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Influence of Biochar-Reinforced Hydrogel Composites on Growth and Biochemical Parameters of Bean Plants and Soil Microbial Activities under Different Moisture Conditions

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Abstract: Hydrogels have shown promise in improving soil quality and alleviating plant drought stress. This study investigated the effectiveness of four hydrogel composites composed of polyvinyl alcohol, sodium alginate, and pine or olive tree biochar in improving bean (*Phaseolus vulgaris*) plant growth and soil microbial activities. The experiment was conducted in natural soil, where biochar–hydrogel composites were applied at a concentration of 0.75% hydrogel per soil weight (*w*/*w*) for 35 days under two different moisture conditions: adequate moisture (70% of water holding capacity (WHC)) and drought stress (40% WHC). The results showed variation between hydrogel composites and, more importantly, between water regimes. Under water deficit conditions, biochar–hydrogel composites consistently caused a decrease in plant weight and in chlorophyll (CHL) CHLa/CHLb ratio. Furthermore, antioxidant enzyme activities and malondialdehyde and protein levels generally increased in contrast to the observations at 70% WHC. Regarding microbial activities, the composites reduced soil respiration (12–38%) while promoting phosphatase activity (42–65%) under both moisture regimes. Overall, the introduction of hydrogel composites did not show consistently positive effects on either plants or soil microorganisms. To thoroughly evaluate the efficacy of these hydrogels as soil amendments, further studies are needed, considering different soil types, plant species, and hydrogel application rates.

Keywords: drought stress; oxidative stress; photosynthetic pigments; soil respiration; soil enzymatic activities

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1. Introduction

The fundamental and first challenge facing agriculture is to produce enough food for a sustainable life. The Food and Agriculture Organization (FAO) insists on developing strategies and establishing practices for modern agriculture in order to protect, use, and sustainably manage natural resources, and ensure public health [1,2]. As the largest consumer of global freshwater, agricultural water uses account for approximately 70% of total freshwater use [3,4]. Aquatic, terrestrial and atmospheric ecosystems around the world are severely affected by changes in the global water cycle. Considering current levels and patterns of water use, it is no longer sufficient to consider water as a renewable natural resource in order to benefit from adequate and sustainable ecosystem services [3]. Therefore, water management, which can be defined as the planned development, distribution, and use of water resources, is one of the most important elements to be evaluated in terms of adaptation to climate change. It is obvious that in today's climatic conditions, societies

need to develop irrigation technologies/products that will minimize agricultural water use. Although there are many methods that can be used to manage water properly, such as rainwater harvesting, saltwater purification, and wastewater reuse, smart technologies have been developed in recent years to monitor these methods [2,5]. In addition to all these methods, the development of processes and products that can minimize agricultural irrigation is one of the subjects that has been studied intensively in recent years. Various technologies, such as low-pressure micro emitters and drip irrigation systems, are now widely used to increase irrigation efficiency. However, they have some disadvantages such as their cost and skill-based nature [6]. These technologies and/or products need to provide maximum crop yield and minimize evaporation or root zone water loss from the soil [7]. A new generation of materials called hydrogels, which are polymeric, porous, and have high water retention capacity, have attracted the attention of researchers in recent years as a sustainable alternative that can facilitate and reduce agricultural irrigation [8,9].

Hydrogels are three-dimensional polymers with a cross-linked structure that allows them to absorb and retain a significant amount of water, acting as an additional water reservoir in the soil. When plants experience water shortage, the water content of the hydrogel is released into the soil and can be taken up by the roots. The remarkable water-absorbing capacity of the hydrogels can be attributed to the presence of hydrophilic functional groups, including carboxyl, amino, and hydroxyl, within the polymer chain of hydrogels and the osmotic driving force in the aqueous environment [8,10]. The basic mechanism behind the swelling capacity of hydrogels can be explained by the hydration of polar hydrophilic groups with water or any aqueous media, which leads to the opening and dilution of the polymeric network structure. This process is driven by hydration forces, capillary action, and osmotic pressure, causing water molecules to penetrate into the hydrogel pores and swell the polar groups [11,12].

Hydrogels can be categorized as natural, synthetic, or a combination of both, depending on the polymers used in their synthesis. Hybrid hydrogels, a combination of synthetic and natural polymers, have gained importance in recent years. The presence of natural polymers makes them biodegradable and eco-friendly, while the synthetic polymer enhances their functional properties in agricultural applications [10]. More recently, a new type of hydrogel composite has been developed. These hydrogel composites include additional materials (fillers) like biochar in their structure. Interactions between polymers and fillers create cross-links in the polymer network, giving the hydrogel composite improved properties. However, few studies have used the biochar–hydrogel composite as a soil amendment to address drought stress issues [13,14].

Furthermore, hydrogels act as effective soil conditioners in agriculture because they stabilize soil structure and improve soil physicochemical and fertility properties [15]. When applied, hydrogels decrease soil bulk density and hydraulic conductivity while increasing soil aggregates, permeability, and porosity [9,16,17]. Hydrogels may also increase the water holding capacity (WHC) of the soil, preventing water loss through evaporation and percolation. However, this increase in WHC is more pronounced in coarse-textured soils when compared to fine-textured soils [8].

Recent studies have shown that using hydrogels in agriculture can improve plant growth and enhance plant resilience to drought [16,18]. For instance, when alginate-based hydrogels were applied, lettuce plants grown in a substrate under drought conditions exhibited an 80% increase in fresh weight compared to control plants [19]. Similarly, maize plants grown in soil treated with lignin-based hydrogel showed improved growth, increased phosphorus uptake, and reduced cell membrane damage compared to plants grown without hydrogel [20]. Using a composite of acrylamide polymer doped with char nanoparticles also mitigated the negative effects of water-deficit stress on tomato growth and increased nutrient levels in plants [21]. However, some studies have noted negative effects of hydrogels, including reduced plant biomass, seedling survival, or flowering [22–24]. The use of hydrogels can also impact soil microbial and enzymatic activities [17]. Although there is less research on the effects of hydrogels on microorganisms compared to plants, some

studies have reported positive effects on soil respiration rates and microbial population sizes [19,21]. Understanding how these microbial communities respond to soil amendment with biochar composites is critical to optimize efforts in improving soil health and maintaining soil functions.

In this study, we used a novel hydrogel composite consisting of the synthetic polymer polyvinyl alcohol (PVA) and the natural polymer sodium alginate, reinforced with biochar. PVA is a non-toxic, water-soluble, and biodegradable polymer with high chemical resistance. Alginate is a glycosidic polymer (anionic) obtained from brown algae, consisting of D-mannuronic acid (M blocks) and L-guluronic acid (G blocks) monosaccharides. The gelation process with three-dimensional network structures occurs by binding divalent or trivalent ions to G blocks in binding regions called egg boxes. These cross-linked ions form a gel structure with chains that bind water and can move freely [25,26]. Thus, it is widely used in hydrogels due to its hydrophilicity, biocompatibility, and biodegradability. Both polymers contribute numerous binding sites to the matrix through their main functional groups, -COOH and -OH. The hydrogels were doped with different concentrations of two types of biochar, derived from pine tree residues (PTB) and olive tree pruning (OTB). This hydrogel composite offers distinctive contributions and benefits in terms of cost-effectiveness, primarily due to the utilization of affordable and widely available materials such as PVA and sodium alginate. Additionally, the incorporation of biochar derived from waste products, such as pine and olive tree residues, not only reduces costs but also provides a sustainable solution by recycling agricultural waste. The production and application processes of these hydrogels are straightforward, resulting in reduced labor and resource costs compared to more complex irrigation technologies or high-tech soil amendments.

Bean plants were selected for this study because they are widely consumed legumes due to their nutritional importance and role as a cost-effective source of protein for both humans and animals. Additionally, bean plants are sensitive to drought stress [27,28]. The plants were grown until the flowering stage, a critical period for water stress susceptibility, as observed by Campos et al. [27].

The main objectives of this research were (i) to evaluate the efficacy of these biochar–hydrogel composites as soil conditioners to promote bean plant growth and improve various physiological parameters related to plant health and (ii) to assess their ability to reduce the negative effects of drought stress on both plants and soil microorganisms. To this end, the effects of these biochar–hydrogel composites on bean plants and soil microorganisms were evaluated in an agricultural soil under two moisture conditions: adequate moisture and drought stress.

2. Materials and Methods

2.1. Soil and Biochar–Hydrogel Composites

Soil samples were taken from the surface layer (0–20 cm) of an agricultural field situated in Madrid, Spain, with GPS coordinates 40°27′18″ N 3°44′55″ W. The soil was subjected to air-drying and sieving using a 2 mm mesh, and subsequently characterized according to the protocols of the Spanish Ministry of Agricultura, Pesca y Alimentación (MAPA) [29]. The primary physicochemical characteristics of the soil were texture (USDA) classified as sandy loam (7.8% clay; 18.8% silt; 73.4% sand), pHw (1:2.5) 7.4, electrical conductivity, ECw (1:5) 194 µS/cm, WHC 50%, and organic matter 4.9%.

OTB and PTB biochar were obtained from the slow pyrolysis of dry pruning obtained from olive (*Olea europaea* L.) and pine trees (*Pinus halepensis*), respectively. The production conditions and full characterization of PTB and OTB can be found elsewhere [30,31]. Four different biochar–hydrogel composites were used in this study: 0.25% PTB-doped hydrogel, 1% PTB-doped hydrogel, 0.25% OTB-doped hydrogel, and 1% OTB-doped hydrogel. These hydrogel composites were prepared according to the procedure described in Doğaroğlu [32]. Briefly, a mixture of 5 g of PVA and 5 g of sodium alginate was added to 100 mL of deionized water and autoclaved at 121 °C for 30 min to ensure complete dissolution. PTB or OTB biochar was then added to the solution and thoroughly mixed to achieve final

concentrations of 0.25% and 1% biochar per hydrogel (*w*/*w*). The solution containing biochar, PVA, and sodium alginate was then dripped into a solution of $CaCl_2$ (5% *w*/*v*) to facilitate cross-linking. Finally, the resulting biochar–hydrogel composites were rinsed with deionized water to remove any residual $CaCl_2$.

The biochar–hydrogel composites utilized in this investigation were extensively characterized by Doğaroğlu [32]. Surface morphological analyses of both the biochar and the biochar-doped hydrogels were conducted via scanning electron microscopy (SEM) (Quanta 650 FEG, FEI, Hillsboro, OR, USA). Furthermore, the chemical bonding patterns were assessed using Fourier-transform infrared spectroscopy (FT/IR-6700, Jasco, Japan). Additionally, the swelling capacities, swelling kinetics, and re-swelling capacities of the biochar–hydrogel composites were systematically evaluated. The size of the hydrogel beads used in our study was 1–2 mm in diameter. This size range was chosen to optimize the water retention capacity and distribution of hydrogels in the soil. The size of the hydrogel beads has a direct effect on properties such as swelling rate and water retention capacity. Smaller beads provide faster swelling and a larger surface area, allowing water to be distributed more homogeneously in the soil. The swelling capacities of the hydrogel composites in different aqueous media and their re-swelling capacities are given in Table 1.

Table 1. Swelling capacities in different aqueous media and re-swelling capacities of hydrogels used.

Biochar-Doped Hydrogel Type	Maximum Swelling Degree in Free Water	Re-Swelling Capacity		
		1st Cycle	2nd Cycle	3rd Cycle
0.25% PTB-doped hydrogel	77.52%	51.5%	57.1%	55.5%
1% PTB-doped hydrogel	141.05%	94.7%	127.2%	124.3%
0.25% OTB-doped hydrogel	87.50%	96.8%	84.9%	82.6%
1% OTB-doped hydrogel	84.06%	87.3%	69.2%	68.3%

2.2. Experimental Design

The biochar–hydrogel composites were swollen in distilled water for four days, until reaching equilibrium. Then, the different hydrogel treatments were individually added to pre-moistened soil, adjusted to a level corresponding to 70% of its WHC, at a rate of 0.75% hydrogel/soil dry weight (DW) (*w*/*w*), equivalent to a field application of 5.6 t per ha, assuming an incorporation depth of 5 cm. The soil and biochar–hydrogel composites were thoroughly mixed by hand and left in the dark at 20 °C for 24 h.

Control (no hydrogel added) and treated soils (600 g, DW) were placed in pots with a capacity of 1.2 L, an average internal diameter of 12 cm, and a height of 11 cm. In each pot, five seeds of common bean (*P. vulgaris* L. cv. Contender) were sown. The pots were randomly distributed and cultivated in a climatic chamber at 20 ± 2 °C, under a photoperiod of 16 h light and 8 h dark. The moisture content was maintained at 70% WHC until the emergence of the seedlings (10 days). Subsequently, the soil was divided into two groups: one group received adequate moisture conditions (70% WHC), while the other group experienced water deficit conditions (40% WHC). A total of 30 pots were employed, with three replicates for each treatment. The pots were weighed every three days, and the water loss was adjusted accordingly.

After 35 days, the shoots were harvested, and their fresh weights were recorded. The seedlings were then washed twice with deionized water. Three leaves from different plants in the same pot (approximately 600 mg fresh weight) were used to determine the relative water content (RWC) according to Zambrano et al. [33]. A portion of the plant leaves was cut and stored in aliquots at −80 °C until the biochemical analysis. Concurrently, soil samples (30 g) were collected in glass containers from the top layer of the pots to assess microbial activities. The soil was manually cleared of plant debris and partially sealed to both minimize water loss and allow for gas exchange.

2.3. Biochemical Analysis of Plant Leaves

Biochemical analyses of the leaves were conducted in accordance with the methods described in Garcia-Gomez et al. [34]. These analyses were carried out in duplicate for each treatment or control group (n = 3 × 2). A brief description of the procedures is provided below.

Chlorophyll a (CHLa), chlorophyll b (CHLb), and carotenoid contents were quantified. Leaf samples (50 mg) were finely ground using a Mixer Mill (Mill Mix 20, DOMEL, Zelezniki, Slovenia) with 80% acetone (10 mL). Subsequently, the absorbance of the resulting extracts was measured at wavelengths of 663.2 nm, 646.8 nm, and 470.0 nm, using an Agilent Spectrophotometer Cary 60 (Agilent Technologies, Santa Clara, CA, USA). The concentrations of CHLa, CHLb, and carotenoids were determined employing the equations detailed by Lichtenthaler [35] for this solvent.

ROS levels in leaf disks with a diameter of 6 mm (three plants per replicate) were quantified following incubation with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) [36]. Malondialdehyde (MDA), a marker of lipid peroxidation, was determined as thiobarbituric acid reactive substances [37]. For this analysis, approximately 0.10 g samples of leaf fragments were homogenized with 10% trichloroacetic acid (TCA) solution (0.665 mL) using a Mixer Mill. The MDA content in the resulting supernatant was determined by reaction with 0.5% thiobarbituric acid solution in TCA. The MDA concentration was calculated based on the difference in absorbance at 532 nm and 600 nm, utilizing an extinction coefficient of 155 mM^{-1} cm^{-1}.

For enzyme and protein analysis, 0.15 g of leaf samples were homogenized in 0.45 mL of sodium phosphate buffer (50 mM, pH 7.8) that included 1 mM EDTA and 1% (*w*/*v*) polyvinylpyrrolidone, utilizing a Mixer Mill. Following homogenization, the extract was centrifuged at 13,000 rpm for 10 min at 4 °C. Catalase (CAT) activity was determined in the supernatants by measuring the consumption of H_2O_2 at 240 nm in an Agilent Spectrophotometer Cary 60 [38]. One unit of CAT activity corresponded to the consumption of 1 µmol of H_2O_2 per minute, calculated using an extinction coefficient of 40 M^{-1} cm^{-1}. Ascorbate peroxidase (APX) and guaiacol peroxidase (GPOD) activities were assessed in the supernatants, using a GENios microplate spectrofluorometer (TECAN Trading AG, Männedorf, Switzerland) in 96-well microplates. APX activity was determined by measuring the rate of ascorbate oxidation with H_2O_2 at an absorbance of 290 nm [39]. GPOD activity was measured by tracking the change in absorbance at 470 nm due to guaiacol oxidation [40]. One unit of APX or GPOD enzyme activity was defined as the amount of enzyme responsible for a change of 0.1 absorbance units per minute. To determine the protein content in each extract, the Bradford method [41] was employed, utilizing bovine serum albumin as a standard.

2.4. Soil Microorganisms

The carbon transformation rate was measured by assessing the quantity of carbon dioxide released from soils after the addition of glucose as a substrate. Soil samples (5 g), with a moisture content between 40% and 60% of their WHC, were amended with 4 mg glucose per g of soil (DW). The release of carbon dioxide was then monitored at a temperature of 22 °C for 12 consecutive hours at 20 min intervals, using a Microbiological Analyzer BacTrac 4000 (SY-Lab, Neupurkersdorf, Austria), and the mean respiration rates were calculated.

Dehydrogenase (DH) activity was assessed following the method developed by Rogers and Li [42]. In brief, DH activity was assessed by incubating 1 g of alfalfa-enriched soil samples with 0.2 mL of 3% *w*/*v* 2,3,5-triphenyltetrazolium chloride and 0.5 mL of 0.5% *w*/*v* glucose in deionized water at 27 °C in darkness for 24 h. After incubation, triphenylformazan (TPF) was extracted with 2.5 mL of methanol, and absorbance at 490 nm was measured using a TECAN spectrophotometer. DH activity was quantified as µg of TPF generated per g of dry soil over a 24 h period.

To assess phosphatase (PHO) activity, 1 g of soil sample was preincubated for 30 min at 27 °C with 3.2 mL of a buffer solution (composed of 50 mL of 1 M NaOH, 1.21 g of tris(hydroxymethyl)aminomethane chlorhydrate (TRIS), 1.16 g of maleic acid, 1.4 g of citric acid, and 0.63 g of boric acid in 500 mL of water) adjusted to pH 6.5 [43]. Subsequently, 80 µL of 0.01 M methylumbelliferyl phosphate in buffer was added, and the samples were incubated in the dark at 27 °C for 2 h. The fluorescence intensity of the generated 4-methylumbelliferone (MU) was measured at 320 nm excitation and 465 nm emission wavelengths using a TECAN spectrophotometer. Activities were quantified as µg of MU produced per g of dry soil.

Ammonium oxidase (AO) activity in soil was determined through the measurement of nitrite production utilizing ammonium ions as the substrate [44]. Briefly, 2 g of soil was mixed with 8 mL of 1 mM $(NH_4)_2SO_4$ and 40 µL of 1.5 M sodium chlorate. After 5 h at 25 °C, the released nitrite was extracted with 2 mL of KCl solution. Nitrite concentration in the supernatant was measured at 520 nm after adding a reagent solution containing 0.25 g of sulfanilamide, 12.5 mg of N-(1-naphthyl)-ethylenediamine hydrochloride, and 2.5 mL of H_3PO_4 in 25 mL of water. Ammonium oxidation activities were quantified as µg of nitrite-N released per g of dry soil over 5 h.

2.5. Statistical Analysis

STATGRAPHICS software (version XVII) was used for statistical analysis of the data set after the verification of normality and homogeneity of variances. For each moisture condition, data were statistically analyzed by one-way analysis of variance (ANOVA) with Fisher's least significant difference procedure (LSD, $p < 0.05$).

The data set, expressed as the percentage effect of the biochar–hydrogel composites compared to their respective controls, was analyzed by a two-way analysis of variance (ANOVA) to compare the effects caused by hydrogels under each moisture regime. The factors in the analyses were the moisture conditions and hydrogel composite type. Pairwise differences and multiple comparisons between the study groups were analyzed by LSD test at a 5% confidence level.

The relationship between toxicological parameters was determined by Pearson correlation analysis. Only significant analyses with a regression fit exceeding R^2 of 0.70 are presented.

3. Results

3.1. Soil pH and Electrical Conductivity

The soil samples (control and biochar–hydrogel composite-treated soils) had initial pH and EC values of 7.41 ± 0.05 and 195 ± 3 µS/cm, respectively, irrespective of treatment. At the end of the experiment, differences in soil pH and EC were observed depending on the moisture level and treatment applied (Table 2). Under adequate moisture conditions, there were no significant differences in soil pH, but EC decreased significantly in the soil treated with 0.25% OTB-doped hydrogel compared to the control soil ($p = 0.0450$). Under drought stress, both 0.25% PTB- and 0.25% OTB-doped hydrogel treatments led to a slight but significant ($p = 0.0083$) decrease in soil pH compared to the control. Additionally, EC significantly increased ($p = 0.0016$) for the 0.25% PTB-doped hydrogel treatment (545 ± 37 µS/cm) compared to the corresponding control (364 ± 28 µS/cm). Soil pH was higher at 70% WHC than at 40% WHC ($p = 0.0000$), while the opposite was observed for EC ($p = 0.0000$).

Table 2. Data of the soil pHw (1:2.5) and electrical conductivity in control soil and soils amended with hydrogel composite doped with biochar, pine tree-derived (PTB) and olive tree-derived (OTB), at the end of the experiment (35 days).

Soil Moisture		pHw (1:2.5)	ECw (1:5) (µS/cm)
70% WHC	Control	7.4 ± 0.1 a	269 ± 19 ab
	0.25% PTB	7.4 ± 0.1 a	287 ± 23 a
	1% PTB	7.5 ± 0.1 a	228 ± 30 bc
	0.25% OTB	7.44 ± 0.05 a	208 ± 14 c
	1% OTB	7.37 ± 0.08 a	227 ± 31 bc
40% WHC	Control	7.30 ± 0.03 AB	364 ± 28 BC
	0.25% PTB	7.09 ± 0.03 C	545 ± 37 A
	1% PTB	7.31 ± 0.05 A	318 ± 15 C
	0.25% OTB	7.1 ± 0.1 C	444 ± 75 B
	1% OTB	7.19 ± 0.06 BC	337 ± 42 BC

EC_w (1:5): electrical conductivity; WHC: water holding capacity; different lowercase letters indicate significant differences among treatments at 70% WHC, while capital letters indicate significant differences at 40% WHC (LSD test, $p < 0.05$).

3.2. Effects of Biochar–Hydrogel Composite and Moisture Regime on Plants

3.2.1. Germination, Plant Growth, and Relative Water Content

Plant germination was unaffected by variations in biochar–hydrogel composite type. Differences in plant growth were observed between the moisture regimes. Biochar–hydrogel composite application tended to reduce plant weight (Figure 1), but differences between the control group and hydrogel-treated plants were not statistically significant ($p > 0.05$) under adequate moisture conditions.

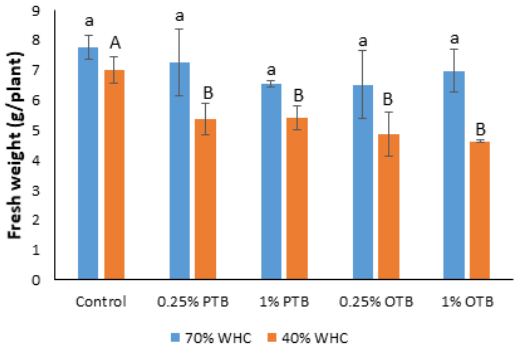

Figure 1. Fresh weight of seedlings measured in bean plants after 35 days of growth in control soil and soils amended with hydrogel composite doped with biochar, pine tree-derived (PTB) and olive tree-derived (OTB), under two different moisture regimes. Different lowercase letters or capital letters indicate significant differences between treatments at 70% WHC and 40% WHC, respectively (LSD test, $p < 0.05$).

Conversely, at 40% WHC, a significant decrease in plant weight was observed in soils amended with biochar–hydrogel composite ($p = 0.0093$). Interestingly, the control plants showed no significant differences between the two moisture regimes. In contrast, the two-way multifactorial analysis of variance revealed that the effects of biochar–hydrogel composites on plant weight depended on the moisture regime (Table 3). Plants grown in biochar–hydrogel composite-treated soils showed a significant reduction in plant weight when subjected to drought stress conditions compared to those in adequate moisture conditions. RWC in the leaves remained consistent across all treatments and moisture regimes, with values averaging around 82 ± 4% at 70% WHC and 81 ± 3% at 40% WHC.

Table 3. Significance levels (*p*-values) resulting from a two-way ANOVA analysis, evaluating the effects of moisture regime and biochar–hydrogel composite treatment, as well as their interactions, on plant and soil microorganisms.

		Moisture Regime	Hydrogel Composite Type	Moisture Regime × Hydrogel Composite Type
Plant weight		0.0072	-	-
Photosynthetic pigments	CHLa	0.0000	-	-
	CHLb	0.0000	-	-
	Carotenoids	0.0235	-	-
	CHLa/b	-	-	0.0147
	Carot/CHLtotal	0.0000	-	-
Stress oxidative biomarkers	ROS	-	-	-
	CAT	0.0000	0.0008	0.0012
	APX	0.0000	0.0025	0.0234
	GPOD	0.0000	0.0045	-
	Protein	0.0000	0.0008	0.0064
	MDA	0.0012	0.0252	-
Soil microorganisms	C transformation	-	0.0102	-
	Ammonium oxidase	0.0000	0.0013	-
	Phosphatase	0.0066	-	-
	Dehydrogenase	0.0142	-	-

- indicates not significant. CHL: chlorophyll, ROS: reactive oxygen species, CAT: catalase activity, APX: ascorbate peroxidase activity, GPOD: guaiacol peroxidase: MDA: malondialdehyde.

3.2.2. Photosynthetic Pigments

In each moisture regime, biochar–hydrogel composite treatments generally led to a reduction in the content of photosynthetic pigments in bean leaves ($p < 0.05$) (Figure 2A–C). Furthermore, the CHLa/CHLb ratio (Figure 2D) generally decreased significantly ($p < 0.05$) with the addition of biochar–hydrogel composite compared to the respective control, but the effects were minor (less than 10% reduction). The carotenoid/CHL total ratio (Figure 2E) decreased when biochar–hydrogel composite treatments were applied ($p = 0.0382$), resulting in an approximately 20% reduction under drought stress, whereas no such effects were observed at 70% WHC.

The comparison of photosynthetic pigment levels in plants grown in control soils at 70% WHC and 40% WHC showed that water deficit significantly decreased CHLa levels ($p = 0.0176$) and the CHLa/CHLb ratio ($p = 0.0017$), while other photosynthetic parameters did not differ between the two moisture regimes. Notably, drought stress significantly reduced the CHLa/CHLb ratio in both control and hydrogel-treated plants, which is associated with an increase in CHLb observed in plants grown at 40% WHC compared to 70% WHC.

The multifactorial analysis revealed that the magnitude of the effects of biochar–hydrogel composite addition on photosynthetic pigments, relative to their respective controls, depended on the moisture regime, except for the CHLa/CHLb ratio (Table 3). The decrease in chlorophyll and carotenoid contents in bean leaves due to the addition of biochar–hydrogel composite was more pronounced under optimal moisture conditions than under drought stress, whereas the effect on the carotenoid/CHL ratio was greater at 40% WHC.

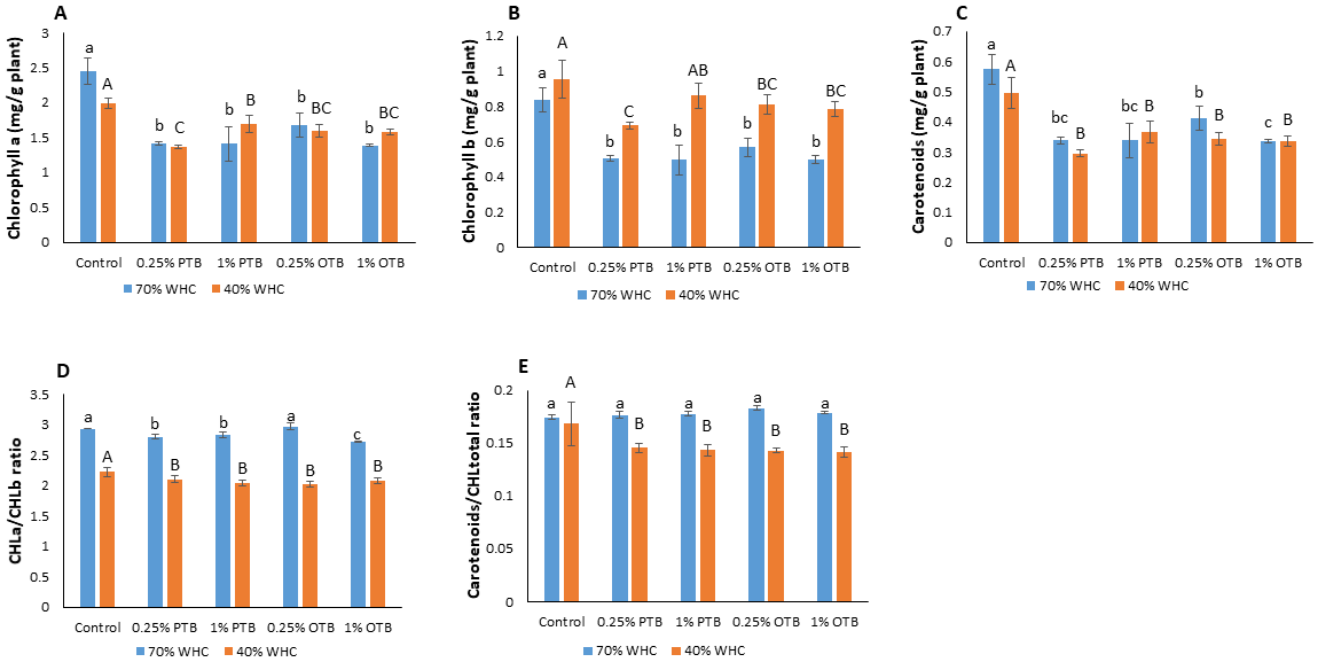

Figure 2. Concentrations of chlorophyll (CHL)a (**A**), CHLb (**B**), carotenoids (**C**), CHLa/CHLb ratio (**D**), and carotenoid/CHL ratio (**E**) measured in leaves of 35-day-old bean plants grown in soils with different biochar–hydrogel composites under two moisture regimes. Different lowercase letters or capital letters indicate significant differences between treatments at 70% WHC and 40% WHC, respectively (LSD test, $p < 0.05$).

3.2.3. Biomarkers of Oxidative Stress

In the control plants, a water deficit resulted in a significant decrease in protein levels ($p = 0.0037$) and GPOD activity ($p = 0.0481$), while APX and CAT activity were unaffected. Conversely, MDA levels increased by 59% at 40% WHC compared to 70% WHC ($p = 0.0088$).

Hydrogel addition showed a tendency to increase ROS generation in bean leaves when compared to their respective controls, irrespective of soil moisture content, but the differences were not significant. The effect of biochar–hydrogel composite addition on antioxidant enzymatic activities (Figure 3A–C) and protein levels (Figure 3D) depended on the moisture regime. CAT proved to be the most sensitive antioxidant enzyme. At 70% WHC, enzymatic activities and protein levels underwent a significant reduction, except for APX activity in soils amended with 1% PTB- and 0.25% OTB-doped hydrogels. CAT showed a substantial decrease in activity for all biochar–hydrogel composite treatments, resulting in inhibition values ranging from 36% to 60% ($p = 0.0002$). Treatment with 0.25% OTB-doped hydrogel showed the least detrimental effect on CAT and GPOD activities, as well as on protein levels in bean leaves. In the presence of adequate moisture conditions, the application of biochar–hydrogel composite had no significant effect on MDA content (Figure 3E).

In contrast, under drought stress, most values increased, with some exceptions depending on the biochar–hydrogel composite type. Notably, the 0.25% PTB-doped hydrogel treatment showed the lowest values in all cases. Application of 1% PTB- and 0.25% OTB-doped hydrogels led to an increase in MDA levels in bean leaves ($p = 0.0001$) by 59 ± 4% and 20 ± 1%, respectively. No significant changes in MDA levels were observed for the other biochar–hydrogel composite treatments.

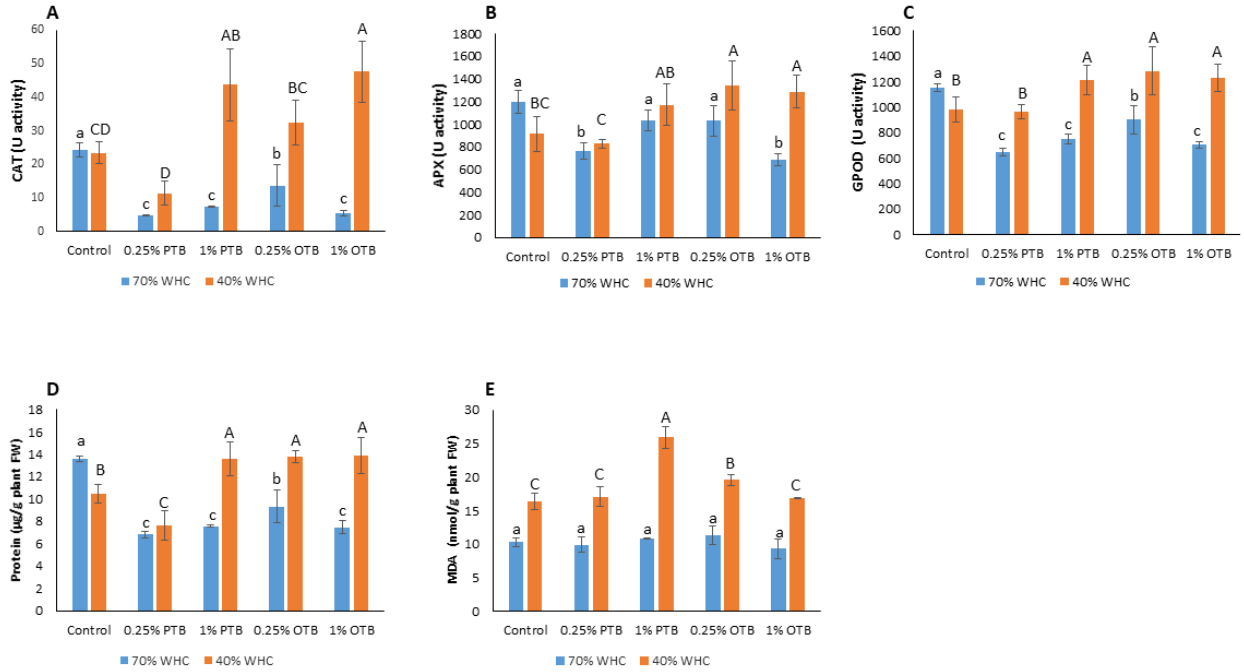

Figure 3. Levels of catalase (CAT) (**A**), ascorbate peroxidase (APX) (**B**), guaiacol peroxidase (GPOD) (**C**), soluble proteins (**D**), and malondialdehyde (MDA) (**E**), in leaves of 35-day-old bean plants grown in soils with different biochar–hydrogel composites under two moisture regimes. Different lowercase letters or capital letters indicate significant differences between treatments at 70% WHC and 40% WHC, respectively (LSD test, $p < 0.05$).

The multifactorial analysis revealed that, with the exception of ROS levels, both the moisture regime and biochar–hydrogel composite type, and to a somewhat lesser extent, their interaction, significantly affected the magnitude of the effects of oxidative stress biomarkers (Table 3). Enzymatic activities and proteins showed notable differences at both moisture regimes, with increases observed under drought stress and decreases under adequate moisture conditions. MDA levels increased significantly under drought stress, compared to plants under adequate moisture conditions, with the greatest differences observed in plants treated with 1% PTB-doped hydrogel. Regarding hydrogel types, 0.25% PTB-doped hydrogel showed differences with the other hydrogel composites, showing the lowest effects on biomarkers of oxidative stress.

3.3. Effects of Biochar–Hydrogel Composite and Moisture Regime on Soil Microorganisms

In the control samples, the drought stress decreased PHO activity ($p = 0.0076$) and stimulated DH activity ($p = 0.0039$), while carbon transformation rates and AO activity were not significantly affected. Under both moisture conditions, all biochar–hydrogel composite treatments decreased carbon transformation rates, with reductions ranging from 12% to 39% compared to the respective control soil (Figure 4A). Under adequate moisture conditions, the inhibition was higher for the OTB-doped hydrogels than for the PTB-doped hydrogels, while under drought stress, no differences were observed between the biochar–hydrogel composite treatments. AO was the least sensitive enzyme and showed a significant decrease ($p = 0.0013$) with the 0.25% and 1% PTB-doped hydrogel treatments at 70% WHC (10.8 ± 0.2 and 15.5 ± 0.3%, respectively) and a significant increase ($p = 0.0342$) with the 1% OTB-doped hydrogel treatment at 40% WHC (22.6 ± 0.8%) (Figure 4B). In contrast to the carbon transformation rates, the well-watered treatments showed that the hydrogel-induced AO inhibition was higher for the PTB-doped hydrogels than for the OTB-doped hydrogels.

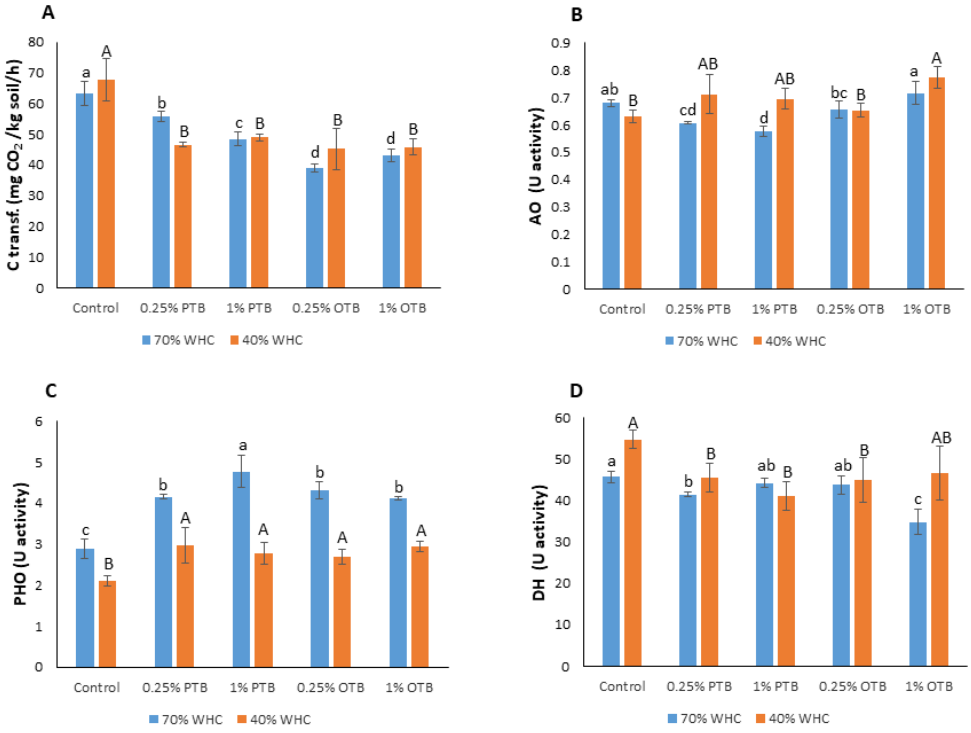

Figure 4. Data of carbon transformation rate (**A**) and the ammonium oxidase (AO) (**B**), phosphatase (PHO) (**C**), and dehydrogenase (DH) (**D**) activities in soils with different biochar–hydrogel composites under two moisture regimes. Different lowercase letters or capital letters indicate significant differences between treatments at 70% WHC and 40% WHC, respectively (LSD test, $p < 0.05$).

PHO activity was stimulated by the addition of biochar–hydrogel composite, resulting in increases of 42% (4.12 ± 0.04) to 65% (4.8 ± 0.3) at 70% WHC ($p = 0.0001$) and 28% (2.7 ± 0.2) to 41% (3.0 ± 0.4) at 40% WHC ($p = 0.0114$) compared to the respective control soil. The highest PHO activity was observed with the 1% PTB-doped hydrogel under adequate moisture conditions (Figure 4C).

The addition of biochar–hydrogel composite either had no effect or decreased DH activity, depending on the hydrogel type and moisture level (Figure 4D). At 70% WHC, the 1% OTB-doped hydrogel treatment showed the most significant inhibition (24 ± 1%) ($p = 0.0014$). Under drought stress, all treatments except the 1% OTB-doped hydrogel reduced DH activity, with inhibition values ranging from 17% to 25% ($p = 0.0407$).

Two-way ANOVA showed that the soil moisture level significantly affected the effects of biochar–hydrogel composites on microbial parameters, except for carbon transformation rates (Table 3). The increase in PHO activity induced by biochar–hydrogel composite treatments was more pronounced under adequate moisture conditions than under drought stress. Meanwhile, the inhibition of DH activity was less severe under drought stress conditions.

Multifactorial analysis also showed that the hydrogel type also influenced the effects on C transformation rate and AO activity (Table 3). An increased respiration rate and an increased AO activity were observed for the 0.25% PTB-doped hydrogel and the 1% OTB-doped hydrogel, respectively, compared to the other hydrogels.

3.4. Correlation Analysis

Under adequate moisture conditions, Pearson correlation analysis revealed a strong relationship among protein concentration, CAT and GPOD activity, and photosynthetic pigment levels in bean leaves (Table 4). However, under drought stress, correlations were exclusively observed among photosynthetic parameters, without any significant association with oxidative stress parameters. A strong correlation was also observed between protein

levels and enzymatic activities, as well as between GPOD and APX. Irrespective of soil moisture content, plant weight did not correlate with biochemical parameters, except for ROS levels in bean leaves, which showed a negative correlation ($R^2 = 0.74$; $p = 0.0047$) with plant weight under drought stress conditions.

Table 4. Pearson's correlation coefficient of plant weight and biochemical parameters measured for plants grown under two different moisture conditions: (A) adequate moisture conditions (70% WHC) and (B) drought conditions (40% WHC) over a 35-day period.

	(A) Moisture Regime 70% WHC						
	CHLa	**CHLb**	**Carotenoids**	**Protein**	**CAT**	**APX**	**GPOD**
CHLa	1	0.99 ***	1.00 ***	0.98 ***	0.98 ***	-	0.94 ***
CHLb	0.99 ***	1	0.99 ***	0.97 ***	0.98 ***	-	0.90 ***
Carotenoids	1.00 ***	0.99 ***	1	0.99 ***	0.98 ***	-	0.95 ***
Protein	0.98 ***	0.97 ***	0.99 ***	1	0.98 ***	-	0.98 ***
CAT	0.98 ***	0.98 ***	0.98 ***	0.98 ***	1	0.72 ***	0.95 ***
APX	-	-	-	-	0.72 ***	1	-
GPOD	0.94 ***	0.90 ***	0.95 ***	0.98 ***	0.95 ***	-	1
	(B) Moisture Regime 40% WHC						
	CHLa	**CHLb**	**Carotenoids**	**Protein**	**CAT**	**APX**	**GPOD**
CHLa	1	0.97 ***	0.96 ***	-	-	-	-
CHLb	0.97 ***	1	0.88 **	-	-	-	-
Carotenoids	0.96 ***	0.88 **	1	-	-	-	-
Protein	-	-	-	1	0.94 ***	0.81 ***	0.81 ***
CAT	-	-	-	0.94 ***	1	-	-
APX	-	-	-	0.81 ***	-	1	0.97 ***
GPOD	-	-	-	0.81 ***	-	0.97 ***	1

WHC: water holding capacity; CHL: chlorophyll; CAT: catalase; APX: ascorbate peroxidase; GPOD: guaiacol peroxidase; - indicates no significant correlation ($p < 0.05$); **, and *** indicate $p < 0.01$, and $p < 0.001$ values, respectively.

Regarding microbial parameters, a correlation between the carbon transformation rate and plant weight was observed at both 70% WHC ($R^2 = 0.76$; $p = 0.0079$) and 40% WHC ($R^2 = 0.92$; $p = 0.0243$).

4. Discussion

This study evaluated the effects of four biochar–hydrogel composites on bean plants grown in natural soil. Independent of the effects of drought stress on plants and microorganisms, the hydrogels themselves significantly affected most of the measured parameters. The percentage of effect of the biochar–hydrogel composite on plants and soil organisms varied depending on the irrigation conditions and, in some cases, the specific hydrogel. The results contribute to the understanding of the role of hydrogels as soil conditioners and their effectiveness in mitigating the impacts of drought stress, encompassing not only plant responses but also soil functionalities.

4.1. Effects of Biochar–Hydrogel Composite and Moisture Regime on Plants

4.1.1. Germination, Plant Growth, and Relative Water Content

The negative effect of drought-induced stress on plant growth has been widely reported in the literature [45,46]. However, how plants respond to such stressors can vary depending on the plant species [47] and can even differ among different plant varieties [48,49]. In the present study, water stress did not result in a statistically significant reduction in the weight of the bean plants at the reproductive stage when they were grown in control soil (no hydrogel applied). This may be due to the controlled environmental growth conditions, which probably balanced the evapotranspiration losses and the soil's capability to retain sufficient moisture for seedling growth. The tolerance mechanisms developed by plants to drought stress could also contribute to the lack of effect, as indicated at the end of this section.

Several studies have shown the positive influence of hydrogels in alleviating the impact of drought stress on plants [18,50]. However, some researchers have reported adverse effects of hydrogels on plants when grown under non-stressed conditions [17,21]. In the present study, the application of biochar–hydrogel composites had no significant influence on seedling emergence, but resulted in reduced plant growth, particularly under drought conditions. Therefore, the hydrogels tested did not mitigate the effects of drought stress in bean plants; instead, their combination with water scarcity exacerbated the impairment of plant growth.

These results showed some discrepancies with those of a previous study using the same hydrogels, which assessed their effects on arugula plants grown on turf [32]. The differences between the two studies highlight the influence of substrate type, plant species, and biochar–hydrogel composite application rates on plant growth responses to biochar–hydrogel composite under water stress [8,51]. In particular, the water retention capacity of hydrogels is closely linked to the characteristics of the soil and the water requirements of plants.

The soil in our study had a coarse texture, which typically enhances the beneficial effects of hydrogels [8,16]. However, it also had a high WHC (50%), which could potentially mask these effects. Nevertheless, previous research [15] has shown that the addition of hydrogel can increase plant growth, even in soils with a high WHC (48%).

Some authors have suggested that the negative effects of hydrogels on plant growth might be related to an increase in soil EC, which can affect the uptake of water and nutrients by plants [22,23]. However, in our study, no correlation between plant weight reduction and increased EC was observed. In fact, plants subjected to water-limited conditions in soils treated with 0.25% PTB-doped hydrogels did not exhibit a more pronounced reduction in plant weight despite having a higher EC value compared to other hydrogel treatments. Thus, the increase in EC values alone does not seem to fully explain the observed toxicity. The effect of hydrogels on plant weight also depends on their application rate and the moisture regime, as shown in previous studies [20,21,32]. For instance, Tomadoni et al. [19] observed an increase in the fresh weight of lettuce plants subjected to drought stress when cultivated in a substrate amended with a 5% alginate-based hydrogel, while doses of 1% had no effect. These findings suggest that, in our present experiment, the amount of biochar–hydrogel composite applied may be insufficient to maintain an adequate water supply in the soil, and higher biochar–hydrogel composite doses may be required to induce beneficial effects on plant weight.

RWC reflects the balance between water absorption and transpiration from plant leaves, and its decrease is a prominent consequence of water deficit conditions [49]. The sustained RWC in the leaf tissues observed in this study, even under water stress conditions, is a notable point of interest. This phenomenon raises intriguing questions about the adaptive mechanisms employed by certain plant species, such as the bean plant in this case, to mitigate the impacts of water deficit. Stomatal closure, a typical plant response to water deficit, may play a crucial role in minimizing water loss and maintaining RWC [52,53]. This adaptability allows the plant to maintain RWC and potentially minimize the reduction in photosynthetic rates, ensuring better tolerance to water stress without significant yield loss [28]. Further research is required to understand the precise mechanisms by which these plants modulate their physiological responses to water stress, especially considering the potential influence of hydrogel applications on these mechanisms.

4.1.2. Photosynthetic Pigments

Drought stress generally leads to a decrease in photosynthetic pigments [52,54,55], which hampers photosynthesis and plant growth. In chloroplast, both photosynthesis and ROS generation occur. Elevated ROS production, as a consequence of water stress, can result in the oxidation and degradation of photosynthetic pigments [56,57]. In this study, the most noticeable effect of water scarcity was the decrease in the CHLa/CHLb ratio, indicating chloroplast damage in both control and treated groups.

Regardless of moisture levels, the addition of biochar–hydrogel composites led to reduced concentrations of photosynthetic pigments. A noteworthy observation in the present study was the increase in CHLb levels in plants grown in biochar–hydrogel composite-treated soils under drought stress in comparison to unstressed plants. This increase resulted in decreased CHLa/b and carotenoid/CHL total ratios.

These findings suggest that the combined effect of biochar–hydrogel composites and water deficit exacerbates the disruption of chloroplast structure and thylakoid membrane integrity. These alterations could lead to a decrease in photosynthesis, which could potentially account for the decrease in plant weight observed in this study when both biochar–hydrogel composite treatment and drought stress were applied together.

4.1.3. Biomarkers of Oxidative Stress

The induction of oxidative stress in plants due to water deficit has been well documented [33,46,56]. This phenomenon was substantiated in the present study by alterations in protein levels, enzymatic activities, and, in particular, differences in MDA levels between plants grown under well-watered and water-deficient conditions. These effects were observed in both control plants and in plants grown in soils treated with biochar–hydrogel composites.

Biochar–hydrogel composite application induced oxidative stress, but the effects varied depending on the moisture regime. Interestingly, hydrogel treatments reduced protein content and enzymatic activities under adequate moisture conditions, but increased these factors under drought stress conditions. Antioxidant enzymes are ROS scavenging systems evolved by plants as a defense mechanism against oxidative stress. However, despite the increase in antioxidant enzyme activities observed in drought-stressed plants grown in hydrogel-amended soils, there was no concomitant decrease in MDA levels. Contrarily, a significant rise in MDA levels was observed when 1% PTB-doped hydrogel ($59 \pm 4\%$) and 0.25% OTB-doped hydrogel ($20 \pm 1\%$) were applied to the soil. MDA levels are indicators of membrane lipid peroxidation induced by oxidative stress, and their increase indicates that these enzymes were unable to effectively protect cell membranes from the oxidative stress. Thus, the substantial increase in antioxidant enzyme activity observed with biochar–hydrogel composite addition did not appear to imply an increase in drought tolerance. Instead, it appears to be a result of the increased oxidative stress resulting from the combined effect of biochar–hydrogel composites and water deficit [46,52].

Proteins respond differently to drought stress at both the genetic and functional levels [58]. Some proteins involved in signaling pathways and stress response are activated during drought, while others, like those linked to photorespiration, may be downregulated. The impact of biochar–hydrogel composites on protein levels appeared to be associated with changes in enzymatic activities, as supported by a strong Pearson correlation between proteins and enzymatic activities at both moisture conditions. Additionally, a strong correlation was found between protein levels and enzymatic activities (CAT and GPOD) with photosynthetic pigments, at 70% WHC. The decrease in chlorophyll levels under stress conditions observed in this study can also be considered a tolerance mechanism in plants. The reduction in photosynthetic pigments limits energy absorption, thereby reducing oxidative stress and, subsequently, leads to a decline in enzymatic antioxidant activity [33,59], which is consistent with the decrease in GPOD and CAT observed in this study.

4.2. Effects of Biochar–Hydrogel Composite and Moisture Regime on Soil Microorganisms

Soil microorganisms play a crucial role in breaking down organic matter and recycling nutrients, essential for plant growth. In our study, we found that soil moisture levels did not significantly affect the soil carbon transformation rate. This lack of effect might be due to the drought conditions not being severe enough to reduce microbial biomass [19]. However, the introduction of biochar–hydrogel composites had a negative impact on this process regardless of soil irrigation conditions. Alginates, as naturally occurring polymers, serve

as substrates that support the growth and development of microbial populations [15,19]. Biochar incorporated into these hydrogels enhances soil microorganism habitat due to its porous structure and high surface area, and provides essential nutrients required for microbial growth [21,60]. Consequently, it would be expected that the addition of an alginate-based hydrogel enriched with biochar to the soil in this study would potentially have a positive effect on the soil microbiota. Therefore, the decrease in carbon transformation rate observed with the application of biochar–hydrogel composites suggests that certain compounds resulting from the degradation of alginate or PVA may have some degree of toxicity to soil microorganisms. Nevertheless, understanding the specific effects of hydrogel components or byproducts on plant growth remains an area for investigation.

Many previous studies have reported that soil enzyme activities decrease with increasing water scarcity [61–63]. This phenomenon can be attributed to the effects of drought on microbial viability and nutrient availability, considering the restricted diffusion of substrates at lower levels of soil moisture. Our study found that the response of soil enzymes to drought stress varied depending on the specific enzyme considered, indicating the intricate balance within microbial communities and suggesting potential shifts in their functional diversity and composition [64]. Notably, PHO was particularly sensitive to drought stress, exhibiting a significant decline in activity in both control and hydrogel-treated soils.

Hydrogels can also alter soil microbial communities, which in turn influence enzymatic activities [17,19,60]. In this study, the addition of biochar–hydrogel composites affected all soil microbial parameters, with AO being the least sensitive enzyme.

Interestingly, the introduction of hydrogels resulted in enhanced PHO activity, especially under normal moisture conditions. PHO enzymes are responsible for breaking down organic phosphorus into inorganic phosphates, which can be directly used by plants. Increased PHO activity is desirable as it is associated with enhanced soil phosphorus nutrient availability, thereby improving plant nutrient uptake. Moreover, the biochar–hydrogel composites used in our study have reactive surfaces featuring hydroxyl and carboxyl functional groups [32], which have the potential to bind phosphate ions and enhance their availability, consequently improving plant nutrient uptake [20]. However, it is important to note that in our study, the rise in PHO activity resulting from hydrogel addition did not correspond to increased plant growth.

Differences were observed between the biochar–hydrogel composite treatments. For instance, under adequate moisture conditions, PTB-doped hydrogels had less adverse effects on the carbon transformation rate, but had more detrimental effects on AO activity compared to OTB-doped hydrogels. In particular, the behavior of the 0.25% PTB-doped hydrogel differed from the others, as it did not induce significant increases in enzymatic activities, protein levels, or MDA concentrations. These variations in the effects of the tested biochar–hydrogel composites on plants and soil microorganisms could be attributed to the characteristics of biochar and its concentration in the hydrogels. Biochar interacts with the hydrogel structure, leading to the formation of various types of bonds that can alter various properties of the doped hydrogel, including its swelling behavior, chemical and mechanical stability, as well as its morphology and surface properties [13,32].

It is noteworthy that although the 1% PTB-doped hydrogel exhibited the highest swelling capacity, it did not show a greater potential to effectively mitigate the adverse effects of drought stress on plants compared to the other hydrogels tested. The increased level of cell membrane damage observed in bean leaves under the application of the 1% PTB-doped hydrogel, as indicated by the increased MDA content, provides insight into the potential trade-offs between water availability and stress mitigation in plant systems. These results are indeed interesting and underscore the complexity of plant–water interactions under specific environmental conditions.

In summary, our results show that hydrogels doped with biochar derived from olive or pine trees have significant effects on plants and soil organisms under both moisture regimes. The use of hydrogel composites as soil conditioners did not enhance the growth of bean plants and had a detrimental impact on carbon transformation rates under optimal

moisture levels. However, it is worth noting that biochar–hydrogel composite application increased PHO activity, which is a positive effect associated with improved phosphorus availability to plants. Unexpectedly, the biochar–hydrogel composite application did not mitigate the negative effects of water deficit; on the contrary, it aggravated some of these effects. The combined exposure of plants to biochar–hydrogel composites and drought stress led to a reduction in plant weight. This reduction may be attributed to disruptions in photosynthetic pigments, as indicated by a decrease in the CHLa/CHLb ratio, and to an increase in oxidative stress, as evidenced by higher MDA levels.

These conclusions emphasize the complex nature of the interactions between hydrogels, soil, and plant species. Expanding the scope of the study to include various soil types, plant species, different composite application rates, and a range of moisture conditions is crucial to gaining a comprehensive understanding of the effectiveness of biochar hydrogels in drought mitigation. Furthermore, the findings of this study underscore the potential risk of the indiscriminate use of hydrogels in agriculture and emphasize the need for a comprehensive ecotoxicological assessment of hydrogels prior to their widespread use in agriculture. This multifaceted approach will provide a more holistic view that is critical for their application in diverse environmental contexts.

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