A consortium of cyanobacteria and plant growth promoting rhizobacteria for wheat growth improvement in a hydroponic system

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Abstract

Biofertilizers containing living organisms are attractive because of their potential positive impact on plant growth and reduced environmental impacts compared to mineral fertilizers. Many studies have reported the potential of plant growth-promoting rhizobacteria (PGPR) and cyanobacteria, applied singly or in combination. In the present study, the combined effect of PGPR and cyanobacteria on *Triticum aestivum* L. was examined in a hydroponic growth system. *Calothrix* sp. and *Anabaena cylindrica* were used as cyanobacterial strains, and *Chryseobacterium balustinum*, *Pseudomonas simiae* and *Pseudomonas fluorescens* were used as PGPR strains. In addition to growth parameters, total nutrients in each treatment and the ability to produce indole acetic acid (IAA) were measured after 17 days. The study results indicate that the consortium of the five isolates gave the best performance in terms of growth parameters. It increased by 36% for plant height in the combination of *Calothrix* sp. and *P. simiae*, and the dry shoot mass was increased by 80%, 77%, and 76% under the combinations of *A. cylindrica* with *C. balustinum*, *P. simiae*, and *P. fluorescens*, respectively. The ability to produce IAA was confirmed in the treatments with cyanobacteria, with PGPR strains, and in treatments combining the different microorganisms, using both colorimetric and chromatographic methods. Thus, biofertilizers containing the consortia cyanobacteria and PGPR used in this study are recommended for improved growth of wheat plants.

Keywords: Bio-fertilizer; hydroponic; cyanobacteria; Plant growth-promoting rhizobacteria; phytohormone

1. Introduction

In order to make agriculture more sustainable, alternative fertilizing systems that allow reduced application rates of chemicals are being sought. Microorganism-based fertilizers (biofertilizers) are considered a way to achieve improved sustainability of food production. Biofertilizers are attractive because of their reduced negative impacts on the environment as compared to chemical fertilizers. Many studies have reported plant growth improvements following inoculation of plant soil with growth promoting rhizobacteria (PGPR) (Datta et al. 2011; Bakker et al. 2007). Generally, plant growth promoting rhizobacteria facilitate the plant growth directly by either assisting in resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents (Glick et al 2007; Glick 2012; Munees and Mulugeta 2014).

Studies have also been carried out on the effect of cyanobacteria as a partial substitute for chemical fertilizers (Osman et al 2010). Cyanobacteria may be the most important nitrogen-fixing agents in many agricultural soils (Nayak et al. 2004; Asari et al. 2007). Reducing chemical inputs in exchange for biological sources helps ameliorate ecosystem stressors, keeping in mind that nitrous gas emissions resulting from contemporary fertilizer use is responsible for multiple environmental hazards including greenhouse gas accumulation, water contamination, and acidification of water (Choudhury and Kennedy 2005; Rai 2006). Cyanobacteria are also known to produce a variety of extracellular substances such as plant growth regulators (Whitton 2000; Prasanna et al. 2010), amino acids (Flynn and Gallon 1990), vitamins (Indira and Biswajit 2012), polysaccharides (Maqubela et al. 2009), and antimicrobial products (Rizk 2006; Tassara et al. 2008) which have direct or indirect impacts on plant growth and subsequent yield.

The success of cyanobacteria as ecological group in a wide range of habitats has been attributed to their unique physiological characteristics, which bestows them with a high adaptive ability under a wide range of environmental conditions (Prasanna et al. 2012). They are a ubiquitous group of prokaryotes, whose evolution (amply supported by fossil and molecular evidence) can be traced back 3.5 billion years (Mundt et al. 2001). Although cyanobacteria are globally important primary producers, exhibiting a worldwide distribution in diverse ecosystems and contributing significantly to the fertility of rice fields, research on their role as biofertilizers is gaining interest since the last decade. Survey of literature indicates their ability to flourish with rice and wheat in aqueous media and help in enhancing plant growth by improving availability of nutrients and colonizing roots (Jaiswalet al.2008; Karthikeyan et al.2009; Babuetal.2015;Bidyarani et al.2015 ; Baglieri et al.2016 ; Chittapun et al.2018 ; Barone et al.2019).

Additionally, hydroponic cultivation faciltates an even distribution of nutrients and water to the plants, thereby reducing wastage and simulating ideal growing conditions (Bharti 2019). Addition of a protein hydrolysate from the cyanobacterium *Spirulina platensis* increased the maize (*Zea mays* L.) plant growth in hydroponic cultivation due to increased supply of nitrogen (Ertani et al., 2019). As well, mutual benefit regarding biomass yield was obtained in a hydroponic co-cultivation of tomato plants and two green algae (*Chlorella vulgaris* and *Scenedesmus quadricauda*) (Barone et al., 2019). However, studies regarding biostimulation in hydroponic cultivation with combined PGPR and cyanobacteria scarce.

The development of biofertilizers using a combination with two or more microorganisms such as algae-bacteria or bacteria-bacteria may help increasing crop productivity. Application of PGPR, singly or in consortia with cyanobacteria, has been shown to produce enhancements in wheat-plant height, dry weight, and grain yield in previous studies (Karthikeyan et al.,2007; Nain et al., 2010; Manjunath et al., 2011). However, the apparent increase in plant growth observed in laboratory controlled experiments has not always had the same results in the field because of the particular microbiome of a given plant variety (Mezzasalma et al., 2018; Walters et al., 2018), which in turn affects the plant exudate that promotes the benefitial influence of the rhizome (Sasse et al., 2017), and, therefore, exploring the effect of diverse synthetic microbial communities (SynComs) is demanded, trying to keep the same environmental origin as the cultured plant (Dal Cortivo et al., 2020; de Souza et al, 2020). Thus, in the present study we evaluated (i) two edaphic cyanobacterial strains and PGPR inoculants, which could influence the growth of wheat including weight, height, chlorophyll content, and (ii) the effect of cyanobacteria inoculation on the contents of C and N in the liquid medium of hydroponic system. We hypothesized that addition of live cyanobateria and PGPR biomass would improve plant growth by way of secreted biomolecules that may act as plant growth promoters.

2. Material and Methods

2.1. PGPRs strains source

The bacterial strains used were *Chryseobacterium balustinum*, *Pseudomonas simiae* and *Pseudomonas fluorescens*. All of them were grown in brain heart infusion (BHI) broth medium to reach 10^8 CFU mL^{-1}. The bacterial strains *Pseudomonas fluorescens*, and *Chryseobacterium balustinum* were provided by the Laboratory of Microbiology of the University of Burgos (Spain). *Pseudomonas simiae* was provided by Plant-Microbe Interactions Group of the Institute of Environmental Biology of the University Utrecht (Netherlands).

2.2. Growth of cyanobacterial strains

A strain of *Anabaena cylindrica*, heterocystous freshwater cyanobacteria, was isolated from the Ap horizon of an irrigated field located at Losar de la Vera (Cáceres, Spain): 40º1'53'' N, 5º36'49'' W. *Calothrix* sp. is one of the potent groups of N_2-fixing cyanobacteria and was collected from the sediment

of a small water stream located at Ros (Burgos, Spain): 42°29'44.1"N, 3°48'18.0"W. For the isolation of the cyanobacterial strains, 1 g of soil was suspended in 100 mL of BG-11 medium (pH 7.5) without $NaNO_3$ as nitrogen source (BG11-N). From this suspension, an aliquot of 100 µL was taken and plated in a Petri dish with BG11-N and 2% agar. The aliquot was distributed homogeneously, closing the plates to prevent drying. Purification of this strain was performed by serial dilution and plating method. After morphological examination (**Figure 1a**) and pigment composition determination by high performance liquid chromatography with photodiode array detection (HPLC-DAD) from a methanol extract (**Figure 1b**), purification and sequencing of ribosomal 16S (*Anabaena*) subunit DNA (rDNA) of a sample of pure culture cyanobacteria using the UltraClean kit (MO BIO Laboratories, Inc.) was conducted for identification of *A. cylindrica*. The DNA sample was amplified by polymerase chain reaction (PCR) using appropriate primers. The amplification product was purified by electrophoresis in agarose gel and its base sequence obtained in an ABI Prism 3100 Sequencer (Applied Biosystem). The similarity of the genetic sequence with respect to those found in the National Center for Biotechnology Information (NCBI) database was assessed using the BLAST analysis application. *Calothrix* sp. was only identified by morphology and pigment composition.

Isolated strains of *Anabaena cylindrica* and *Calothrix* sp. were cultured in 250 mL BG11-N medium in photo-bioreactors in a growth chamber with a 16:8 photoperiod, photon density of 100 µmol m^{-2} s^{-1} and 28°-18°C during light-dark periods and forced aeration; thereafter it was transferred to 500 mL Erlenmeyer flasks. The flasks were exposed to a light/dark cycle of 16:8 h, respectively, at room temperature, with regular microscopic examination in order to monitor the culture for contamination. The evolution of the stock cyanobacterial cultures was monitored by measuring absorbance of 1 mL aliquot at 750 nm on a UV-Vis spectrophotometer (GENESIS 2, Milton Roy), as well as turbidity (HI93703 Turbidity Meter, Hanna). Finally, the volumetric dry biomass of the culture was evaluated gravimetrically by extracting 1 mL aliquots and drying in an oven at 60 °C.

2.3. Experimental set-up

A total of 13 different experimental treatments were employed in order to test the plant growth stimulating properties of the cyanobacteria and mixtures of these with the different chosen bacteria; an overview of these treatments and their codes are shown in **Table 1**. Basically, these treatments included either (i) cyanobacteria (*A. cylindrica* and *Calothrix* sp.) which were were cultivated in BG11 to reach a final concentration of 1.8 g DM L^{-1}, (ii) PGPR (*C. balustinum*, *P. simiae* and *P. fluorescens*) after cultivation in BHI media to reach a concentration of 10^8 UFC mL^{-1} of bacterial suspension, or (iii) organism mixture (1:1, v/v). Finally, three experimental uninoculated controls were included: deionized water, BG11 (the cyanobacteria growth medium) and BGI (the bacterial growth media). All of these controls were previously sterilized.

A hydroponic system was chosen for evaluating the plant growth promoting properties of the different microorganism treatments. Hydroponic systems which are considered as appropriate experimental setups for biofertilization studies, since they facilitate visualization and measurement of plant root-related biochemical parameters (Gantar et al. 1995). A hydroponic setup was designed using truncated plastic Eppendorf microtubes in order to permit root growth of germinated seedlings through tips. All materials used in the experiment were sterilized before adding the seedlings and the cyanobacteria or PGPR cultures, and five replicates of each treatment were prepared. Seeds of wheat (*Triticum aestivum L* Var. CAMARGO) were sterilized by immersing in 5% NaClO for 2 min, thereafter they were thoroughly rinsed several times with distilled water, and then germinated between two layers of filter paper. Two-day germinated wheat seedlings were placed in test tubes containing 40 mL sterile distilled water. Then they inoculated with 10 mL of different treatment solutions. Once prepared, the entire setup was kept in a growth chamber with the conditions described above.

After 17 d, all plants were harvested and various physiological parameters were determined: root length, shoot height, and dry and fresh weights. Chlorophyll contents were measured on all plant stems with a handheld chlorophyll content meter (CCM-200 plus, Opti-Sciences, Hudson, USA). Samples of the liquid media from all replicates were kept in sterilized flasks and stored in freezer at -20 °C until their analysis. Different chemical parameters were measured in liquid samples: pH, Electrical conductivity (EC), turbidity, total carbon C, total N and inorganic C contents with an automatic combustion analyzer (SHIMADZU (TOC-V CSN)), and NO_3^--N, NH_4^+-N and PO_4^{-3}-P with a segmented flow auto-analyzer (SAN++).

2.4. Determination of IAA-like compounds by colorimetry

Four milliliters of the aqueous media were mixed with 2 mL of L-Tryptophan (4g L^{-1}) and incubated in darkness at 30°C for 48h. After incubation, 3 mL of this mixture were added to test tube and treated with 2 mL of the Salkowski reagent (2 mL of 0.5 M $FeCl_3$ were mixed with 98 mL of 35% $HClO_4$, Gordon and Weber, 1951). The mixture was allowed to stand for 30 min for color development. The intensity of the color developed was measured at 535 nm by using a spectrophotometer (GENESIS 2, Milton Roy). The amount of L-tryptophan-derived auxin content in liquid of each treatment was reported as IAA-equivalents after correlation curves were developed using standard IAA solutions (i.e. 0, 20, 40, 100 and 200 mg L^{-1}).

2.5. Analysis of auxins by UHPLC-MS

Samples of aqueous media (2 mL) were analyzed for indole acetic acid (IAA) contents by ultra-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS). A volume of 500 µL of the aqueous extract was mixed with 500 µL of cold methanol in an Eppendorf-like tube; the mixture was agitated and then centrifuged 15 min at 4 ºC and 12,838 *g*. An aliquot of 900 µL of the supernatant was withdrawn and evaporated to dryness in a speed-vac concentrator (SAVANT SPD111V, Thermo Scientific), the pellet was immediately resuspended in 100 µL of methanol:water (9:1, v/v), and 6.0 µL of this solution analyzed by UHPLC-MS. Indoleacetic acid molecules eluted at 1.73 min in the chromatographic system and were detected in negative mode as the $[M-H]^-$ ion with m/z 174.0555 by mass spectrometry. An Acquity UPLC® HSS T3 column (10 × 2.1 mm, 1.8 µm particle size), with the corresponding guard pre-column (10 × 2.1 mm, 1.8 µm particle size) (Waters, Milford, USA) was connected to the electrospray ionization (ESI) source of a SYNAPT HDMS G2 QToF mass spectrometer (Waters, Manchester, UK). Gradient elution was conducted at a flow rate of 0.5 mL min^{-1}, using as solvent (A) a mixture of methanol:water (1:1, v/v) and solvent (B) a mixture of acetonitrile:methanol (1:1, v/v), both solvents with 5 mM ammonium acetate; the gradient was: initial, 99.9% A; 0.5 min, isocratic; 1.5 min, 20% A; 3.0 min, 20% A; 4.0 min, 0.1% A; 8.0 min, isocratic; 10.0 min, 99.9% A. Parameters of the mass spectrometer were: 100 – 1200 Da in resolution mode; capillary voltage 2.0 kV; source temperature, 90 ºC; desolvation temperature, 300ºC; cone gas, 10 l/h; desolvation gas, 700 l/h; and scan time, 0.2 s. As reference for the mass correction leucine-enkephalin was used (m/z 554.2615). For quantification, 3-indoleactic acid (CAS No. 87-51-4, $C_{10}H_9NO2$) from SIGMA-ALDRICH was used to draw a calibration curve ([Indoleacetic acid] = 0.0052 × chromatographic peak area + 0.0163, $R^2 = 0.99$) with five different concentrations. The presence of other phytohomones such as gibberelines, kinetine or abscisic acid was only determined qualitatively.

2.6. Enzyme activity

Extracellular enzyme activity was measured in the liquid media according to the method of Marx et al. (2001), which is based on the use of fluorogenic MUB or AMC-substrates, in 96-microtiter plates. Samples were analysed for acid (AcPA) and alkaline phosphatases (AlkPA) (EC 3.1.3.2 and EC 3.1.3.3/AlkPA, respectively), β- (bGA) and α-glucosidases (aGA) (EC 3.2.1.20 and EC 3.2.1.21, respectively), N-acetyl-β-glucosaminidase (bNAG) (EC 3.2.1.30), β-xylosidase (bXyl) (EC 3.2.2.27), leucine-aminopeptidase (LeuAMP) (EC 3.4.11.1) and arylsulfatase (AS) (EC 3.1.6.1) using 4-methylumbelliferone (MUB) or amino-4-methylcoumarin (AMC) derivatives as substrates. Homogenous aliquots of 50 µL were withdrawn and dispensed into a 96 well microplate (eight analytical replicates per sample). Buffer (50 µL) (100 mM MUB buffer, pH 6.1 for carbohydrases and AcPA, pH 7.8 for LeuAMP and 10 for AlkPA) and 100 µL of the different 1 mM substrate solutions were added giving a final substrate concentration of 500 µM. Plates were kept for 180 min at 30 °C under agitation (150 rpms); the

reaction was stopped by the addition of 50 µL of Tris-Buffer at pH 12 and immediately read in a fluorometric plate-reader (TECAN, Switzerland) using excitation-emission filters of 360-450 nm, respectively. Fluorescence was converted into amount of MUB (4-methylumbelliferone) or AMC (7-amino-4-methylcoumarin) according to specific standards (0 to 1500 pmol) prepared on each plate in order to take into account the degree of fluorescence quenching through aqueous extract particles and organic matter. Enzymatic activities were converted to specific activity normalized to the TOC carbon content of each fraction.

2.7. Statistical analysis

The results obtained for each variable were analyzed for statistical differences between treatments using SPSS v.18.0 (SPSS 2009). After checking for normality and homogeneity of variances using the Kolmogoroff and Levene tests, respectively, the data were subjected either to the (parametric) one-way ANOVA with significance defined at $p \leq 0.05$. Thereafter, the Tukey's *post-hoc* test was used to compare treatments. Non-parametric analyses were used if data were not normally distributed and could not be transformed. The Mann-Whitney (U) Test was used to detect significant ($\alpha = 99\%$) differences between treatments in the latter case.

3. Results

3.1. Effects on physiological plant parameters

As shown in Figure 2, in which the panels show example plants from each experimental treatment, the different combinations of microorganisms had large effects and very dissimilar effects on the growth of wheat. For organizational purposes, we separate the detailed consideration of the results of the plant growth experiment by first examining the effects of the cyanobacteria alone on the plant, and then the cyanobacteria + bacterial consortia: Application of cyanobacteria alone (treatment A) significantly increased various biometric parameters (**Figure 4**) as determined by one-way ANOVA comparing treated plants and control (BG11). Fresh shoots ($F[3.16]=16.75$, $p < 0.05$) and roots ($F[3.16]=21.87$, $p < 0.05$) were substantially enhanced in the Ana treatment (**Fig. 4A**). Dry root biomass was also higher in the Ana treatment as compared to the controls (BG11 and water); dry shoot biomass was also notably increased in the *Calothrix* sp. treatment (C), but no statistically significant differences with respect to the BG11 treatment were obtained for this parameter in the plants inoculated with *A. cylindrica* (**Fig. 4B**). Similarly, root and shoot lengths were statistically equivalent to those of the control (BG11) in the Cal and Ana treatments, respectively, but the opposite situation was found for the shoot ($F[3.16]=11.01$, $p< 0.05$) and root lengths ($F[3.16]=22.23$, $p < 0.05$) (**Fig. 4C**). Treatment C had the highest significant increases as compared to the controls, namely 32.84% in plant height, 28% in dry shoot weight and 21 % in dry root

weight. No significant differences were observed in the chlorophyll content between treatments apart from the water control (**Fig. 4D**).

The inoculation of plants with consortia PGPRs plus cyanobacteria led to increases in various biometric parameters as compared to control and/or to PGPR treatments alone (Figure 4). Fresh root biomass was augmented in all treatments as compared to the control (BHI), and combinations *Calothrix* sp. with bacteria (CB1-CB3) had significantly higher root biomass than innoculations with Anabaena (AB1-AB3). In contrast, there were no statistically significant differences for fresh shoot biomass, though all treatments were higher than the control (**Fig. 5A**). Both dry root and shoot biomasses had statistically significant higher values than the control (BHI), and the mixture treatments AB1, AB2 and CB3 had higher biomass values than the respective PGPR treatments alone (Fig. 4B, Post-hoc tests). The statistical test revealed significant differences for *Triticum aestivum* L. root length with regard to the control exclusively for the treatment CB2 ($F[9,40]=2.8$, $p=0.012$), with no significant differences between inoculated plants, whereas all the inoculated plants except those from the treatments B2 and AB3 had greater shoot lengths than the control ($F[9,40]=4.72$, $p\leq0.001$), again without significant differences among treatments (**Fig. 5C**). Root and shoot lengths of the plants inoculated solely with cyanobacteria were higher than the root and shoot lengths obtained in the combined treatments (**Figs. 4C and 5C**). No significant differences between treatments and the control were observed for chlorophyll contents but the values in the treatments B2, AB2 and AB3 were lower than in other treatments (**Fig. 5D**).

Comparing fresh shoot biomass of treatments combining cyanobacteria and bacteria (AB, CB) and cyanobacteria alone (A, C), the results were similar (**Figs. 5A and 4A**). This was also generally the case for fresh root biomass, with the exception that AB treatments were lower than A treatments (**Figs. 5A and 4A**). Finally, the combined treatments AB and CB had lower dry plant biomass than the relative plants solely inoculated with the cyanobacteria (**Figs. 5B and 4B**).

3.2. Effect on chemical properties

Electrical conductivity (EC) of the culture medium was significantly (*p=0.013*) affected by treatment (**Table 2**); it declined from 0.036 in the control to 0.02 dS/m in the cultures with *A. cylindrica* and *Calothrix* sp. The EC values also decreased significantly (*p=0.003*) in the combined cultures of PGPRs with *Calothrix* sp. (CB1, CB2 and CB3) as compared to the control value (3.14 dS/m), but they were close similar to the values measured for the PGPR inoculated cultures. Conversely, the EC values for the combined cultures with *A. cylindrica* were similar to those of the control. Notably, the EC values in the culture medium of inoculated plants with PGPRs alone or together with the cyanobacteria were much higher than those of the BG11 and cultures only inoculated with cyanobacteria. Results for pH and turbidity were very similar to those obtained for EC in the sense that lower values were obtained for A

and C than AB and CB, whereas pH values in B, AB and CB cultures were relatively homogenous and equivalent to the control (**Table 2)**. The highest turbidity values were shown by the PGPR inoculated cultures, whereas AB cultures along with the control (BHI) displayed the lowest turbidity values and intermediate values were measured in the CB cultures (Table 2).

Inoculation did not influence nitrogen (either total nitrogen or ammonium) or phosphorus concentrations in treatments A or C (**Table 3**). No statistically significant differences were observed for the nitrogen (either total nitrogen or ammonium) content treatments ABx or CBx , which were much lower than the nitrogen content values of the B treatments (PGPR alone) and the control. Among the treatments with inoculation by a single PGPR strain, that with *Pseudomonas fluorescens* (B3) showed the highest mean concentrations of TN and NH_4^+ with increased values of 14% and 7%, respectively, as compared to control (BHI). Similar results were obtained for phosphorus content even though significant differences were found between the treatments CB1 and CB3 (**Table 3**). The *post-hoc* tests indicated significant differences for TOC concentrations ($F [3.16] = 5.597$, $p = 0.018$) between C and BG11. For ABx CBx, TOC was significantly decreased ($F[9,40]=3.238$, $p=0.005$) in CB3 and AB2 as compared to control.

3.3. Production capacity of IAA like compounds

All treatment cultures were screened for their ability to produce and release the plant growth regulator indole acetic acid (IAA) to the liquid medium. Varying capacities for the production of IAA-like products between treatments were seen (**Figure 6**; ANOVA $p=0.009$). The highest IAA concentration in cyanobacteria alone treatments was obtained with *A. cylindrica*, which had an average IAA concentration of 22.83 ± 4.82 μg mL^{-1} whereas 6.00 ± 0.53 μg mL^{-1} were measured in the control. On the other hand, there were significant differences in this parameter for the combinations of cyanobacteria and bacteria AB2, AB3 and CB3, but only the combination AB2 exhibited a significantly higher value than the bacterial only treatment B2. According to the colorimetric method, IAA concentrations ranged between 57.41 ± 3.6 μg mL^{-1} (control) and 75.21 ± 5.4 μg mL^{-1} (CB3). IAA concentration in the liquid medium was also determined by UHPLC-MS (**Figure 7**)**;** according to these measurements, a relative different picture to that shown by the colorimetric method was pointed out. Thus, the treatment B2 had the highest IAA concentration whereas concentrations lower than in the control were found in treatments B3, CB1 and CB3. With regard to the bacteria only treatments, the combined bacteria-cyanobacteria treatments apart from those of *P. fluorescence* (B3) exhibited lower values of IAA (**Figure 7**). No IAA was detected by this latter method in the only cyanobacteria inoculated cultures (**Table 4**), but the presence of other auxins is illustrated in **Table 4**.

3.4. Enzyme activity

Global differences in extracellular enzyme activities were found between treatments (**Figure 8)**. There was notable enhanced activity as compared the control for the enzymes LeuAMP, AlkPA, bNAG and bGA in the *A. cylindrica* inoculated culture (A, **Figure 8A**), but only AlkPA activity was enhanced in the *Calothrix* sp. inoculated culture (C, **Figure 8A**), it reaching the maximum relative value (1.95 nmol mg^{-1} TOC min^{-1} is the enzymatic activity value that corresponds to the maximum relative value in the figure, which is 350). Activities higher than the control were found for B1-3 for AS (**Figure 8B**), whereas only the *P. fluorescence* (B3) inoculated culture exhibited augmented activity as compared to control in other enzymes, namely aGA and bNAG. Substantially increased AS activity ($F[9.40] = 4.66$, $p = 0.007$, Welch test) was observed in the three PGRP combined cultures of *A. cylindrica* (1.24 nmol mg^{-1} TOC min^{-1} for AB3) and *Calothrix* sp. (1.43 nmol mg^{-1} TOC min^{-1} for CB2) in comparison to that of the control (**Figures 8C,D**), but no enhanced activity or even reduced activity in regard to control was pointed out for other enzymes in the AB and CB treatments apart from LeuAMP and AcPA in the CB1 treatment. In the AB and CB combined treatments, enzyme activity was significantly lower than in the control for bNAG, bGA and AlkPA, which contrasted with the cyanobacteria and bacteria alone treatments (in particular in the A and B3 treatments) for bNAG.

4. Discussion

Along with cyanobacteria, native and applied PGPR are reported to play an important role in better crop growth resulting in 50–70% increase in crop yield (Lucy et al., 2004); and specifically for wheat plants, improvement of plant height, dry weight and grain yield has been demonstrated (Karthikeyan et al., 2007; Nain et al., 2010; Manjunath et al., 2011). However, there are very few studies which have assayed the effects of cyanobacteria and PGPR on cereal growth in a liquid medium (hydroponic culture)(Manjunath et al., 2011). In the present study, two heterocystic cyanobacterial strains, *Anabaena cylindrica* and *Calothrix* sp. were used to test their biofertilizing capacity due to its nitrogen fixation activity (Bergman et al., 1997; Whitton and Potts, 2000) and potential influences on plant growth in liquid media, either alone or in combination with three microbial inoculants.

Visual inspection of images shown in **Figure 1** shows that plants inoculated with cyanobacteria had enhanced root and shoot development as compared to uninoculated plants (control). The positive effect of cyanobacteria inoculation on measured parameters, namely root and shoot weight and length, was even improved in some of the treatments combining cyanobacteria with PGPR (**Figures 4** and **5**). The positive results of biofertilization for plant biometric parameters observed in this study are in agreement with results of other authors: for instance, in one study, rice plants were cultivated with three selected bacterial strains (*Providencia* sp., *Brevundimonas* sp., *Ochrobacterium* sp.) and three cyanobacterial strains (two

Anabaena sp. and *Calothrix* sp.) (Prasanna et al., 2012). In this study, enhancement of seed germination and plant growth due to inoculation was observed.

Hydroponic cultivation has been shown to render higher yield in regard to plant biomass with lower water consumption but at much higher energetic cost, at least for lettuce, in comparison with conventional ground cultivations (Barbosa et al., 2015). Furthermore, the availability of land suitable for cultivation is reaching a threshold, a fact that prompts crop production by the hydroponic methodology as an attractive alternative to conventional methodology, in addition to the capability of continuous production throughout the entire year. However, there are uncertainties regarding the potential beneficial effect of biofertilization in this type of cultures because of the principles that are known or believed to act at the ground level in such a regard cannot be extrapolated directly to the hydroponic system, at least a priori. The positive effect of biofertilization with PGPRs has been demonstrated for a number of cultivars, mainly rice and wheat, and the formation of a complex biofilm structure with a microbe-plant particular interaction seems to underlie such positive effect (Courty and Wipf, 2016; Crouzet et al., 2019; Dal Cortivo et al., 2020), whereas in liquid media the biofilm structure cannot be expected to have a comparable organization level as in soil (van Gestel et al., 2015). Even though the biofertilization effect on wheat seeds using two cyanobacteria and PGPRs, either alone or in combination, was analysed here separately, the results of biometric parameters point out a major beneficial effect of cyanobacterial inoculation alone as compared to PGPR alone inoculation, with a slight improvement in the PGPR+cyanobacteria inoculated plants in regard to fresh (AB inoculations) or dry (CB inoculations) plant biomass as compared to cyanobacteria alone inoculated plants (**Figs. 4A, B and 5A, B**). Haney et al. (2015) demonstrated using hydroponic cultures that plant accessions in *Arabidopsis thaliana* with positive fitness for the plant were restricted to a few specific fluorescence Pseudomonad strains, others negatively affecting plant health, with plant capability to underpin bacterial growth being a selective factor in that trait. As to whether selective accession can also be applied to cyanobacteria remains to be unveiled. Favorable accessions seem to be discriminated by root released signals through plant exudates (Hartmann et al., 2009; Lareen et al., 2016; Jacoby et al., 2017; Sasse et al., 2018); and exudates have been measured in hydroponic cultures for *A. thaliana* and *Zea mays* (Jacoby et al., 2017; Oburger et al., 2013; Strehmel et al., 2014), they being shown to contain a number of secondary metabolites which include hormones (indoles), aminoacids, organic acids and flavonoids primarily, but with plant genotypic variation in the secondary metabolite composition. Phytosiderophores were also reported in soil-grown wheat by Oburger et al. (2014). Regarding wheat, it was early shown that the strain SA-1 of *Anabaena variabilis* enhanced plant growth with increased nitrogenase activity over the strain SA-0 when they were cultured in association (Spiller and Gunasekaran, 1990), thus it pointing out that accession selectivity for cyanobacterial strain is likely running on as for PGPRs. In this study, weakly better results concerning plant biomass, shoot length and chlorophyll were obtained under co-cultivation with *Calothrix* sp. than

with *A. cylindrica* (**Figs. 4**). Root associated microbiota has been shown by different studies to be plant genotype or even phenotype specific (Jacoby et al, 2017; Coskun et al., 2017). Through their root exudates plants select the rhizobacteria, and possibly also fungi, that better fit their nutrient requirements, with the specific microbioma modulating the nitrogen cycle in the root microenvironment (Coskun et al, 2017). Nitrogen fixing cyanobacteria may contribute positively to the nitrogen cycle control through symbiotic interations with the root associated bacteria, but this positive association may be dependent on specific plant-bioma consortia (de Souza et al., 2020). Therefore, the results of this study point to *Calothrix* sp. is likely a better partner than *A. cylindrica* for the PGPRs used plus wheat consortium. Even though both cyanobacterial strains were isolated from local soil environment where the wheat variety is cultured, but with different location, the particular composition of the soil may have influenced the better results obtained for *Calothrix* sp. due to a better adaptative trait as it was shown for a selective biostimulation of rhizospheric organic acids (Macías-Benitez et al., 2020).

Even though results of measurements of indoleacetic acid are somewhat dissimilar between the colorimetric and the UPLC-MS methods in this study, it being likely due to the fact that L-tryptophan was added in the colorimetric measurement, phytohormone release is clearly pointed out in this study, with higher values being measured in the *A. variabilis* inoculated cultures (**Fig. 6A**). Kinetin was also observed in the cyanobacteria plus PGPRs cultures especially for *Calothrix* sp. co-cultivations (Table 4), this fact suggesting that phytohormones can selectively be released either by the plant or by the cyanobacteria (Ali et al., 2009a; Manjunath et al., 2011; Suresha et al., 2019). Nonetheless, release of cytokinins and auxins is known to be dependent on the plant growth cycle (Singh, 2014), a fact that may lead to varying results between studies and measurement methods. Mazhar et al. (2012) reported in a study on *Triticum aestivum* (var. Uqab-2000) using an hydroponic growth system that cyanobacterial strains isolated from crop fields varied in their auxin production as indicated by colorimetric screening and determined by GC–MS analysis. Furthermore, IAA synthesis by the microorganisms growing in the vicinity of plant surfaces is accounted by tryptophan-like compounds excreted by plants (Idris et al., 2007).

Alkaline phosphatase is a fundamental enzyme of the phosphorus cycle, associated to P availability and soil organic matter content (Prasanna 2012). Earlier reports link activity of this enzyme with microbial biomass carbon and, hence, this enzyme is considered one of the most relevant indicators of the microbial activity in soils (Richardson et al., 2009; Nain et al., 2010). In the present study, *Calothrix* sp. and *A. cylindrica* exhibited high values for the activity of this enzyme, which would contribute to increase plant growth. A similar enhancement of alkaline phosphatase activity has been reported by Manjunath et al. (2016) with a *Calothrix* sp. strain alone and with a consortium of *Anabaena sp*. and *Azotobacter sp.* Grzeck et al. (2015) reported in a study with cyanobacteria and algae that alkaline phosphatases were synthesized intensively when there was a deficit of phosphorus, and the abundance of

phosphorus caused the disappearance of their synthesis or activity. In agreement with these previous reports, in the present study lower alkaline phosphatase activity was found in the combination of bacteria and cyanobacteria than in the cyanobacteria alone inoculated cultures, this fact being coincident with reduced content of P and TOC in the latter cultures (**Table 3**). Conversely, enhanced activity of sulfatase (AS) was found in AB3, CB2 and CB3 treatments as compared to the cyanobacteria or bacteria alone. This enzyme plays a central role in many physiological processes and for cycling sulphur, and is produced by PGPR, as was shown for *P. aeruginosa* SL-72 (Dimanthi, 2018). Enhanced activity of this enzyme was also reported by Naseby and Lynch (1988) for cultures inoculated with *P. fluorescens* F113. Knauff et al. (2003) related arylsulfatase activity increases in the rhizospheres of S-deficient plants like S*inapis album*, *Lolium perenne* , *Triticum aestivum* and *Brassica napus*). This is also in agreement with the results of Kertesz et al. (1993), who reported that the reduction in the source of sulfates resulted in the specific production of arylsulfatase in *Pseudomonas* sp. by up to 140 times. In this study, it is interesting to note that cyanobacteria increased the activity of this enzyme when combined with bacteria, which could be explained by increased microbial activity in those treatments. Also, Vong et al. (2010) reported similar results for arylsulfatase activity, which was closely correlated with microbial activity. Even though sulfur content was not measured in the present study, the increased activity of this enzyme in the PGPR plus cyanobacteria cultivations, even as compared with PGPR alone cultivations, is likely driven by sulfate deficiency or enhanced demand by cyanobacterial metabolism. The uptake of nutrients by the cyanobacteria for supporting their own metabolism may be extended to nitrogen as contents of TN and NH_4^+ in the consortia were significantly lower than in the bacteria inoculations and the control (**Table 3**).

Conclusion

The present study has shown that the consortium of cyanobacteria and PGPR strains improved the growth of wheat plants in a hydroponic growth system. One of the main results of the study is the positive effects of cyanobacteira-PGPR mixtures on plant growth, which apparently enhanced nutrient availability and plant biometrics.

However, the more widespread utilization of PGPR and cyanobacteria will necessitate that a number of issues be addressed going from laboratory and greenhouse experiments, to field trials to large scale commercial field use and that will require a number of new approaches for the growth and a better understanding of how different bacterial strains and cyanobacteria work together for the synergistic promotion of plant growth. Additional studies need to be conducted on the effectiveness of different and novel inoculant delivery systems.

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Figure 1 Filaments of *Anabaena cylindrica* and *Calothrix* sp. under Leica microscope view, 40× Obj. Cyanobacteria turbidity was 373 and 351 FTU respectively

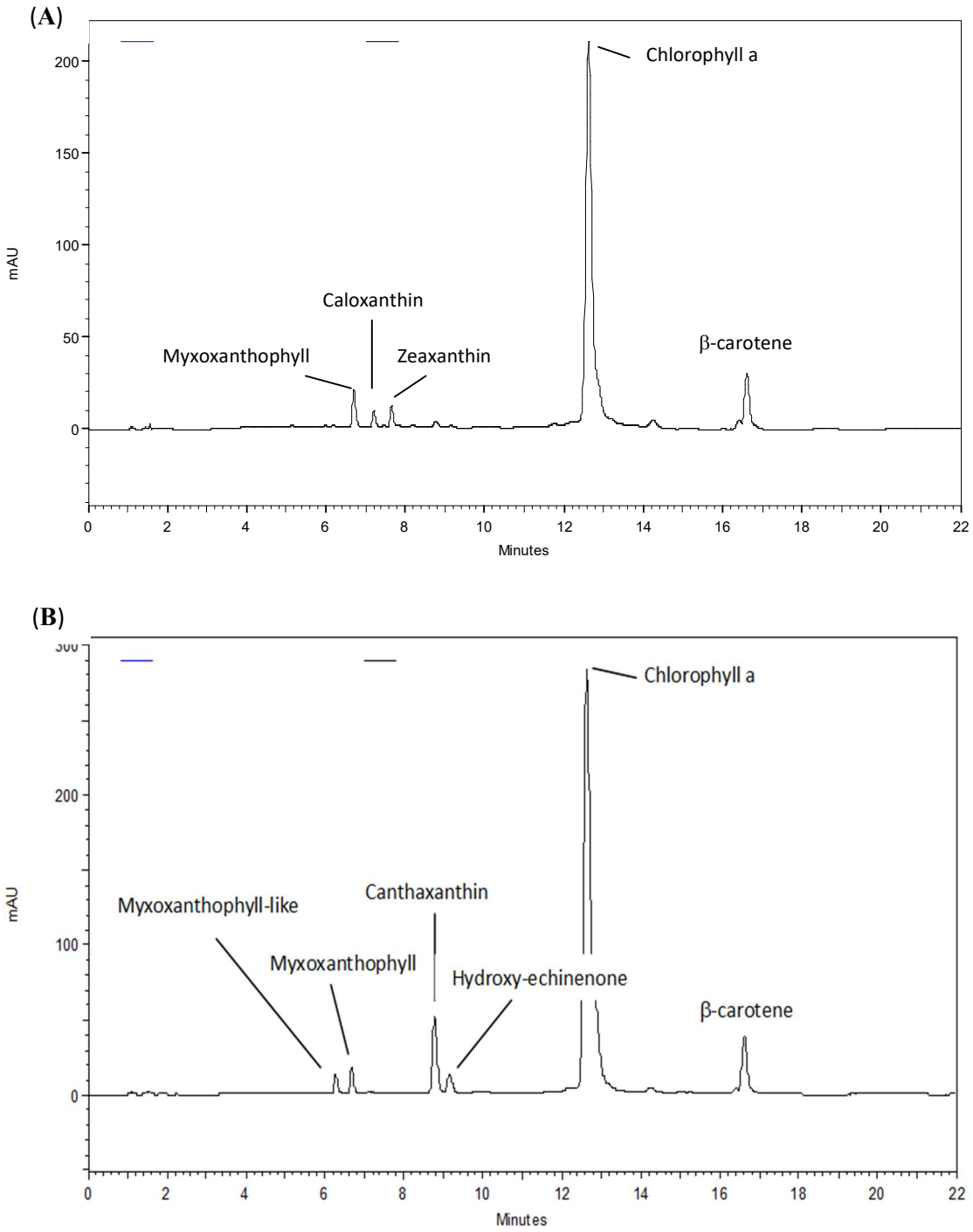

Figure 2: Pigment Chromatograms of *Calothrix* sp. (A) and *Anabaena cylindrica* (B).

643 **Table 1.** Basic chemical parameters of different treatments used in the hydroponic experiment. All
644 temperatures were 25°C.
645

Treatments	Strains used	EC (dS/m)	pH
BG11	Uninoculated control	0.20	8.84
Ana	*Anabaena cylindrica*	0.30	8.02
Cal	*Calothrix* sp.	0.29	8.32
BHI	Uninoculated control	16.11	7.2
B1	*Chryseobacterium balustinum*	16.39	7.13
B2	*Pseudomonas simiae*	16.44	7.20
B3	*Pseudomonas fluorescens*	16.93	7.29
AB1	*A. Cylindrica* + *C. balustinum*	9.03	7.19
AB2	*A. Cylindrica* + *P. simiae*	9.40	7.18
AB3	*A. Cylindrica* + *P. fluorescens*	9.28	7.20
CB1	*Calothrix* sp. + *C. balustinum*	9.17	7.15
CB2	*Calothrix* sp. + *P. simiae*	9.26	7.26
CB3	*Calothrix* sp. + *P. fluorescens*	8.76	7.22

646

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Figure 3 Changes in growth of wheat root and shoot after 17 days of treatments. W1, Control water; BG11.5, Control for cyanobacteria; A4, *A. cylindrica*; C5, *Calothrix* sp.; BHI.4, Control for bacteria; B.5, Bacteria; AB.2.3, *A. cylindrica* and bacteria; CB2.4, *Calothrix* sp. and bacteria.

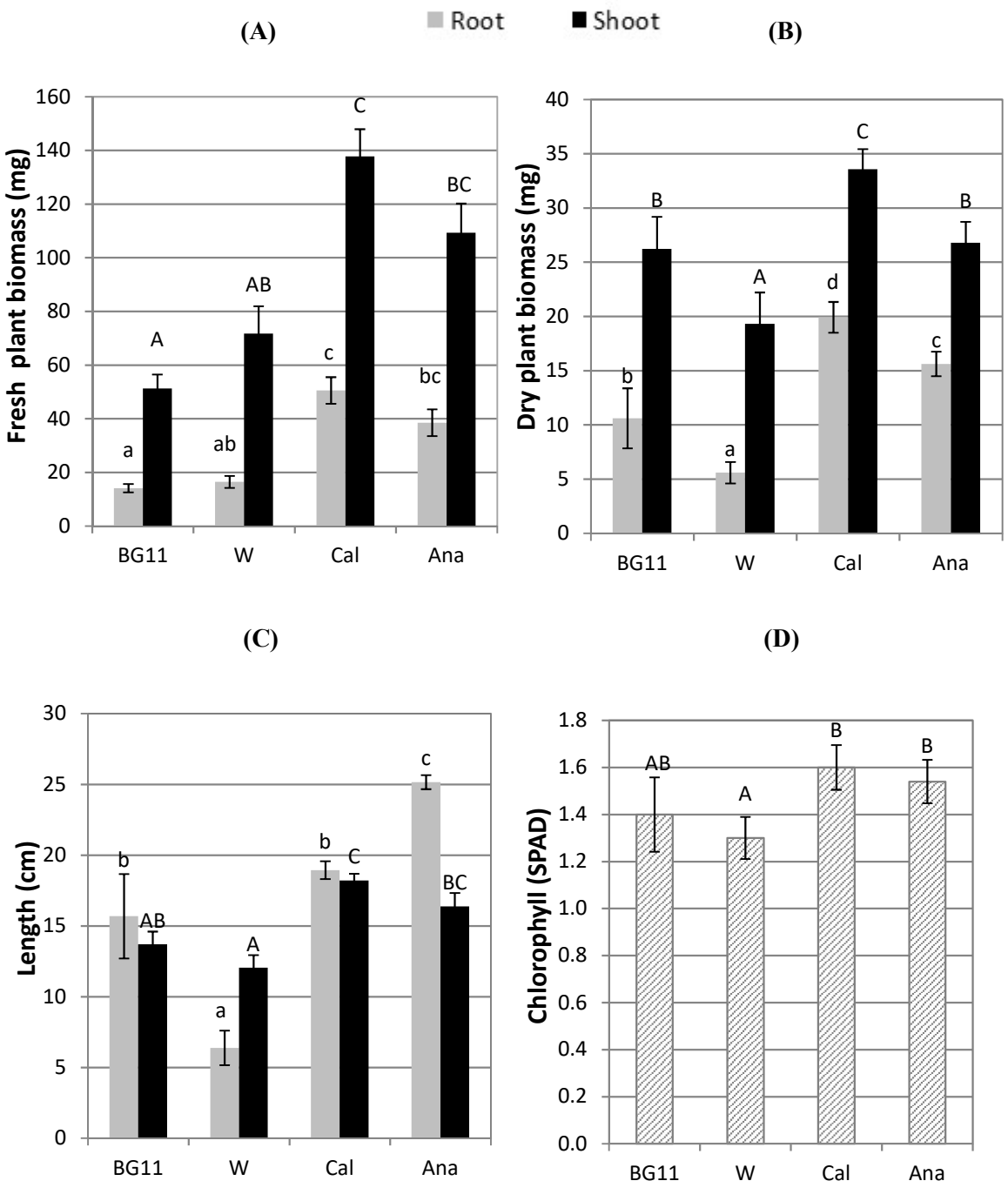

Figure 4 The biomass of root and shoot: Fresh weight, (**A**); dry weight, (**B**); root length and shoot height, (**C**); and chlorophyll SPAD value of wheat seedlings, (**D**); of wheat grown with cyanobacteria treatments in hydroponic systems with different treatments after 17 days. W, Control water; BG11, Control for cyanobacteria; Ana, *A. cylindrica*; Cal, *Calothrix* sp. Statistically significant differences between treatments are indicated by different letters.

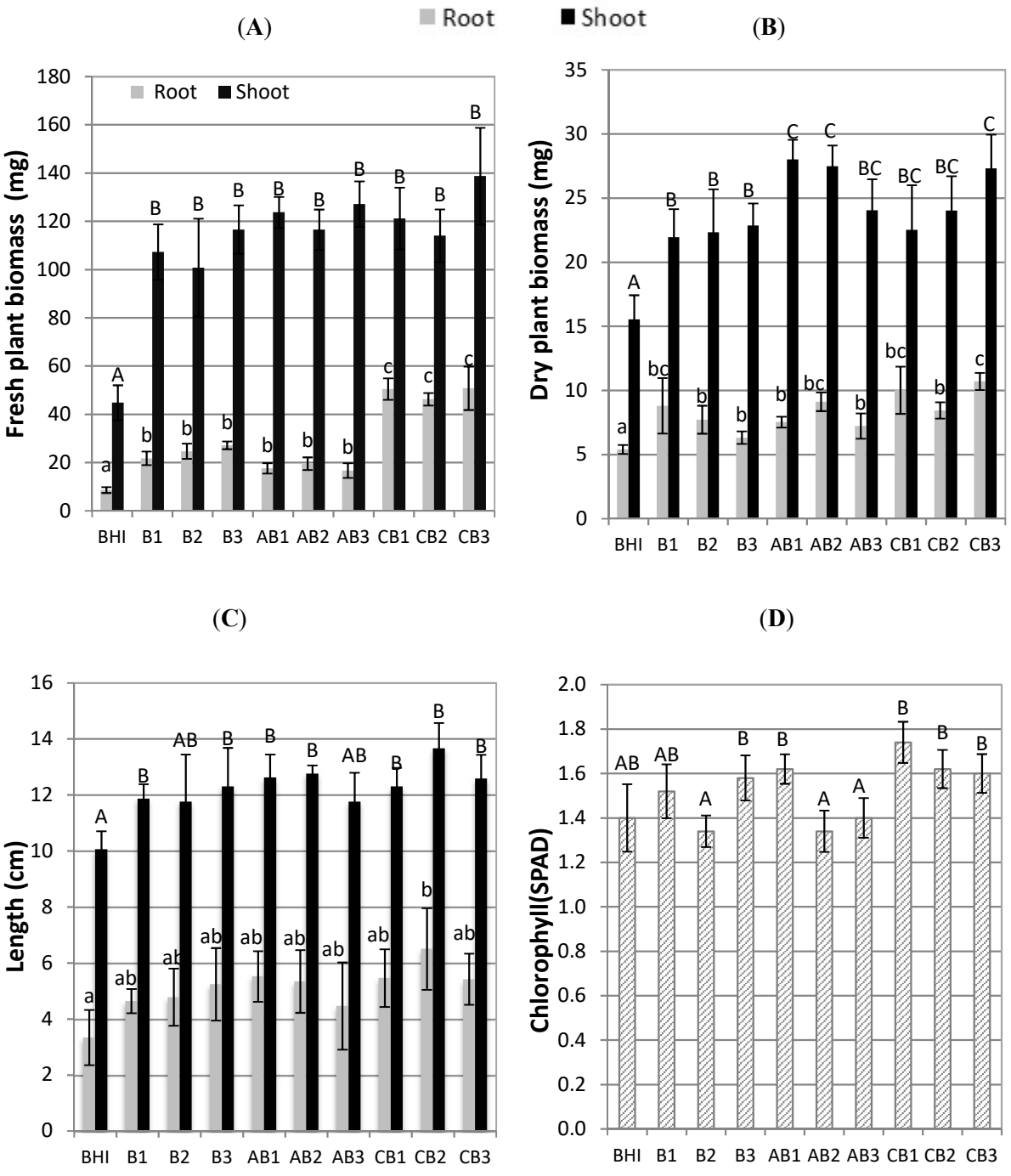

Figure5 The biomass of root and shoot: Fresh weight, (**A**); dry weight, (**B**); root length and shoot height,(**C**); and chlorophyll SPAD value of wheat seedlings, (**D**); of wheat grown with cyanobacteria treatments in hydroponic systems with different treatments after 17 days with PGPR-cyanobacteria treatments. BHI, Control; B1, *C.balustinum*; B2*, P.simiae*; B3, *P. fluorescens*; AB1, *A. cylindrica* and *C.balustinum*; AB2, *A. cylindrica* and *P.simiae;* AB3, *A. cylindrica* and *P. fluorescens*; CB1, *Calothrix* sp. and *C.balustinum;* CB2, *Calothrix* sp. and *P.simiae;* CB3, *Calothrix* sp. and *P.fluorescens* . Statistically significant differences between treatments are indicated by different letters.

679

680 **Table 2** Acidity (pH), electrical conductivity (EC), and turbidity of the culture medium after 17 days
681 of wheat growth in a hydroponic system with PGPR and cyanobacteria inoculation treatments. Data
682 represent mean ± SE (n= 5).

Treatments	pH	EC dS/m	Turbidity FTU
Cyanobacteria			
BG11	6.90 ± 0.03	0.036 ± 0.001	3.47 ± 0.56
Cal	6.80 ± 0.04	0.02 ± 0.001	48.51 ± 1.1
Ana	6.32 ± 0.1	0.02 ± 0.001	72.4 ± 2.92
PGPR-Cyanobacteria			
BHI	8.20 ± 0.01	3.144 ± 0.061	106.4 ± 5.43
B1	8.16 ± 0.05	1.656 ± 0.024	334.6 ± 22.64
B2	8.10 ± 0.02	1.536 ± 0.034	298.2 ± 20.47
B3	7.93 ± 0.03	1.427 ± 0.107	312.6 ± 18.06
AB1	8.23 ± 0.02	3.382 ± 0.098	83.0 ± 4.55
AB2	8.30 ± 0.01	3.158 ± 0.061	110.2 ± 6.59
AB3	8.26 ± 0.01	3.262 ± 0.091	101.6 ± 5.96
CB1	8.10 ± 0.01	1.556 ± 0.112	206.2 ± 39.88
CB2	8.00 ± 0.04	1.535 ± 0.043	167.2 ± 21.28
CB3	8.11 ± 0.04	1.501 ± 0.026	172.8 ± 24.51

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687 **Table 3** Concentrations of nutrients in each liquid media treatments after 17-daysof wheat growth in
688 hydroponic systems. Treatments are as in Table 1. All the values are means of 5 replicates (n=5) ± SE.

Treatment	mg TN L^{-1}	mg NH_4^+-N L^{-1}	mg PO_4^{-3} L^{-1}	TOC mg L^{-1}
Cyanobacteria				
BG11	1.588^{a} ± 0.069	0.091^{a} ± 0.025	ND	28.154^{b} ± 3.401
Cal	1.768^{a} ± 0.253	0.181^{a} ± 0.041	ND	4.770^{a} ± 0.775
Ana	3.436^{a} ± 1.145	0.141^{a} ± 0.040	ND	22.898^{b} ± 8.190
PGPR-Cyanobacteria				
BHI	25.770^{a} ± 1,728	15.082^{b} ± 0.041	7.862^{c} ± 0.016	2.682^{ab} ± 0.880
B1	26.854^{a} ± 0,99	15.734^{b} ± 0.863	7.879^{c} ± 0.058	6.890^{b} ± 2.058
B2	27.204^{a} ± 1,206	15.902^{b} ± 0.614	7.880^{c} ± 0.049	5.380^{ab} ± 2.096
B3	29.258^{a} ± 0,491	16.576^{b} ± 0.509	7.914^{c} ± 0.032	5.676^{ab} ± 1.008
AB1	9.971^{b} ± 0,622	5.204^{a} ± 0.261	6.360^{ab} ± 0.031	1.228^{ab} ± 1.825
AB2	8.371^{b} ± 0,47	4.39^{a} ± 0.294	6.338^{ab} ± 0.053	0.508^{a} ± 0.135
AB3	13.648^{b} ± 2,822	6.978^{a} ± 1.451	6.370^{abS} ± 0.025	2.004^{ab} ± 0.868
CB1	10.502^{b} ± 0,966	5.442^{a} ± 0.501	6.479^{b} ± 0.054	1.918^{ab} ± 0.966
CB2	10.784^{b} ± 0,474	5.696^{a} ± 0.307	6.338^{ab} ± 0.044	1.92^{ab} ± 0.465
CB3	9.526^{b} ± 0,358	5.238^{a} ± 0.211	6.257^{a} ± 0.031	0.646^{a} ± 0.394

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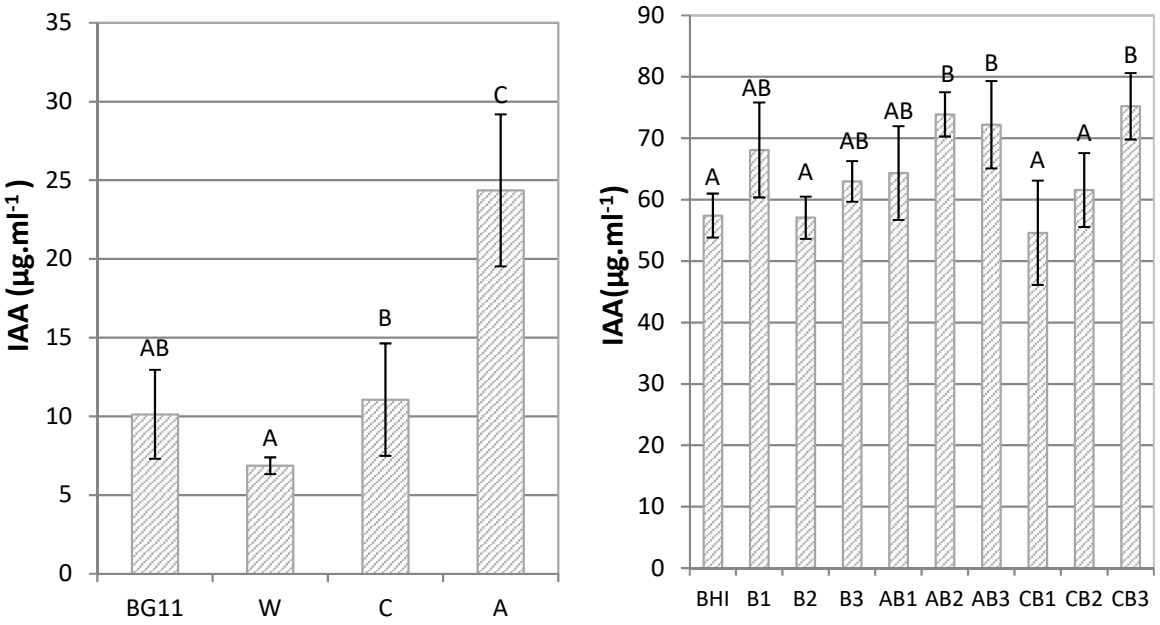
693 (**A**) (**B**)



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695 **Figure 6** IAA-like products evaluated colorimetrically in liquid suspensions by cyanobacteria (**A**),
696 bacterial isolates and combinations (**B**). All the values are means of 5 replicate (n=5) ± SE. ANOVA
697 significant at $p \leq 0.05$. Statistically significant differences between treatments are indicated by different
698 letters.

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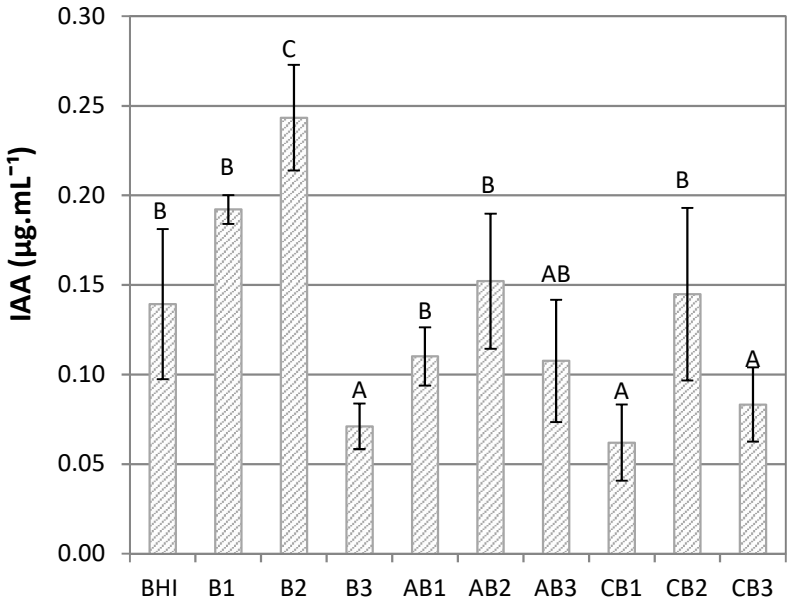

702 **Figure 7** IAA presence in liquid suspensions cuantified with HPLC-MS in bacterial isolates and

703 combinations. All the values are means of 5 replicate ± SE. ANOVA significant at $p \leq 0.05$. Statistically
704 significant differences between treatments are indicated by different letters.
705
706 **Table 4** Auxin production by cyanobacteria, bacteria and combinations with HPLC-MS Method.
707 Positive or negative test is given by symbols. + seems to be lighter, with (+) doubtful, and (–)not
708 recognizable.
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Treatments	Indoleacetic acid	Gibberellic acid	Kinetin	Abscisic acid
W	-	-	-	-
BG11	-	-	-	-
BHI	+	-	+	-
B1	+	-	(+)	-
B2	+	-	(+)	-
B3	+	-	+	-
Cal	-	-	-	-
Ana	-	-	-	-
AB1	+	-	+	-
AB2	+	-	(+)	-
AB3	+	-	(+)	-
CB1	+	(+)	+	-
CB2	+	-	+	-
CB3	+	-	+	(+)

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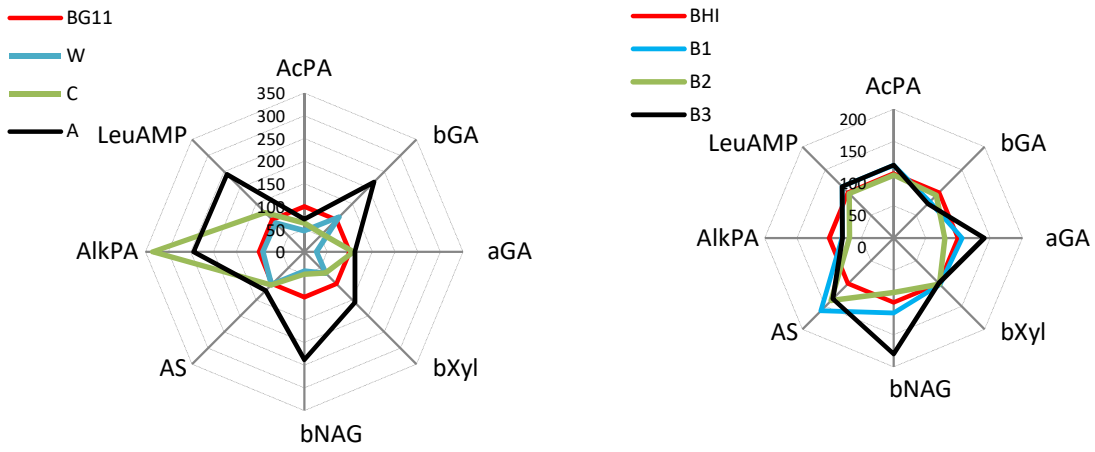
Pane A



Panel B

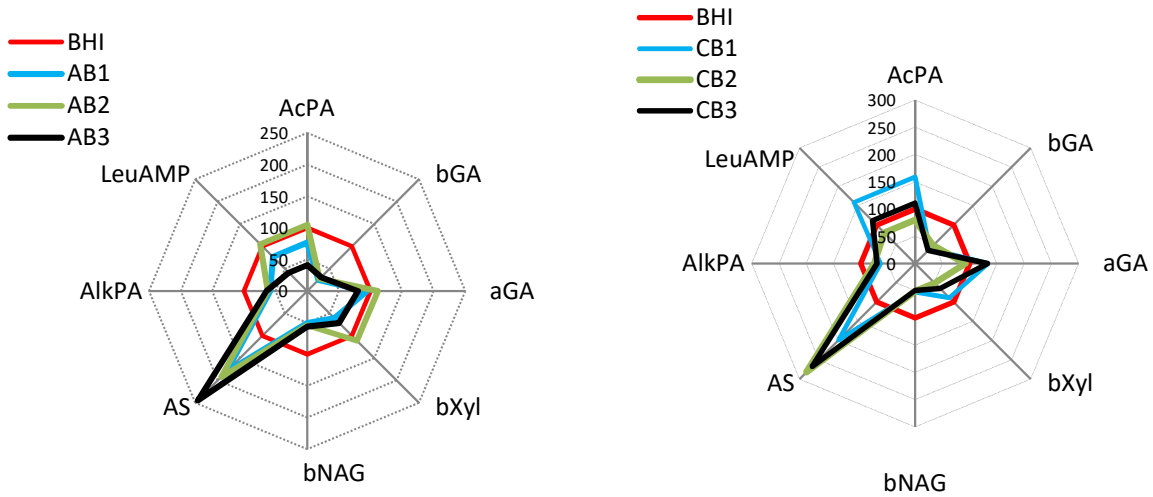


Figure 8 Radar graphic of enzymatic activities within each treatment. Treatment codes in bold as described in **Table 1**. The units correspond to nmol MUB or AMC mg^{-1}TOC min^{-1} and are scaled from 0 to 350 encompassing the whole range of enzymatic activities in the study. Enzyme codes are as follows: acid and alkaline phosphatases, AcPA and AlkPA respectively; β- and α-glucosidases, aGA and bGA respectively; N-acetyl-β-glucosaminidase, bNAG; β-xylosidase, bXyl; leucineaminopeptidase, LeuAMP; and sulfatase, AS.