-induced environmental stress (Cramer et al., 2018). In Italy, the second-largest producer of durum wheat, this challenge has always been associated with increasing environmental and economic risks due to the increased consumption of chemical fertilizers (Gazzani, 2021). In 2020, 2.09 million tons of chemical fertilizers were used in Italy, a slight increase of 5.7 % since 2015 (ISTAT, 2021). Increased demand for agrochemicals has played a significant role in the upward movement in their negative impacts on the environment (e.g., land degradation and ecosystem deterioration), water (e.g., degradation of surface water and groundwater), and food product quality (e.g., accumulation of harmful substances) (Paladino et al., 2020). However, during this period, the consumption of chemical-free fertilizers has increased by 54%, but this amount is still small compared to chemical fertilizers (ISTAT, 2021).

Among the alternative fertilization approaches for sustainable development, the use of plant growth-promoting bacteria (PGPB), as bio-stimulants, is becoming a more widely accepted technique for improving agricultural productivity and plant stress tolerance (Bakhshandeh et al., 2020; Saberi Riseh et al., 2021). Recent evidence suggests that various pathways are activated by these beneficial bacteria, producing growth regulators (Khan, 2021), inducing the solubilization of insoluble minerals and biological fixation of nitrogen (Pii et al., 2016), improving antagonistic potential against phytopathogens (Wang et al. 2021), stimulating the plant antioxidant defense system (Ha-Tran et al., 2021), and improving plant tolerance to heavy metal stress (AbdElgawad et al., 2021).

In recent years, many attempts have been made to investigate the bio-fertilization, bio-protection and bio-remediation aspects of PGPBs (Yaghoubi et al., 2018a; Crecchio, 2020; Manoj et al., 2020). Applications of PGPB, as an alternative to traditional fertilizers, could affect the primary and secondary metabolisms in the wheat grain. A considerable amount of literature has been published on the effect of environmental stress on biochemical processes for the synthesis of both major (starch, proteins and polysaccharides) and minor (e.g. lipids, phenolic, vitamins, minerals, etc.) components of the mature cereal grain (Sehgal et al., 2018; Călinoiu & Vodnar, 2018; Sakr et al., 2021). Nevertheless, relatively little

attention has been paid to the drivers of specific changes in metabolomic profile responses in the grains in response to bio-inoculation, especially under stress conditions.

Abiotic stresses are increasingly recognized as a serious and worldwide concern in sustainable wheat production by declining the grain yield and quality via the reduced end-use functional properties such as the content of carbohydrates and proteins (Riaz et al., 2021). Besides the genetic effects, there is a consensus among researchers that grain yield and quality in cereals is influenced not only by changes in the content of proteins, starch, and lipids in the grains and their interactions under stress, but also depends on the content of primary and secondary metabolites (Chen et al., 2020; Graziano et al., 2020). Using metabolite profiling to analyze the metabolite composition of complex plant matrices, researchers have been able to describe the biological and biochemical composition of grains and to understand the impact of various biological conditions (Beleggia et al., 2013; Zhen et al., 2016).

Years ago, we started a comprehensive research work with the aim to provide an exciting opportunity to advance the knowledge of the relationship between soil biological fertility levels and the communities of beneficial soil bacteria. As a part of this project, we isolated several beneficial bacterial strains from durum wheat fields at Lavello (Southern Italy, Basilicata region) and identified the four most beneficial among them as *Acinetobacter pittii*, *Acinetobacter oleivorans*, *Acinetobacter calcoaceticus*, and *Comamonas testosteroni* (Yaghoubi et al., 2021c). These bacterial strains showed a promising ability not only in transforming the insoluble complexes of phosphate, potassium, and zinc to soluble forms and biological fixing of nitrogen *in vitro* conditions, respectively (Yaghoubi et al., 2021c), but also in improving some agronomic and physiological parameters of durum wheat plants in a greenhouse experiment (Yaghoubi et al., 2021a). Also, it was determined how the application of these beneficial bacterial strains, as bio-inoculant, shaped rhizosphere and root-associated bacterial communities under stress (Yaghoubi et al., 2021b). Apart from the previous investigation on the physiological and molecular changes in the roots and leaves in stressed plants in response to the bio-inoculation, the debate about plant grains, as an important source of dietary nutrients, has also gained fresh prominence, especially since their metabolic compounds in response to biofertilizer have not been addressed in a comprehensive study, so far. Therefore, the key questions of the present study, focusing on grains, were as follows: (i) are there any changes in the grain of plants treated with microbial/chemical fertilization treatments under stress conditions? (ii) If so, have these changes been made to stimulate increased plant stress tolerance, or were they the subsequent results of the plant's response to stress?

In this regard, our research aims to advance our understanding of the interaction between plant growth conditions (optimal or stress), fertilization (chemical or native PGPB consortium as bio-inoculants) and metabolite composition of durum wheat grains. Attempts were also extended to find a logical relationship between metabolic compounds and nutrient concentrations in grains. We hypothesize that applying the

biofertilizer consortium and traditional chemical fertilization and stress conditions profoundly influence the metabolic composition of durum wheat grain. Moreover, these changes in the grain induce stress tolerance, which prevents loss of grain yield.

2. Material and Methods

2.1. Greenhouse experiment

As fully described by Yaghoubi et al. (2021a), durum wheat seeds (var. Furio Camillo) and the clay loam soil were collected for the greenhouse experiment from the same durum wheat fields where the PGPBs were isolated. Plants were grown in constant light (14h light and 10h dark) and a temperature (20 °C) regime. Briefly, fertilization treatment was defined in four levels, which included (i) Co: no fertilization (control); (ii) BC: seed inoculation with the biofertilizer consortium of four PGPB strains and pot inoculation by the bacterial suspension (10^{-6} CFU ml^{-1}) every three weeks; (iii) CF: Soil treated by a combination of chemical fertilizers before planting, such as mono ammonium phosphate (52% P_2O_5 and 11% N; 115 Kg ha^{-1}), potassium sulfate (44% K_2O; 75 Kg ha^{-1}) and zinc oxide (75% Zn; 10 Kg ha^{-1}) as well as ammonium sulfate (21% N; 290 Kg ha^{-1}; divided into three parts and added before planting, at tillering and flowering stages); and (iv) BC+ ½CF: a combination treatment of biofertilizer consortium and half dose of chemical fertilizers. Stress treatment was established on three levels, including (i) non-stress control, (ii) drought stress at 40% of field capacity (a result of less watering), and (iii) salinity stress at 150 mM NaCl, by applying saline solutions every three days from the 63 DAS (booting stage) until 81 DAS. Grain samples were harvested from each pot (Totally thirty-six pots; four fertilization treatments × three stress levels × three replications) at 124 days after sowing (DAS) for further analyses.

2.2. Determination of N, P, K and Zn concentration in durum wheat grain.

Using an S2Picofox TXRF Spectrometer, the concentrations of P, K, and Zn in durum wheat grains were determined using total-reflection X-ray fluorescence spectrometry (TXRF) (Bruker Nano GmbH, Berlin, Germany). The total nitrogen in the grain was also determined using the Kjeldhal technique (Model UDK 149 Automatic Kjeldhal Distillation Unit, VELP Scientifica, Italy).

2.3. Metabolite profiling

2.3.1. Carbohydrate extraction and estimation

Soluble sugars were separated in ethanol (80% v/v) at 80°C for 60 minutes, then added newly made anthrone reagent (150 mg anthrone in 100 ml H2SO4 (72%), heated in a water bath at 100°C for 10 minutes, and then cooled in an ice bath for 5 minutes. The starch concentration of the remaining pellet following soluble sugar extraction was determined (Galtier et al., 1995). To extract starch, the starch solution was

hydrated and gelatinized (90 percent) with dimethyl sulfoxide, precipitated and rinsed with ethanol, centrifuged, vacuum-dried at 30 °C, and processed with a mixture of -amylase and amyloglucosidase. A multi-mode microplate reader (Synergy Mx, Biotek, Santa Clara, USA) was used to determine total soluble and insoluble sugar by reading their absorbance at 625 nm (de Sousa et al., 2017).

2.3.2. Measurement of soluble and total protein and amino acid profile

Extraction of soluble and insoluble proteins was carried out according to the method described by Hartree (1972) with some modifications (AbdElgawad et al., 2014). Briefly, ground grain samples (100 mg) were homogenized in 0.05 M K-phosphate buffer (pH 7.0) and centrifuged (14,000 rpm, 4°C, 20 min). To precipitate the soluble protein, 10 percent w/v trichloroacetic acid (TCA) was added to the supernatant and redissolved in 1 N NaOH. After washing with ethanol (80 percent v/v), TCA (10 percent w/v), ethanol: chloroform (31% v/v), ethanol: ether (31% v/v), and ether to remove phenolic chemicals, the remaining pellet was utilized to detect insoluble proteins. The washed pellet was re-dissolved in 1 N NaOH at 80°C for 1 h, and finally, soluble and insoluble protein content was measured by reading the absorbance at 650 nm.

Ground grain samples (100 mg) were used for amino acids extraction by homogenizing in 80% aqueous ethanol for 1 min at 7000 rpm, spiking with norvaline, followed by centrifuging at 14,000 rpm for 20 min. The particle was re-suspended in chloroform after the clear supernatant was vacuum-evaporated. During this time, the residual was re-extracted with HPLC grade deionized water, centrifuged again, and the supernatant was combined with the pellet suspended in chloroform. The aqueous phase obtained by centrifugation was filtered using a Millipore micro filter (0.2 M pore size) (14,000 rpm, 10 min). A Waters Acquity UPLC-tqd system (Milford, Worcester County, MA, USA) with a BEH amide column was used to measure amino acids quantitatively (Zinta et al., 2018).

2.3.3. Assessment of total lipid content and fatty acid profile

Total lipid analysis was done using a modified protocol of Bligh and Dyer (1959). Briefly, a mixture of chloroform-methanol (1:2 v/v) and distilled water were added to 100 mg of ground samples, followed by homogenizing the suspension and adding chloroform and water. The bottom layer (organic phase) achieved by centrifugation was transferred into new pre-weighed tubes. Meanwhile, the upper liquid phase was mixed with chloroform and acetic acid, and then the bottom phase was added to the first organic phase after the centrifugation. Finally, the solvent was evaporated and the tube was weighed again to estimate the lipid content by gravimetric analysis.

Fatty acids extraction and quantification were performed according to the protocol described by Torras-Claveria et al. (2014). Briefly, methanol was added to 100 mg of grain samples at room temperature

until the discoloration of the samples, followed by adding codeine and nonadecanoic acids as internal standards. The analysis of gas chromatography-mass spectrometry (GC–MS) was carried out on a Hewlett-Packard 6890, MSD 5975 mass. Fatty acids were identified using the NIST 05 database and Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de).

2.3.4. Organic acid analysis

According to AbdElgawad et al. (2014) a known weight of ground grain samples (ca 100 mg) was utilized for the quantitative assessment of individual organic acids (2021). Organic acids were extracted in phosphoric acid (0.1 percent) supplemented with butylated hydroxyanisole, then centrifuged at 14,000 rpm for 30 minutes at 4 °C. The supernatants were filtered through Millipore microfilters (0.2 M pore size) and submitted to HPLC isocratically with 0.001 N sulfuric acid, set at 210 nm, and a flow rate of 0.6 mL min1. The Ultimate 3000 RSLC nano HPLC system was used for the assay. Similarly, the separation was carried out at 65 °C using an Aminex HPH-87 H (310 mm 7.7 mm) column with a Bio-Red IG Cation H (30 4.6) pre-column.

2.3.5. Determination of tocopherol content and antioxidant capacity

Tocopherols were extracted in n-hexane solvent and quantified by HPLC (Shimadzu, Hertogenbosch, The Netherlands) using normal phase conditions (Particil Pac 5 µm column material, length 250 mm, i.d. 4.6 mm), based on the methods described by AbdElgawad et al. (2015). Dimethyl tocol (DMT; 5 ppm) was also used as an internal standard. Data were analyzed with Shimadzu Class VP 6.14 software provided by the HPLC system.

The ferric reducing antioxidant power (FRAP) was measured to evaluate total antioxidant capacity in durum wheat grains, as fully described by AbdElgawad et al. (2021). Briefly, the extraction was done by adding ethanol (80% v/v) and centrifuging at 14,000 for 20 min. For 30 minutes at room temperature, FRAP reagent (20 mM $FeCl_3$ in 0.25 M acetate buffer, pH 3.6) was combined with a known volume of the produced extract. A multi-mode microplate reader was used to measure the absorbance at 517 nm.

2.3.6. Estimation of polyphenol and proline content

Polyphenols were extracted in ethanol (80% v/v), centrifuged, washed the pellet by ethanol (80% v/v), and finally quantified by a Folin–Ciocalteu assay according to Zhang et al. (2006) at 625 nm using a multi-mode microplate reader. Gallic acid also was used as a reference standard for plotting calibration curve (0–25 µg ml^{-1}).

Proline content was measured by homogenizing the ground grain samples in aqueous sulfosalicylic acid (3%), centrifuging at 10,000 rpm for 30 min, elutriating the supernatant, and twice washing the pellet

with aqueous sulfosalicylic acid (3%). Finally, the supernatants were enriched by toluene and the ninhydrin acid reagent, and measured calorimetrically at 520 nm using a multi-mode microplate reader (AbdElgawad et al., 2015).

2.4. Statistical analysis

SigmaPlot (SigmaPlott® v11.0, Systat Software Inc., London, UK) was used to perform statistical analyses such as a two-way analysis of variance (ANOVA) and Tukey's HSD (honestly significant difference) test, and to also draw the graphs. The NCSS program was used to perform Ward's clustering analysis (Version 21.0.3. Kaysville, Utah, USA).

3. Results

3.1. Grain yield

As reported earlier (Yaghoubi et al., 2021a), fertilization treatments increased grain yield under both non-stress and stress conditions. The grain yield reached the highest value in non-stress, drought and salinity when treated with respectively BC+½CF (1.05 g/plant), BC (0.46 g/plant), and BC (0.61 g/plant). These results demonstrate that the biofertilization treatment is especially effective under stress conditions, whereas chemical fertilization has a stronger effect under optimal conditions (Yaghoubi et al., 2021a).

3.2. Soluble sugar and starch content

To understand how these treatments affected the composition of the grains, we first analyzed their carbohydrate composition. There was a strong interaction between the effect of fertilization and drought and salinity stress: In unfertilized plants, drought and salinity had a non-significant effect on the amount of starch and soluble sugars in the grain (Fig. 1). In contrast, CF and BC+ ½CF resulted in significantly higher soluble sugar contents compared to the controls under non-stress (+64.1 and +69.7%), whereas the increase by BC alone was smaller and not significant (Fig. 1a). Under drought and salinity stress, the effect of CF and BC+ ½CF on soluble sugar levels was strongly enhanced, whereas starch levels tended to be reduced significantly in salinity-treated plants and non-significantly in drought-treated plants. These results show that chemical fertilizers have a strong impact on the carbohydrate composition, particularly under stress conditions.

3.3. Soluble and total protein and amino acid profile

Next we determined the effect of the treatments on protein and amino acid composition. Again in unfertilized plants, the stress had non-significant impact on seed soluble and insoluble protein levels (Fig. 2). However, BC+ ½CF treatment significantly increased the amount of total protein in drought-stressed

grains compared to the control (+31.7%), this increase was not significant compared to CF and BC treatments which were 16.2 and 9.8% higher than control (Fig. 2a, b). As nitrogen is a major constituent of proteins, and a regression analysis allowed us to demonstrate an expected close correlation between nitrogen content in grains total proteins ($R^2 = 79$; $P < 0.01$) and soluble protein ($R^2 = 35$; $P < 0.05$) contents (Fig. 2c).

Next we determined the composition of soluble proteins. Globulins formed the major class of storage proteins, contributing from 6.1 ug.g^{-1} (in no-fertilization under salinity) to 16.6 ug.g^{-1} (in BC+½CF fertilization level in non-stress condition), followed by albumin (4.8 – 7.4 ug.g^{-1}), prolamin (1.8 – 5.5 ug.g^{-1}), and glutelin (0.1 – 0.2 ug.g^{-1}). The maximum concentrations of these storage proteins were occurred in BC+½CF, which were 24.2, 51.8, 66.1, and 45.4% in non-stress, 26.7, 32.3, 39.7, and 14.7% in drought, and 22.0, 103.2, 121.5, and 52.3% higher than those in no-fertilization level, respectively (Fig. 2d).

We focused on the specific changes in amino acid compositions in the grains, as a substantial nutritional quality trait in durum wheat plants. Glutamine and proline were the most abundant amino acids in grains, which varied from 3.3 and 1.9 to 6.1 and 5.8 (mg 100 mg^{-1} of protein), followed by ornithine (2.2 – 5.7 mg 100 mg^{-1} of protein) and glutamate (1.4 – 5.1 mg 100 mg^{-1} of protein). The concentration of almost all amino acids in grains was affected by biofertilizer consortium/chemical fertilization and stress treatments (Fig. 3). Higher concentrations of specific amino acids (e.g. serine, asparagine, lysine, alanine, and histidine) were found in the grains of plants treated with BC or BC+½CF, while higher concentrations of other amino acids (e.g. leucine, aspartate, and tyrosine) were detected in plants treated with CF. Ward's clustering method, using euclidean distance, revealed that the fertilization levels clustered into different groups in terms of the amino acid compositions, in which the distance among them varied from about 1.2 – 3.8 (Fig. 3).

The proline content was also measured as essential proteinogenic amino acid and a known stress defense molecule. Accordingly, when plants were stimulated with BC, no significant difference in the content of proline was detected compared to unfertilized control plants. Moreover, following the application of CF and, BC+½CF a non-significant increase in the proline content was recorded about 27.6 and 46.7% in drought, and 20.3 and 14.8% higher than no fertilization treatment (Fig. 4a). These results show that stress in combination with fertilization increases protein content as well as protein and amino acid composition of the grains.

3.4. Antioxidant capacity and polyphenol content

The general response of plants to abiotic stress conditions is an up regulation of enzymatic and non-enzymatic antioxidant defense mechanisms. To determine if this response extends to the grains, we therefore first analyzed their total antioxidant levels. The concentration of ferric reducing antioxidant power (FRAP)

was not significantly affected by stress or fertilization, although there was a consistent tendency to be lower in response to BC or BC+½CF (Fig. 4b).

Polyphenols content was consistently increased in the grains of stress-treated plants, whereas fertilization had no significant impact (Fig 4c). We observed no significant effect of stress treatments on tocopherols in unfertilized seeds or seeds from plants supplied with chemical fertilizer (Fig. 5). Interestingly, total tocopherol levels were significantly reduced when biofertilizer ($P < 0.05$) alone or in combination with chemical fertilizer was applied under stress conditions.

3.5. Organic acid levels

The present research also sought to find any change in organic acids composition, as critical functions in many cellular processes. In this regard, we detected six organic acids in all samples: succinate, citrate, lactate, malate, oxalate and trans-aconitic, respectively (Table 1). Fertilization treatments increased oxalate concentrations at all levels of stress, while almost all fertilization levels reduced citrate and lactate concentrations (with some exceptions) (Table 1).

3.6. Total lipid content and fatty acid profile

Lipid content and fatty acid levels were determined to have a clear idea of their possible changes in response to the fertilization treatments and to make comparisons with nutrient status in the grains. In this regard, the results showed that lipid content was not affected by stress, but BC, CF, and BC+ ½CF treatments consistently increased levels (Fig. 4d). 18 fatty acids were detected in grains, the most important of which were palmitic acid (hexadecanoic; C 16:0) as a major saturated fatty acid, as well as linolenic acid (octadecatrienoic; C 18:3), and oleic acid (octadecenoic; C 18:1) as the major unsaturated fatty acids, which accounted for about 76 to 80% of the fatty acid concentrations. Consistent with the overall lipid contents, fatty acid levels were not affected by the stress conditions, but fertilization led to a considerable increase in overall levels (Fig. 6). Application of biofertilizer consortium, alone (BC) or in combination with a half dose of chemical fertilizers (BC+½CF), had the greatest effect on increasing the concentration of fatty acids (e.g. Octadecenoic (18.1 and 18.3)) under both non-stress and stress conditions. Strong evidence of the difference between the effect of biofertilizer and chemical fertilizer on the composition of fatty acids was obtained from ward's clustering analysis, which showed that these treatments were clustered into two different groups in each stress treatment (Fig. 6).

3.7. Nutrient concentrations

Nutrient status in the grains was determined to reveal their effectiveness from the fertilization and stress treatments, and to assess their relationship with metabolic parameters. Accordingly, nutrient levels in grains

were affected by fertilization and stress treatments (Table 2). Interestingly, both drought and salinity increased the nutrient accumulation in grains in comparison with the non-stress condition. Moreover, the concentration of total nitrogen reached the maximum values in the combined treatment of biofertilizer consortium and half dose of chemicals (BC+ ½CF) in each stress level, which were 28.9, 27.9, and 14.5 % higher than those in unfertilized control plants in non-stress, drought and salinity conditions, respectively (Table 2). Although almost similar results were obtained for phosphorus, zinc, and potassium under non-stress and salinity treatments, the results were slightly different under drought stress; K content was higher in grains from plants under chemical fertilization. The highest concentrations of these nutrients in grain under drought treatment were obtained from chemical fertilizer (CF) in non-stress, biofertilizer consortium (BC) under drought, and CF treatments in salinity level, although they were not significantly different from other fertilizers levels (Table 2).

3.8. Correlation analysis

Finally, we used a Pearson correlation analysis to investigate the relationship between nutrient accumulation in grains and metabolic parameters. Significant positive correlations were found between grain nutrient concentrations and total protein, soluble sugar and proline. In addition, there was a significant positive relationship ($P < 0.05$) between the concentration of total nitrogen in grains and soluble protein. Moreover, grain lipid content showed a significant correlation ($P < 0.05$) with the accumulation of phosphorus in grains (Table 3).

4. Discussion

Our earlier study showed that under non-stress conditions PGPB inoculation enhanced grain yield to a smaller degree than treatment with chemical fertilizers, while under stress conditions they tended to be at least as effective. These results suggested that, in contrast to chemical fertilization, the microbial consortium is able to activate stress tolerance mechanisms (Yaghoubi et al., 2021b).

In order to answer the key questions of this study, the nutritive values and metabolic compounds in the grain and their relationship with grain yield were investigated, some of which were sugar and protein content in the grain. As Fig. 1, 2, and 4a revealed, increasing in the content of soluble sugar, soluble protein, and proline under stress was recorded in all fertilization levels, but such increasing occurs differently in response to the biofertilizer consortium and chemical fertilizers. Accordingly, the production of these osmolytes in grains were non-significantly increased by biofertilizer consortium. There are similarities between the responses expressed by consortium-inoculated plants in this study and those described by Wang et al. (2022), Ilyas et al. (2020), and Upadhyay and Singh (2015), who reported that biofertilizers can stimulate carbohydrate metabolism, and improve the accumulation of soluble sugars, proline and soluble protein in

wheat plants upon exposure to drought and salinity. Synthesizing and accumulating such compatible solutes can contribute to maintain turgor pressure, improving the water holding capacity of cells and stabilizing subcellular structures, by acting as osmotic regulators and reactive oxygen species scavengers under stress (Ilyas et al., 2020). Furthermore, it has already been reported that beneficial bacteria can act as osmolytes and consequently, help plants to resist osmotic stress by accumulating a considerable amount of compatible solutes inside their cells (Parida & Das, 2005).

On the other hand, what we observed was a higher production of soluble sugars (significantly) and proline (non-significantly) in plants treated by CF and BC+½CF as compared to those inoculated by BC, which could possibly indicate the greater impact of chemical fertilizer on these parameters. In contrast, it seems that further increases in soluble protein content in PGPB-inoculated plants (BC and BC+½CF treatments) indicated a greater effect of beneficial bacteria on the accumulation of soluble protein, as shown in Fig. 2b. It is difficult to explain this result, but it might be related to their differences in correlation with nutrients. Accordingly, while soluble sugar and proline were significantly correlated with the concentration of all four measured nutrients (N, P, K, and Zn) in the grain, the soluble protein was correlated only with nitrogen accumulation. In this regard, Triboï et al. (2003) and Sehgal et al. (2018) have already reported that changes in protein content and protein fraction composition under stress are primarily owing to changes in the amount of nitrogen accumulated during grain filling. Moreover, it has been proved that N acquisition can be linked to protein content, especially proteins associated with N assimilation in plants (Sehgal et al., 2018). In the present research, increased accumulation of amino acids involved in N assimilation (e.g. glutamine, glutamate, aspartate, and asparagine) in biofertilizer-inoculated plants compared to those treated with chemical fertilizers, can somehow confirm this justification. Accordingly, the accumulation of these amino acids in BC treatment was higher than those in CF, equal to 3.2, 27.5, 9.5 and 46.3% in non-stress, 15.7, 68.3, 15.3 and9.7% in drought, and 33.3, 16.1, 18.9 and 68.4% in salinity conditions.

The results of Table 2 clearly indicate the changes in nutrient accumulations in durum wheat grains in response to the application of PGPB bacterial consortium. This finding was reasonably expected, since our beneficial bacteria, including *Acinetobacter*, and *Comamonas* genera, had already shown a great ability in converting the insoluble phosphate, potassium, and zinc complexes to soluble forms, biological fixing the nitrogen, and producing indole acetic acid (IAA) *in vitro* conditions (Yaghoubi et al., 2021c). Prior studies have proved the importance of PGPB in enriching the harvestable and reproductive parts of the plant with macro and micro nutrients in non-stress (Yaghoubi et al., 2018b) and stress conditions (Meena et al., 2017). What is new and very interesting is that there was a tendency of higher nutrient acquisition under stress as compared to non-stress conditions. In particular, this increase was accompanied by a decrease in grain yield in stressed plants, which ultimately led to a non-significant correlation between the nutrient accumulation in grain and grain yield. This finding are contrary to the previous research that reported nutrient availability

in the soil, and their acquisition, assimilation, distribution within the plant tissues are gravely declined by environmental stress (Feller et al., 2018; Etienne et al., 2018). A possible explanation may be the increment of root biomass or absorption surface area in the root, as a mechanism for stress tolerance in crops, which can result in the uptake of more dissolved nutrients from the soil solution (Studer et al., 2017). Especially, since the physiological demand for nutrient uptake under stress conditions can be greater than needed for high yield (Haneklaus et al., 2018). Moreover, the presence of adequate calcium ions in the soils in southern Italy, where lands are covered by carbonate and calcareous soils (Lo Papa et al., 2020), can alter the balance in adsorption between potassium and sodium ions under stress conditions in favor of potassium, and finally improve the accumulation of potassium, calcium, and nitrogen in the plants (Tuna et al., 2007).

Increased concentrations of storage proteins, including globulin, albumin, prolamin, and glutelin, upon exposure to BC+½CF treatment in each stress level, might be also related to the accumulation of these N-containing amino acids in grains, whose crucial functions in protein translocation and storage in plants have already been reported (Zhen et al., 2016). Prior studies have noted the importance of some amino acids, in particular glutamine, asparagine, lysine, and alanine that was more accumulated under stress conditions in fertilized plants, in contributing to proteins synthesis and acting as signaling molecules to regulate the expression of key transcription factor genes involved in stress responses in plants (Kan et al., 2015; Galili, 2002; Parthasarathy et al., 2019). Moreover, fertilization levels did not increase the glycine accumulation, as one of the most abundant amino acids in grain samples, under non-stress conditions. A possible explanation might be its easier and faster absorption and transfer in the plants compared to other amino acids, because of the lower microbial demand for glycine (Yang et al., 2017). However, increasing glycine concentration in plants treated with fertilization treatments (especially BC+½CF) under stress can improve plant stress tolerance through boosting the scavenging system of reactive oxygen species and promoting the accumulation of soluble sugar (Liu et al., 2016).

Fig. 1b illustrated a non-significant reduction in grain starch content in nearly all fertilization levels when the plant was exposed to stress, which resulted in a reduction in grain yield since about 70% of the grain weight is composed of starch. However, this reduction could not be the only reason for the significant reduction in grain yield, because of not only the possible considerable reduction in other factors of yield components (e.g. tiller number, grain numbers per spike, number of spikes per plant), but also the lesser effect of stress (particularly drought) on starch content due to the potential of remobilization and translocation of carbon reserves from vegetative tissue to grains (Bhusal et al., 2017; Prathap et al., 2019). However, the results obtained in the grain yield are similar to those of starch accumulation in terms of greater impacts of biofertilizer consortium than other fertilization levels under stress conditions. Previous research has indicated that PGPB can contribute to catalyzing the transformation of glucose-1- phosphate and ATP to form ADP glucose, as a substrate for starch syntheses, by inducing the enzyme ADP-glucose

pyrophosphorylase (AGPase) (Meena & Rai, 2017). Also, the differences in starch content between biofertilizer consortium and other fertilization levels in salinity was more pronounced than that in drought. This result could be related to the greater ability of our beneficial bacteria, particularly N_2 fixer *Comamonas testosteroni*, to growth in saline conditions *in vitro* (1% NaCl concentration) (Yaghoubi et al., 2021c), in comparison to the common PGPB strains. Probably, this might be a possible explanation why the grain yield in salinity was higher than that in drought, when the PGPB consortium was applied.

It has already reported that the degradation of lipids and alteration in its compositions in wheat plants are closely related to stress conditions (Wang et al., 2020). As shown if Fig. 4d, lipid concentration in stress-treated plants remained high in response to fertilization treatments. In fact, it seems that producing amino acids (e.g. proline) due to higher absorption and accumulation of nutrients (especially nitrogen) in fertilized plants or breaking down proteins in those unfertilized, reduced lipid oxidation (Wang et al., 2016). Based on cluster analysis, biofertilizer treatment, alone or in combination with a half dose of chemical fertilizer, was placed in a separate group compared to chemical fertilization and no fertilization. According to Fig. 6, application of biofertilizer consortium (BC and BC+½CF treatments) increased the accumulation of most unsaturated fatty acids (7 out of 8) such as octadecenoic (C 18:1), octadecatrienoic (C 18:3), dodecanoic (C 12:0), hexadecanoic (C 16:1), hexadecadienoic (C 16:2), hexadecatrienoic (C 16:3), and tetracosenoic (C 24:1). In contrast, 5 saturated fatty acids, including tetradecanoic (C 14:0), pentadecanoic (C 15:0), hexadecanoic (C 16:0), octadecanoic (C 18:0), and hexacosanoic (C 26:0), had the highest concentration in chemical and no fertilization treatments, and the other 5 saturated fatty acids did not show a specific reaction to fertilization treatments. These results accord with other studies, which showed that the application of PGPB can enhance the accumulation of unsaturated fatty acids in plant cells, and consequently maintain membrane stability and ensure the metabolism of other substances in cells, especially under stress conditions (Chen et al., 2022; Akhtar et al., 2021; Rezaei-Chiyaneh et al., 2020).

From the data in Fig. 5, it is apparent that increases in β-tocopherol and γ-tocopherol contents in plants treated with BC and BC+½CF treatments were associated with a simultaneous reduction in α-tocopherol. As a result, a decrease in the grain total tocopherol was observed not only in the plants treated with biofertilizers under both stresses but also in those with chemical fertilizers in salinity conditions. These findings do not support the previous research by Sonbarse et al. (2020), who reported that the application of PGPB can result in improving the tocopherols, as the main anti-oxidative molecules. In this regard, the plant seems to activate certain mechanisms during the stress in response to the applied treatments, one of which is the production of polyphenols as non-enzymatic antioxidants in the plants, which can provide more protection against potential oxidative damage and enhance the stability of cell membranes (Sarkar et al., 2021). Beneficial bacteria indirectly help restrain the function of oxidizing enzymes by stimulating the

accumulation of polyphenols, as polyphenols can form complexes with metals that catalyze oxygenation reactions (Notununu et al., 2022).

From the Table 1 we can see that the responses of organic acids to biofertilizer and chemical fertilizers were different. Although extensive research has been carried out on alteration in organic acid profile in vegetative parts of plants, no single study exists which examines the effect of PGPB and stress on the organic acid contents of mature grain. An increase in the secretion of organic acids such as oxalate, citrate and malate in plants under abiotic and biotic stresses has been previously reported (Tahjib-Ul-Arif et al., 2021; Lou et al., 2016), but these data must be interpreted with caution since their function and accumulation in the grain may be different from other organs. One of the possible implications of N uptake and its accumulation in the grain in response to biofertilizer and chemical fertilization can be an increase in the malate accumulation in the grain, since a positive correlation has been reported between malate accumulation in plants and net N assimilation and nitrogen reductase activity (Miyagi et al., 2019). It has been previously reported that plants growing in alkaline soils secrete organic acids, particularly citrate, from their roots to absorb nutrients such as phosphorus and iron by lowering the pH of the rhizosphere (Tahjib-Ul-Arif et al., 2021). This can explain the higher citrate levels in no fertilization and chemical fertilizer treatments since the soils of southern Italy are slightly alkaline (pH > 8) (Yaghoubi et al., 2021b). The observed decrease in citrate content in treatments containing biofertilizers (BC and BC+½CF), could be attributed to the nativeness of our beneficial bacteria and their adaptation to the conditions of high pH calcareous soils, which, by providing the necessary nutrients, eliminates the need for the plant to produce more of these organic acids. If we accept this justification for citrate, then the reduction in succinate in the grains of biofertilizer-treated plants is not so unexpected; an effect of the lower concentration of citric acid and, consequently, of a reduced Krebs cycle, the key stage of cellular respiration, will be a lower or no production of succinate in such plant cells. In fact, although succinate acts in several catabolic and anabolic metabolic pathways, it is mainly involved in the citric acid cycle as a product of substrate-level phosphorylation materialized (Tretter et al., 2016).

5. Conclusion

Increased accumulation of nitrogen in the grains of biofertilizer-inoculated plants was directly related to the protein content of the grains and finally led to an increase in amino acids, especially those involved in nitrogen assimilation, such as glutamine, glutamate, aspartate, and asparagine. The occurrence of these phenomena, in turn, not only resulted in an increment in concentrations of storage proteins, including globulin, albumin, prolamin, and glutelin, but also led to an increment in the accumulation of most unsaturated fatty acids and some organic acids (e.g. malate and oxalate). Moreover, stimulation of carbohydrate metabolism, especially under stress, occurred in response to the PGPB bacterial consortium

inoculum and the consequent increased nutrient accumulation in grains. Changes in metabolic compounds and nutrient concentrations in durum wheat grains, including changes in amino acids, organic acids, and fatty acid profiles, might be one of the mechanisms by which PGPB ameliorate grain yield under stress, particularly in comparison with the no fertilization and chemical fertilizers. Finally, our results provide reliable evidence regarding the application of the native beneficial bacteria, as a biofertilizer consortium, and the possibility of replacing or reducing the need for traditional chemical fertilizers, constituting a useful and sustainable alternative management of fertilization plans.

Author contributions

Carmine Crecchio, Gerrit T.S. Beemster, Hamada AbdElgawad, and Mohammad Yaghoubi Khanghahi conceived and designed the experiments.Mohammad Yaghoubi Khanghahi drew the figures and wrote the manuscript. Mohammad Yaghoubi Khanghahi and Hamada AbdElgawad performed the experiments, analyzed and summed all the data. Hamada AbdElgawad, Erik Verbruggen, Gerrit T.S. Beemster, and Carmine Crecchio directed the experiments and revised the manuscript. Carmine Crecchio, Gerrit T.S. Beemster, Shereen Magdy Korany, and Emad A. Alsherif contributed reagents/materials/analysis tools.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

FIGURE 1 The effect of chemical and biofertilization on carbohydrate composition of durum wheat grain under optimal and stress conditions. Soluble sugar (a) and starch (b) content in grain under different fertilization and stress conditions. Means (± standard error; n = 3) followed by similar letter(s) are not significantly different at 5% probability level (Tukey's HSD test).

FIGURE 2 The effect of chemical and biofertilization on protein composition of durum wheat grain under optimal and stress conditions. Soluble protein (a), and total protein (b) content, and their relationships with nitrogen accumulation in grain (c), as well as the content of storage proteins (d) under different fertilization and stress treatments. Means (± standard error; n = 3) followed by similar letter(s) are not significantly different at 5% probability level (Tukey's HSD test). * and ** Significant at $P < 0.05$ and $P < 0.01$ level. Co: No fertilization (control); BC: Biofertilizer consortium of four PGPB strains; CF: Soil treated by chemical fertilizers; BC+ ½CF: A combination treatment of biofertilizer consortium and half dose of chemical fertilizers

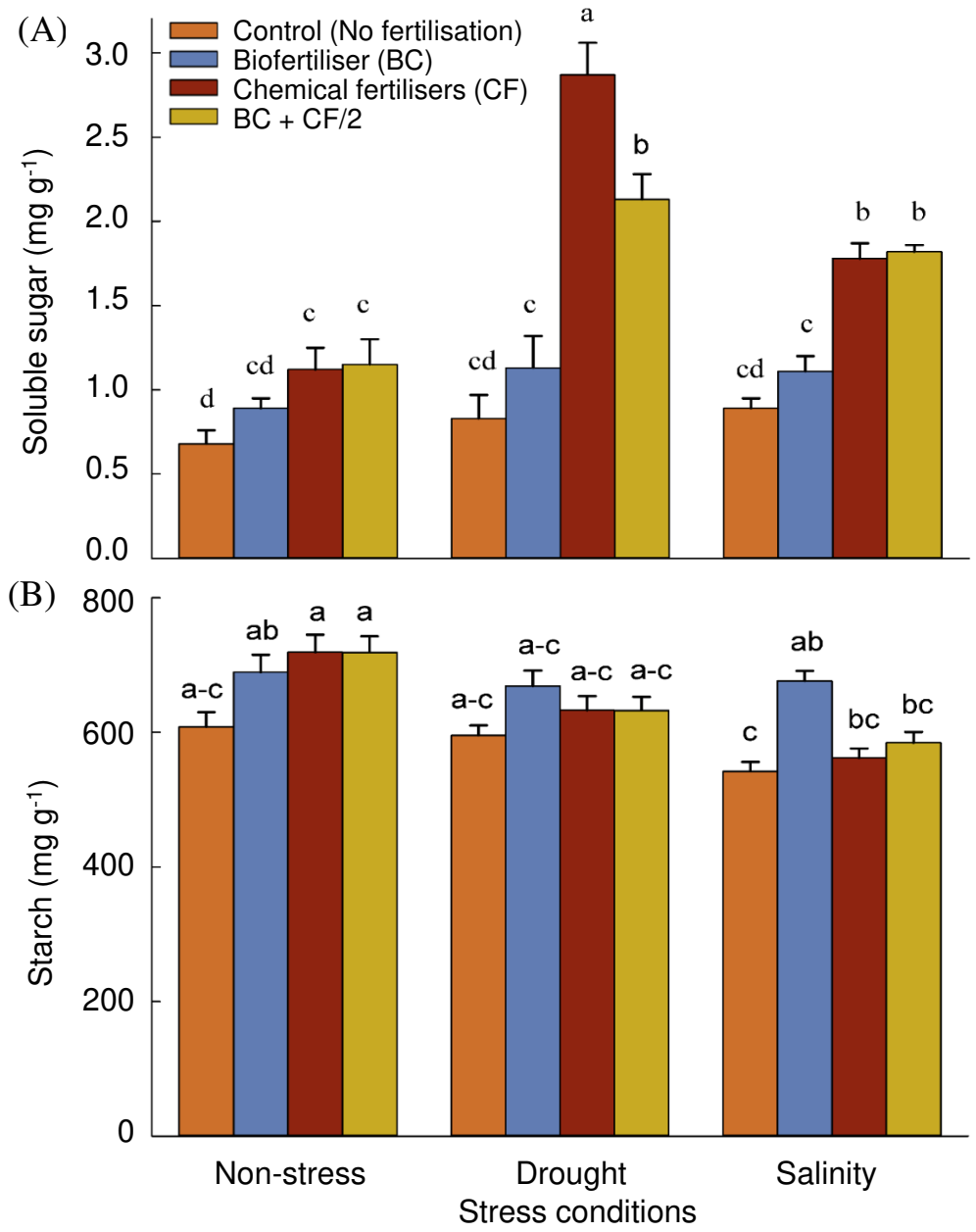
FIGURE 3 The effect of chemical and biofertilization on amino acid composition of durum wheat grains under optimal and stress conditions. Co: No fertilization (control); BC: Biofertilizer consortium of four

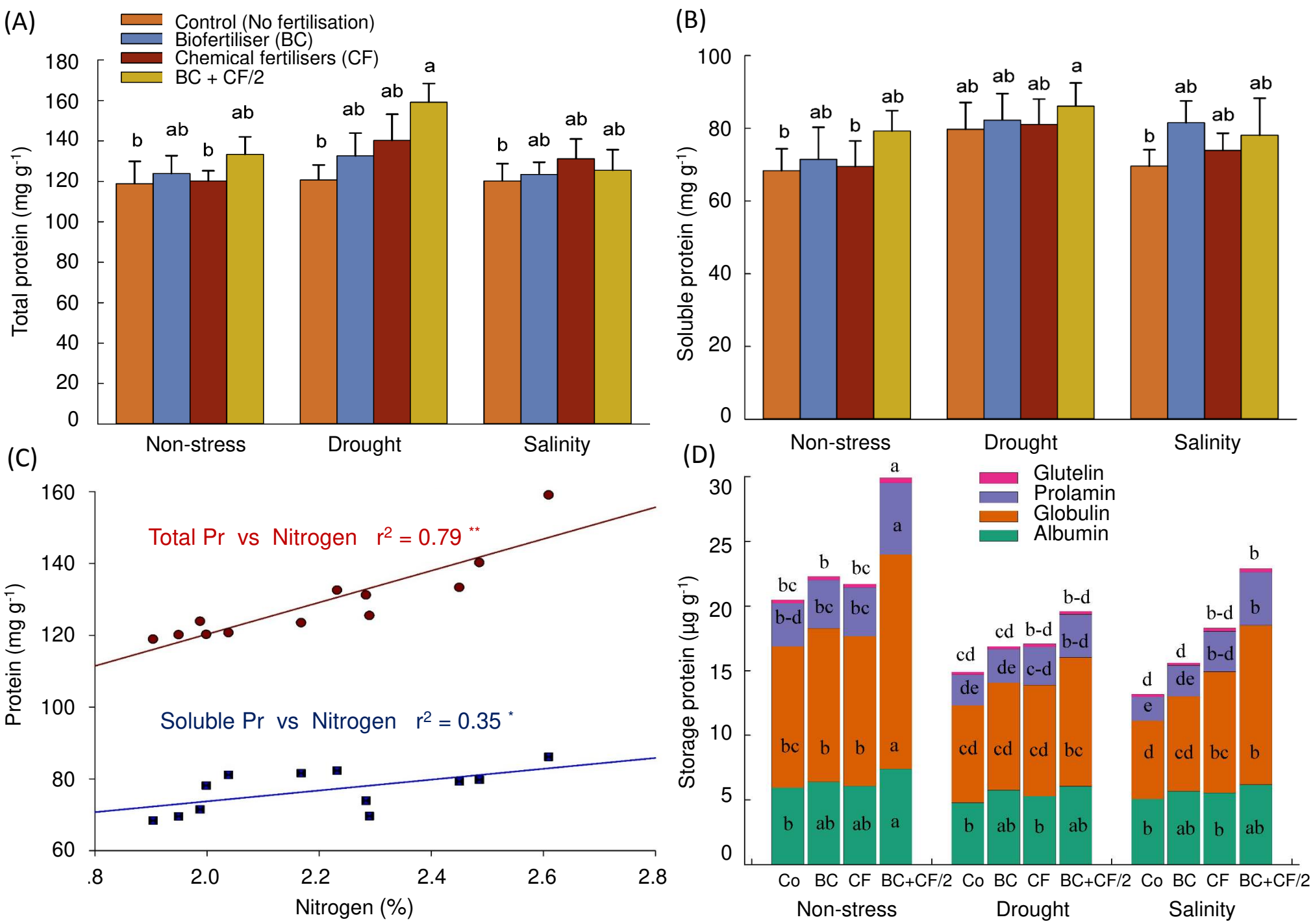
PGPB strains; CF: Soil treated by chemical fertilizers; BC+ ½CF: A combination treatment of biofertilizer consortium and half dose of chemical fertilizers

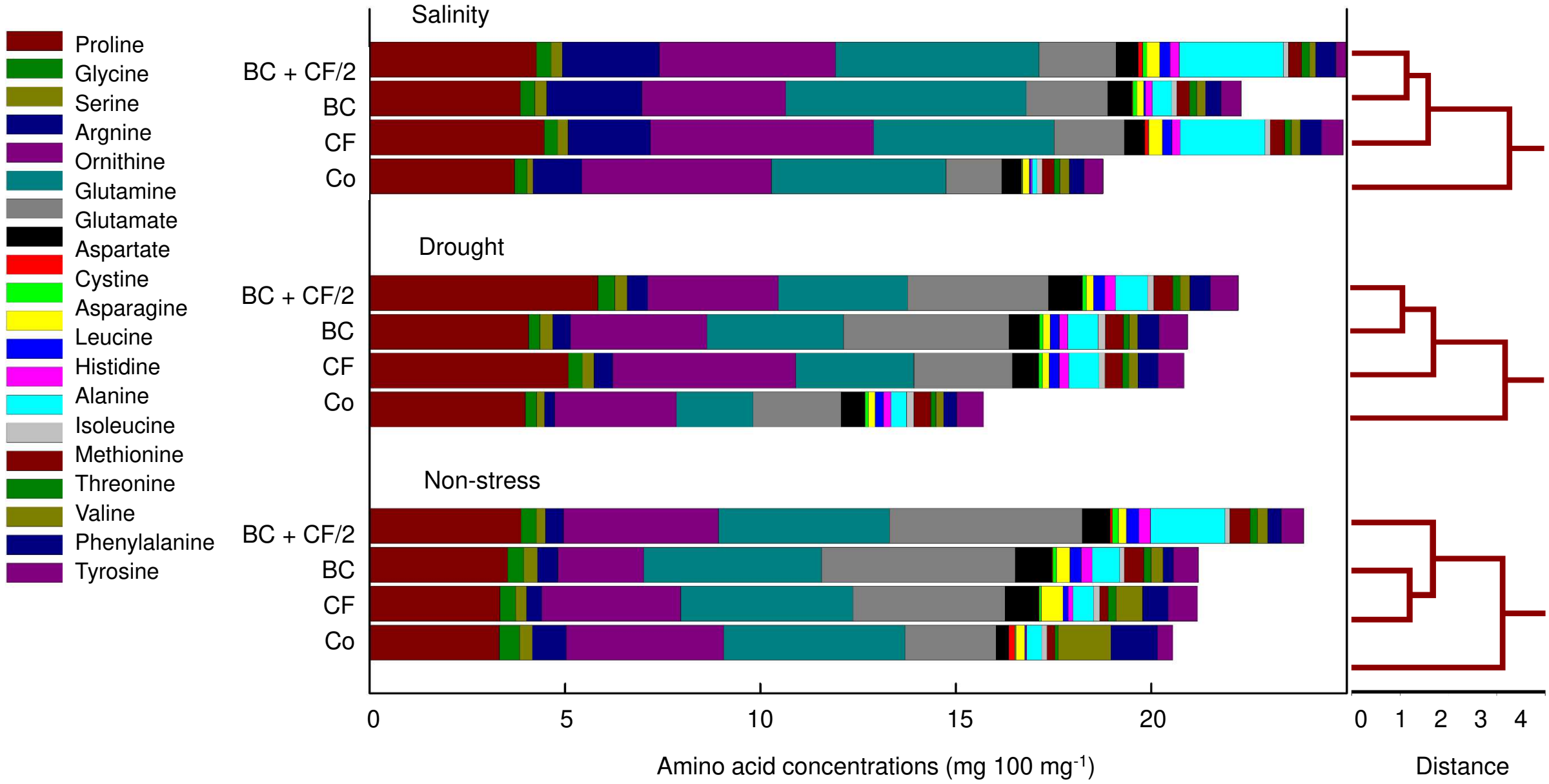
FIGURE 4 The content of proline (a) the ferric reducing antioxidant power (FRAP) (b), polyphenols (c), and lipid (d) in grains under different fertilization and stress treatments. Means (± standard error; n = 3) followed by similar letter(s) are not significantly different at 5% probability level (Tukey's HSD test)

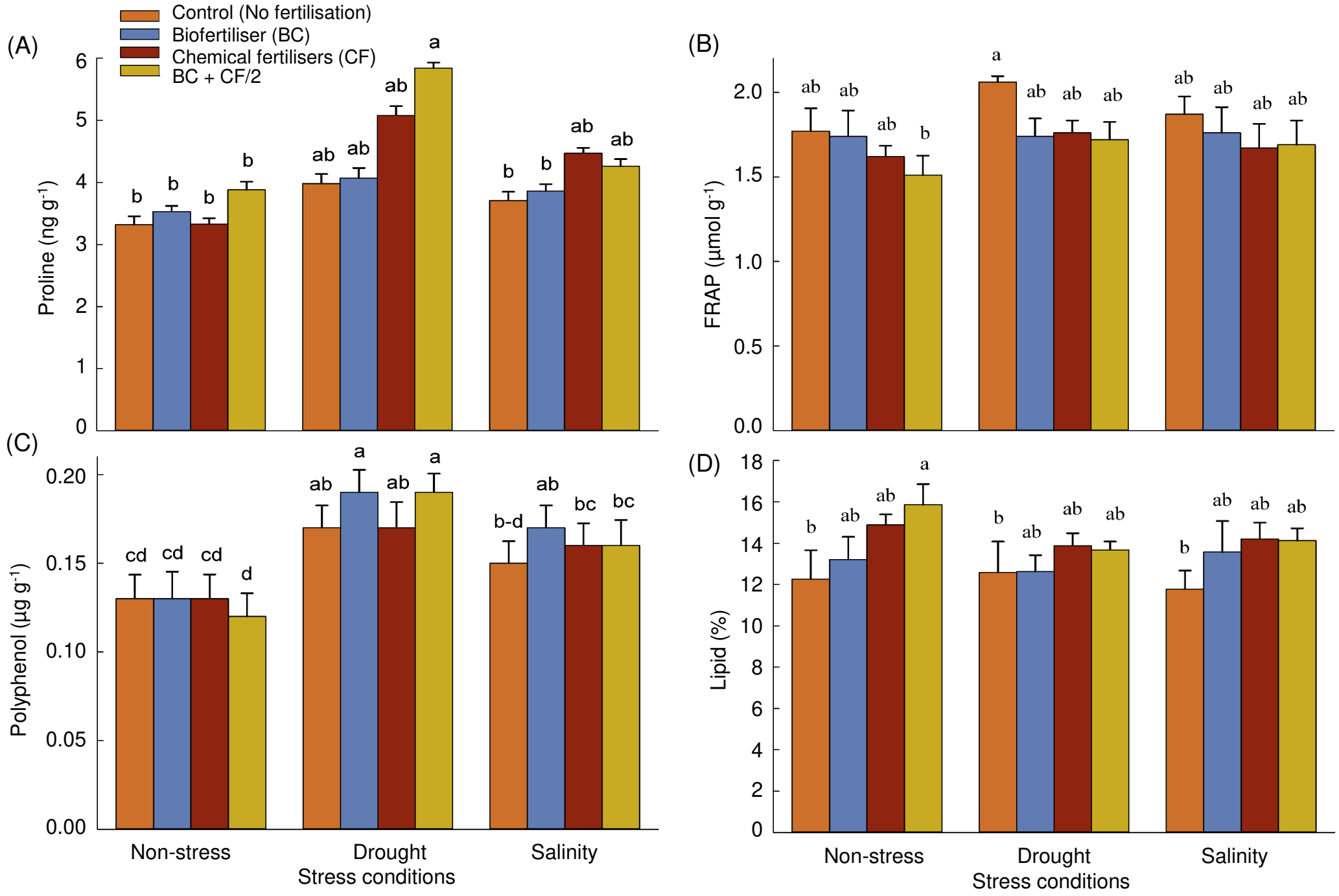
FIGURE 5 The effect of chemical and biofertilization on tocopherol levels of durum wheat grain under optimal and stress conditions. Means in each parameter followed by similar letter(s) are not significantly different at 5% probability level (Tukey's HSD test). Co: No fertilization (control); BC: Biofertilizer consortium of four PGPB strains; CF: Soil treated by chemical fertilizers; BC+ ½CF: A combination treatment of biofertilizer consortium and half dose of chemical fertilizers

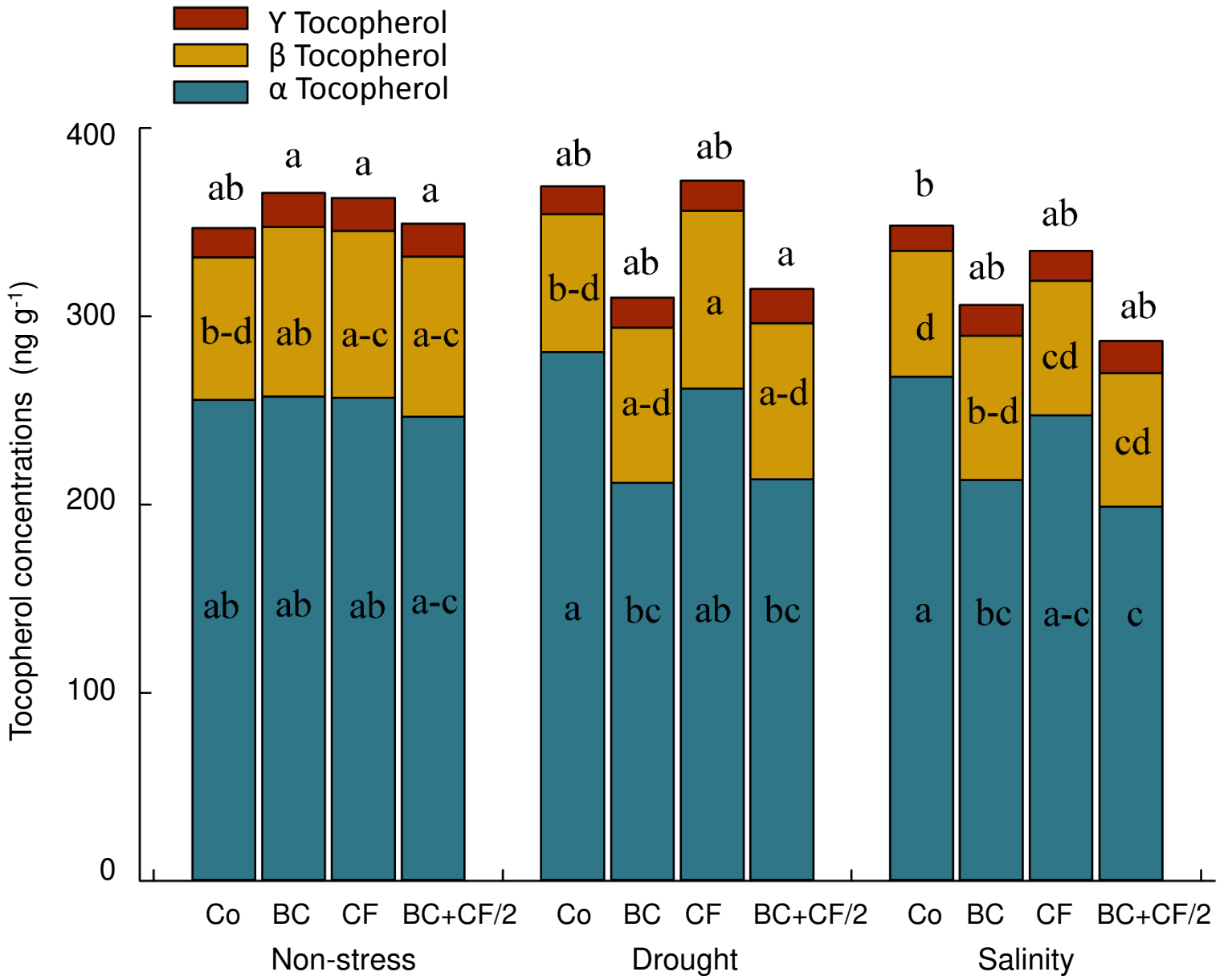
FIGURE 6 The effect of chemical and biofertilization on fatty acid composition levels of durum wheat under optimal and stress conditions. Co: No fertilization (control); BC: Biofertilizer consortium of four PGPB strains; CF: Soil treated by chemical fertilizers; BC+ ½CF: A combination treatment of biofertilizer consortium and half dose of chemical fertilizers


(A)
Control (No fertilisation)
Biofertiliser (BC)
Chemical fertilisers (CF)
BC + CF/2
Soluble sugar (mg g-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
d
cd
c
c
cd
c
a
b
cd
c
b
b
(B)
Starch (mg g-1)
800
600
400
200
0
a-c
ab
a
a
a-c
a-c
a-c
a-c
c
ab
bc
bc
Non-stress
Drought
Salinity
Stress conditions


(A)
Control (No fertilisation)
Biofertiliser (BC)
Chemical fertilisers (CF)
BC + CF/2
Total protein (mg g-1)
Non-stress
Drought
Salinity
(B)
Soluble protein (mg g-1)
Non-stress
Drought
Salinity
(C)
Total Pr vs Nitrogen r2 = 0.79 **
Soluble Pr vs Nitrogen r2 = 0.35 *
Protein (mg g-1)
Nitrogen (%)
(D)
Glutelin
Prolamin
Globulin
Albumin
Storage protein (µg g-1)
Co BC CF BC+CF/2
Non-stress
Drought
Salinity


Proline
Glycine
Serine
Argnine
Ornithine
Glutamine
Glutamate
Aspartate
Cystine
Asparagine
Leucine
Histidine
Alanine
Isoleucine
Methionine
Threonine
Valine
Phenylalanine
Tyrosine
Salinity
BC + CF/2
BC
CF
Co
Drought
BC + CF/2
BC
CF
Co
Non-stress
BC + CF/2
BC
CF
Co
0
5
10
15
20
Amino acid concentrations (mg 100 mg-1)
0
1
2
3
4
Distance


Control (No fertilisation)
Biofertiliser (BC)
Chemical fertilisers (CF)
BC + CF/2
(A)
Proline (ng g-1)
(B)
FRAP (µmol g-1)
(C)
Polyphenol (µg g-1)
(D)
Lipid (%)
Non-stress
Drought
Salinity
Stress conditions


γ Tocopherol
β Tocopherol
α Tocopherol
Tocopherol concentrations (ng g-1)
400
300
200
100
0
ab
a
a
a
b-d
ab
a-c
a-c
ab
ab
ab
a-c
ab
b-d
ab
ab
a
a-d
a
a-d
a
bc
ab
bc
b
ab
ab
d
b-d
cd
a
bc
a-c
cd
Co
BC
CF
BC+CF/2
Co
BC
CF
BC+CF/2
Co
BC
CF
BC+CF/2
Non-stress
Drought
Salinity

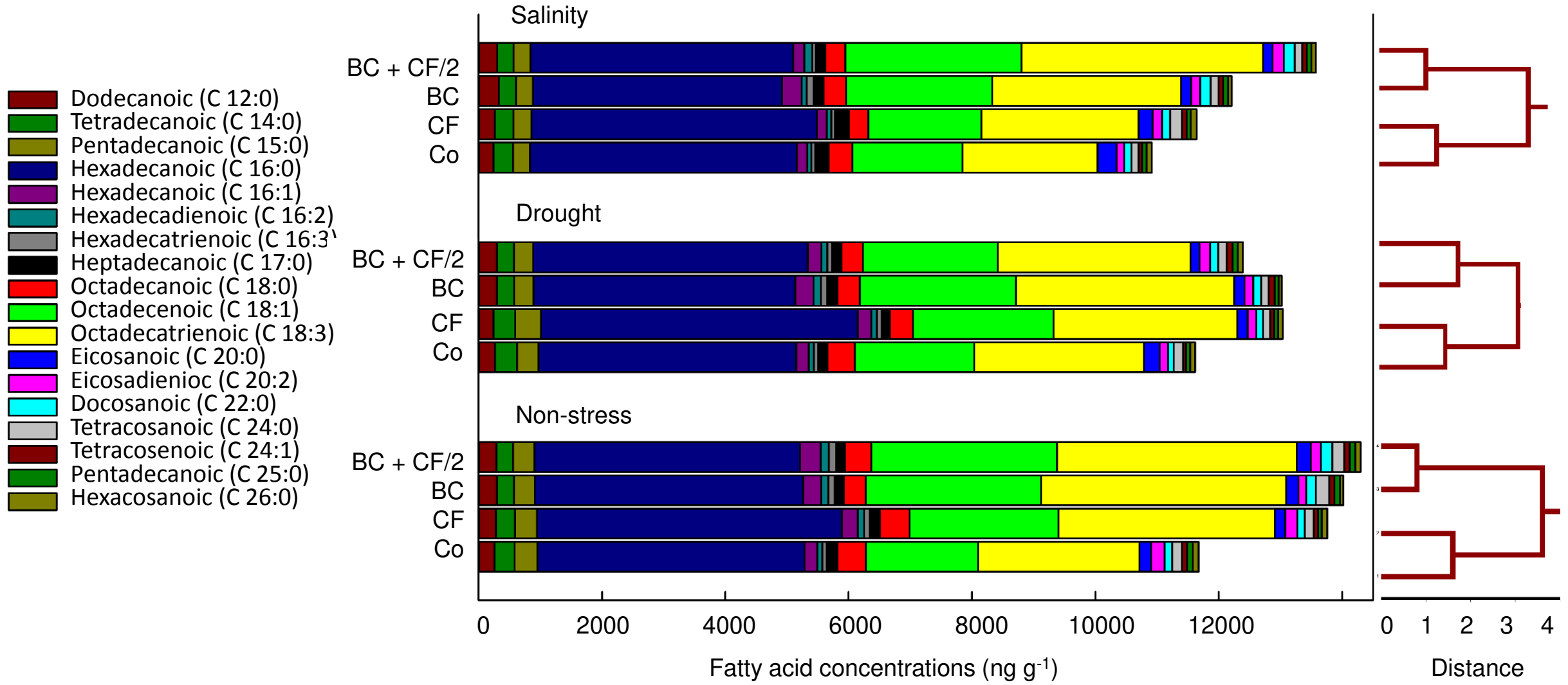

Dodecanoic (C 12:0)
Tetradecanoic (C 14:0)
Pentadecanoic (C 15:0)
Hexadecanoic (C 16:0)
Hexadecanoic (C 16:1)
Hexadecadienoic (C 16:2)
Hexadecatrienoic (C 16:3)
Heptadecanoic (C 17:0)
Octadecanoic (C 18:0)
Octadecenoic (C 18:1)
Octadecatrienoic (C 18:3)
Eicosanoic (C 20:0)
Eicosadienioc (C 20:2)
Docosanoic (C 22:0)
Tetracosanoic (C 24:0)
Tetracosenoic (C 24:1)
Pentadecanoic (C 25:0)
Hexacosanoic (C 26:0)
Salinity
BC + CF/2
BC
CF
Co
Drought
BC + CF/2
BC
CF
Co
Non-stress
BC + CF/2
BC
CF
Co
0
2000
4000
6000
8000
10000
12000
Fatty acid concentrations (ng g-1)
0
1
2
3
4
Distance

TABLE 1
The effect of chemical and biofertilization on organic acid concentrations of durum wheat grains under optimal and stress conditions.

Stress	*Fertilization*	Succinate (ng g^{-1})	% Change	Malate (ng g^{-1})	% change	Citrate (ng g^{-1})	% change	Lactate (ng g^{-1})	% change	Trans-aconitic (ng g^{-1})	% change	Oxalate (ng g^{-1})	% change
Non-stress	Co	307.19 ab	-	96.93 ab	-	184.15 a-c	-	176.34 a-c	-	29.80 bc	-	60.70 ab	-
	BC	347.64 ab	+ 13.2	106.27 a	+ 9.6	151.44 d	– 21.6	142.63 d	– 23.6	31.06 a-c	+ 4.2	64.81 a	+ 6.8
	CF	297.52 ab	– 3.15	108.37 a	+ 11.8	191.83 a-c	+ 4.2	182.83 a-c	+ 3.7	28.59 bc	– 4.2	60.82 ab	+ 0.2
	BC+ ½CF	327.98 ab	+ 6.8	98.74 ab	+ 1.9	181.60 a-c	– 1.4	172.79 a-c	– 2.1	32.13 a-c	+ 7.8	66.55 a	+ 9.6
Drought	Co	360.83 ab	-	95.89 ab	-	196.45 ab	-	188.43 ab	-	33.07 ab	-	63.33 ab	-
	BC	266.89 b	– 35.2	92.62 ab	– 3.5	148.86 d	– 32.0	141.15 d	– 33.5	33.04 ab	– 0.1	66.63 a	+ 4.7
	CF	385.44 a	+ 6.8	110.41 a	+ 15.1	208.80 a	+ 6.3	200.76 a	+ 6.5	27.10 c	– 22.0	63.63 ab	+ 0.5
	BC+ ½CF	290.12 ab	– 24.4	94.91 ab	– 1.0	163.36 cd	– 20.2	154.21 cd	– 22.2	29.28 bc	– 12.9	68.10 a	+ 7.5
Salinity	Co	337.02 ab	-	85.62 b	-	184.90 a-c	-	176.71 a-c	-	26.70 c	-	51.28 b	-
	BC	305.06 ab	– 10.5	82.05 b	– 4.3	167.01 b-d	– 10.7	159.78 b-d	– 10.6	36.07 a	+ 35.1	67.47 a	+ 31.6
	CF	315.86 ab	– 6.7	86.36 b	+ 0.8	171.41 b-d	– 7.9	164.68 b-d	– 7.3	29.18 bc	+ 9.3	55.94 ab	+ 9.1
	BC+ ½CF	269.96 b	– 24.8	87.25 b	+ 1.9	151.07 d	– 22.4	143.03 d	– 23.5	33.66 ab	+ 26.1	58.08 ab	+ 13.3

Means in each column followed by similar letter(s) are not significantly different at 5% probability level (Tukey test).
Co: No fertilization (control); BC: Biofertilizer consortium of four PGPB strains; CF: Soil treated by chemical fertilizers; BC+ ½CF: A combination treatment of biofertilizer consortium and half dose of chemical fertilizers. (n = 3)

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TABLE 2
The effect of chemical and biofertilization on nutrient concentrations of durum wheat grain under optimal and stress conditions.

Stress	*Fertilization*	N (%)	% change	P (µg g^{-1})	% change	K (µg g^{-1})	% change	Zn (µg g^{-1})	% change
Non-stress	Co	1.90 d	-	4577.11 b	-	6698.42 e	-	45.40 c	-
	BC	1.99 cd	+ 4.6	5906.83 ab	+ 29.1	7969.54 c-e	+ 19.0	52.47 bc	+ 15.6
	CF	1.95 cd	+ 2.6	7370.27 a	+ 61.0	8933.29 b-d	+ 33.4	51.34 bc	+ 13.1
	BC+ ½CF	2.45 ab	+ 28.9	7491.67 a	+ 63.7	8852.97 b-d	+ 32.2	59.71 ab	+ 31.5
Drought	Co	2.04 cd	-	5921.56 ab	-	7291.14 de	-	51.10 bc	-
	BC	2.23 b-d	+ 9.4	7389.27 a	+ 24.8	9432.57 a-c	+ 29.4	70.83 a	+ 38.6
	CF	2.49 ab	+ 21.9	8544.60 a	+ 44.3	11464.94 a	+ 57.2	70.15 a	+ 37.2
	BC+ ½CF	2.61 a	+ 27.9	8076.81 a	+ 36.4	10390.39 ab	+ 45.5	67.55 a	+ 32.2
Salinity	Co	2.00 cd	-	6083.65 ab	-	7862.21 c-e	-	50.44 bc	-
	BC	2.17 b-d	+ 8.4	6504.37 ab	+ 6.9	8431.18 b-e	+ 7.2	58.13 a-c	+ 15.2
	CF	2.28 a-c	+ 14.1	6637.40 ab	+ 9.1	8053.02 c-e	+ 2.4	53.86 bc	+ 6.8
	BC+ ½CF	2.29 a-c	+ 14.5	7925.17 a	+ 30.3	9718.84 a-c	+ 23.6	62.57 ab	+ 24.1

Means in each column followed by similar letter(s) are not significantly different at 5% probability level (Tukey test).
Co: No fertilization (control); BC: Biofertilizer consortium of four PGPB strains; CF: Soil treated by chemical fertilizers; BC+ ½CF: A combination treatment of biofertilizer consortium and half dose of chemical fertilizers.(n = 3)

TABLE 3

Correlation coefficients (r) between nutrient concentration in grain and some metabolic parameters in response to the fertilization and stress treatments (n = 12).

	N	P	K	Zn
Total protein	**0.89** **	**0.73** **	**0.81** **	**0.73** **
Soluble protein	**0.59** *	0.19 ns	0.34 ns	0.42 ns
Soluble sugar	**0.79** **	**0.76** **	**0.83** **	**0.61** *
Lipid	0.48 ns	**0.63** *	0.48 ns	0.31 ns
Starch	0.32 ns	0.24 ns	0.21 ns	0.19 ns
FRAP	– 0.38 ns	– 0.56 ns	– 0.47 ns	– 0.36 ns
Polyphenols	0.46 ns	0.21 ns	0.29 ns	0.42 ns
Proline	**0.87** **	**0.65** *	**0.73** **	**0.60** *
Grain Yield	0.35 ns	0.29 ns	0.37 ns	0.31 ns

* and ** Significant at $P < 0.05$ and $P < 0.01$ level, respectively. ns: Not Significant