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Farmyard Manure Enhances Phytoremediation and Mitigates Pb, Cd, and Drought Stress in Ryegrass

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Abstract: Here, a pot experiment was designed to evaluate the phytoremediation potential of ryegrass (*Lolium perennne* L.) for Pb- and Cd-polluted soils under various drought levels in the presence of farmyard manure (FYM). Three levels of Pb (0, 300, and 600 mg kg^{-1}), Cd (0, 100, and 200 mg kg^{-1}), and drought (field capacity 100, 50, and 30%) as well as two levels of FYM (0 and 1%) were used in this experiment. Results from this study showed a significant decrease (up to 84%) in the overall growth and physiology of ryegrass. A substantial increase in antioxidants (SOD, CAT, and POD) was observed under HMs and drought stress. By the application of FYM, antioxidant activities were significantly reduced. The ryegrass accumulated higher amounts of Pb (up to 150 mg kg^{-1} in shoots and 193 mg kg^{-1} in roots) and Cd (up to 71 mg kg^{-1} in shoots and 92 mg kg^{-1} in roots) in plant tissues; however, an FYM addition significantly reduced the accumulation of both metals. Furthermore, the results of this research indicated that ryegrass has a promising ability to phytoremediate Pb and Cd, and the addition of FYM may be helpful in enhancing metal stabilization and plant growth despite water constraints.

Keywords: heavy metals; phytotoxicity; rhizoremediation; grassland; drought; organic matters

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1. Introduction

Heavy metals (HMs) pollution has become a threat and challenge in the world due to rapid industrialization. The inefficient and uncontrolled use of agricultural inputs (such as pesticides and fertilizers) and the application of wastewater for irrigation increases the levels of HMs in the food chains [1]. HMs are very toxic even in small concentrations and ultimately affect the health of human beings [2]. According to the United States Environmental Protection Agency, lead (Pb) is one of the primary bio-toxic HMs present in various environments [3]. The main sources of Pb pollution in environmental spheres are Pb-based industries, transportation, mining activities, extensive and improper fertilization, etc. [4]. Major sources of cadmium (Cd) include the processing and smelting of non-ferrous metals, production of phosphatic fertilizers, manufacturing of batteries, and recycling of electronic devices [5]. Pb exposure to plants causes impairments in the morphological, physiological, and biochemical traits of the plants. Moreover, leave chlorosis, root defaming, impeded photosynthesis processes, modification of hormonal functions, disturbed permeability, and shape of the membrane are characterized by higher contents of Pb [6]. Similarly, higher levels of Cd negatively affect the growth and physiology of plants. It has been found that Cd can harm many biological, chemical, and physiological processes in the plant, such as respiration, photosynthesis, and nitrogen absorption [7–9]. However, the severity of toxicity depends on the Cd concentration present in the soil or water, as well as the duration of exposure [10]. Thus, it is important to remove Pb, and Cd, from the environment for the sustainability of the ecosystem.

Numerous physical, biological, and chemical methods are used to clean the soil from HMs [11,12]. However, due to the formation of toxic byproducts and higher costs, these physicochemical techniques are not widely accepted [13]. Phytoremediation technology, especially the use of hyper-accumulator plants to remove organic and inorganic (i.e., HMs) pollutants from contaminated soils, is a green, environmentally friendly, and cost-effective option. In phytoremediation, plants absorb higher amounts of HMs and translocate them to various parts for accumulation [12,14,15]. A variety of grasses are being used for the phytoremediation of HMs from polluted soils [15,16]. Ryegrass (*Lolium perenne* L.), a promising crop for phytoremediation, is typically known for its fast growth, higher tolerance to abiotic stresses, and capability to grow in various soils [17,18]. Previous studies have also reported a higher intake of Pb and Cd by ryegrass as it can tolerate higher levels of these pollutants [17,19,20]. Furthermore, due to its phytoextraction and bioaccumulation abilities, perennial ryegrass has demonstrated a higher potential for the remediation of Cd from contaminated soil [21]. Thus, the perennial ryegrass is deemed to be the most suitable option for the reclamation of HMs-contaminated soils.

The world is running out of water due to sudden changes in climatic patterns. This causes higher rains in some regions while the minimum occurring in other regions through the years causes droughty conditions. Drought is the main component that affects agricultural yields throughout the world [22]. Globally, in the last 40 years, drought stress has decreased crop production by 10% [23]. It is predicted that prevalent droughts will decrease yields by up to 50% by 2050 [24]. In drought conditions, turf grasses are the most important land vegetation that covers the lower contaminated surfaces and produces substantial biomass [25].

The addition of organic material has several advantages over inorganic fertilizers including higher nutrient use efficiency, increased rhizospheric microbes, and improved soil aeration and water-holding status of normal or degraded soils. In recent times, a variety of organic materials such as biochar, manures, and press mud have been employed to increase the phytoremediation potential of plants grown under HMs contamination [26,27]. The farmyard manure (FYM), like other organic amendments such as compost and charcoal, provides a much higher pool of nutrition for healthy soil and proper plant growth [28]. Literature has shown that organic amendment techniques can help to remove HMs like Cd, As, Cr, Pb, Cu, and Zn from soil [29]. From the above-mentioned information, we can conclude that ryegrass was easy to establish, produced higher biomass much faster, and was well adapted to hazardous habitats like those with drought, salinity, and HMs [30].

2. Materials and Methods

2.1. Soil Sample Collection and Lab Analysis

The FYM and soil were collected from the Agronomy farm and postgraduate research area of the Department of Forestry and Range Management (DFRM), respectively, University of Agriculture, Faisalabad (UAF). The soil was air-dried and sieved using a 2 mm mesh to ensure uniformity. A composite sample from the collected soil was properly labeled and packed in zipper bags and subsequently transported to the Water and Soil Testing Laboratory, Ayyub Agriculture Research Institute, Faisalabad. Here, comprehensive analyses of the FYM and soil's physicochemical properties were done, and the results are presented in Table 1. Electrical conductivity and pH were measured using an EC meter and pH meter, respectively (31). The USDA soil textural triangle was used to determine the soil textural class. Nitrogen (N), phosphorus (P), and potassium (K) were measured by following standard procedures described elsewhere [31,32]. Total N was evaluated with the Kjeldahl digestion method using the Kjeldahl apparatus (DF-4S Mitamura Riken Kogyo Inc., Tokyo, Japan). The P and K were estimated using the wet digestion method and the P in digested samples was measured through a UV-visible spectrophotometer at 430 nm wavelength. The K^+ was determined with a flame photometer (FP7, Jenway, Essex, UK) [32].

Table 1. Physiochemical properties of soil used during the experiments of this study.

No	Characteristics	Units	Concentrations Soil	Concentrations FYM
1	Sand	%	53.0 ± 0.6	-
2	Silt	%	22.5 ± 0.5	-
3	Clay	%	24.5 ± 0.4	-
4	pH		7.75 ± 0.34	8.22 ± 0.15
5	EC	dSm^{-1}	1.32 ± 0.03	1.39 ± 0.08
6	Organic matter	%	0.87 ± 0.07	85.8 ± 3.5
7	Total organic carbon	%	-	14.5 ± 0.55
8	Total nitrogen	%	0.82 ± 0.19	1.17 ± 0.03
9	Available phosphorus	mg/kg	44.0 ± 3.53	4608 ± 220
10	Available potassium	mg/kg	371 ± 21.43	8258 ± 397
11	Total Pb	mg/kg	ND	ND
12	Total Cd	mg/kg	ND	ND
13	Extractable Pb	mg/kg	ND	ND
14	Extractable Cd	mg/kg	ND	ND

EC, electrical conductivity; Pb, lead; Cd, cadmium; ND, not detectable.

2.2. Experimental Procedure

A pot trial was conducted in the research area of DFRM, UAF. The 3 kg of soil, collected from the DFRM, UAF, was added to each pot. For spiking, sufficient amounts of lead nitrate [Pb $(NO_3)_2$] were added to soil to achieve a concentration of 300 and 600 mg kg^{-1} of Pb in respective treatments. Similarly, the cadmium nitrate [Cd $(NO_3)_2.4H_2O$] was also applied in respective pots to achieve a concentration of 100 and 200 mg kg^{-1} of Cd. The Pb and Cd spiked soils (in pots) were then left undisturbed for 60 days in a warehouse for aging purposes. Three levels of Pb (i.e., 0, 300, and 600 mg kg^{-1}), Cd (i.e., 0, 100, and 200 mg kg^{-1}), and drought (i.e., at field capacity (FC) 100%, 50%, and 30%)) as well two levels of FYM (i.e., 0% and 1%) were used in this experiment. Treatments, each with four replicates, were arranged by following the completely randomized design (CRD). A shed was prepared with the help of iron rods and plastic sheets to cover the plant during rainy days and to maintain the moisture level in pots. For drought maintenance in respective treatments, water was added thrice a week in each pot to maintain the FC levels of 30%, 50%, and 100% for ryegrass.

2.3. Seed Germination and Growth Traits of Ryegrass

Fifteen ryegrass seeds were planted at equal distances, and after 12 days of growth, a consistent stand was achieved by retaining five seedlings in each pot. Ryegrass plants were harvested after 92 days of seed sowing. The growth traits such as shoot and root lengths were recorded with a measuring tape by uprooting the plants at the time of harvest. Similarly, the root and shoot fresh and dried weights were obtained through a portable electric weight balance. For the determination of root and shoot dry masses, respective samples were dried at 65 °C till the achievement of a constant weight. Moreover, the number of tillers per plant was noted after 30 days of seedling emergence, and the manual counting of leaves (per plant) was done at harvesting time [31].

2.4. Physiological Traits of Ryegrass

The determination of chlorophyll contents, including chl a, chl b, total chl, and carotenoids, was done following established standard methods [32,33]. The SPAD value was checked by using a SPAD meter (SPAD-502 Konica, Minolta, Osaka, Japan) [32]. Similarly, the electron transport rate (ETR), quantum yields (YII), fluorescence yield (Ft), and

photosynthetically active radiation (PAR) were obtained through a photosynthetic yield analyzer (MINI-PAM-II) (WALZ mess und Regeltechnik, Effeltrich, Germany) by using standard methodologies [34]. Moreover, the photosynthetic assimilation rate, transpiration rate, and water use efficiency of ryegrass were measured by an infrared gas analyzer (IRGA) (Analytical Development Company, Hoddeson, UK).

The membrane stability index of ryegrass was evaluated by following the methods of Sairam et al. [35]. In detail, 0.1 g of fresh leaves were properly cleaned and washed with distilled water. Thereafter, two sets of each treatment were developed by adding distilled water (i.e., 10 mL) in test tubes. One set of leaf materials was heated in a water bath for 30 min at 40 °C, and after that, the EC1 was measured with an EC meter. Then the second set of leaf samples was heated for 10 min at 100 °C and then the EC2 was noted. The MSI values were calculated by the following Equation (1).

$$\text{MSI (\%)} = (1 - \text{EC1}/\text{EC2}) \times 100 \quad (1)$$

The relative water content (RWC) of ryegrass was determined by taking flag leaves and then the fresh weight of these leaves was noted. Thereafter, the leaves were placed in test tubes and distilled water was added to them. These tubes were then stored in a shady place for 24 h to maximize the turgidness of the leaves. After recording the turgid weight, the samples were kept at 70 °C for 72 h in the oven until a constant weight was gained. After recording the oven dry weight, the RWC was obtained by applying Equation (2) [34].

$$\text{RWC (\%)} = [(\text{FW} - \text{DW})/(\text{TW} - \text{DW})] \times 100 \quad (2)$$

where TW is turgid weight, FW is fresh weight, and DW is dried weight.

2.5. Antioxidant Enzyme Activities and H_2O_2

To examine catalase (CAT) activities, a procedure described by Ali et al. [31] was used. To achieve the desired objective, a reaction mixture of 10.4 mL was prepared. This combination consisted of 200 µL of enzyme extract, 200 µL of hydrogen peroxide (0.3 M), and 10 mL of phosphate buffer (0.05 M) with EDTA (0.002 M) at a pH of 7.0. The decrease in absorbance was measured at a wavelength of 240 nm using a spectrophotometer, following the complete decomposition of hydrogen peroxide (H_2O_2). The methodology used by Beauchamp and Fridovich [36] was applied to assess the activity of superoxide dismutase (SOD). This was achieved by measuring the reduction in absorbance of nitroblue tetrazolium chloride at a wavelength of 560 nm. To assess peroxidase (POD) activity, the experimental procedure described by Angelini et al. [37] was utilized. This method involved monitoring the conversion of guaiacol to tetra-guaiacol by measuring the absorbance at 436 nm.

The hydrogen peroxide's H_2O_2 contents were detected from leaf material (0.2 g) by blending in 3 cm^3 of poured $C_2HCl_3O_2$, 0.1% (m/v) in an ice bath to form an even blend and centrifuging it for 15 min at 12,000 rpm [38]. An aliquot (0.5 cm^3) of the supernatant was blended with 0.5 cm^3 of a 35-phosphate buffer (pH 7.0) and 1 M potassium iodide (KI). The absorbance of the mixture was tested at 390 nm. The percentage of the scavenger activity of the H_2O_2 radical is calculated using Equation (3).

$$\text{Inhibition (\%)} = [(\text{Abs Cont} - \text{Abs Sample})/\text{Abs Cont}] \times 100 \quad (3)$$

where Abs Cont is the absorbance of the control reaction, Abs Sample is the absorbance of the sample.

2.6. Determination of Lead and Cadmium in Soil and Plants

Ammonium bicarbonate-DTPA procedures were used to determine the levels of Pb and Cd in soil samples [39]. Using a one-liter solution made by melting 1.97 g of DTPA beside 79.06 g of NH_4HCO_3, the AB-DTPA extracting solution was set and the pH was

maintained at 7.60 by adding NH_4OH and/or HCl. The 20 mL prepared solution was added to each flask, containing 10 g of soil. Then, the flask was shaken for 15 min in a reciprocating shaker at a rate of 180 rpm. The soil extracts were subsequently filtered using Whatman No. 542 filters, followed by analysis to determine bioavailable Cd and Pb using atomic absorption spectrometry (AAS) (Hitachi Polarized Zeeman AAS, Z-8200, Tokyo, Japan).

For plant analysis, the oven-dried grass samples were crushed in a grinder and then sieved with a 0.5 mm sieve to produce homogenous plant samples for digestion. A total of 0.5 g of grass sample was added to a 50 mL digestion flask. Then, 10 mL di-acid solution of HNO_3 and $HClO_4$ (2:1, v/v) was added to each flask. Flasks were then kept overnight and, after that, digested on a hotplate in a closed chamber until white dense fumes were observed in samples. Thereafter, a known amount of deionized water was poured in each flask to achieve a 50 mL volume. These samples were then filtrated through filter paper and transferred to plastic bottles of the same capacity. At last, these samples were analyzed through AAS for the determination of Pb and Cd in different plant parts.

2.7. Translocation and Bioconcentration Factors

The translocation factor (TF) and the bioconcentration factor (BCF) were calculated using Equations (4) and (5) [40] and used to assess the accumulation of metals in plants and their translocation from the roots to the aerial parts of plants.

$$\text{TF}: \frac{\text{Metal accumulation in shoots } (\text{mg/kg})}{\text{Metal accumulation in roots } (\text{mg/kg})} \times 100 \quad (4)$$

$$\text{BCF}: \frac{\text{Metal accumulation in harvested plant material } (\text{mg/kg})}{\text{Metal concentration in soil } (\text{mg/kg})} \times 100 \quad (5)$$

2.8. Statistical Analysis

The data obtained through the experiment were statistically evaluated using STATISTIX 8.1 software. The analysis of variance (ANOVA, South St. New Providence, NJ, USA) with Tuckey's honest significance difference (HSD) test was performed on the obtained data.

3. Results

3.1. Physicochemical Attributes of Soil and Farmyard Manure

The physicochemical analysis of the soil indicated that the soil used in this study was a sandy loam with a neutral pH, with less than 1% organic matter and undetectable Pb and Cd (Table 1). Results of the analysis of the selected properties of the FYM showed the presence of sufficient amounts of essential macronutrients (i.e., N, P, and K), with a slightly alkaline pH, and organic matter, and the absence of Pb and Cd (Table 1).

3.2. Growth Traits of Ryegrass

In the current study, the results indicated a significant reduction in most of the biomass attributes of ryegrass when grown in Cd- and Pb-contaminated soil (Figures 1 and 2). The root length (16.7% and 23.4%), shoot length (9.9% and 12.8%), root fresh weight (28.6% and 33.3%), root dry weight (10.6% and 13.3%), shoot fresh weight (18.4% and 20.1%), shoot dry weight (21.9% and 34.9%), number of tillers per plant (11.6% and 12.8%), and number of leaves per plant (12.6% and 24.0%) were decreased in the presence of Pb (300 mg kg^{-1} and 600 mg kg^{-1}), respectively. At 100 mg kg^{-1} Cd and 200 mg kg^{-1} of Cd contamination, up to 82.5% and 83.4% reduction in biomass attributes was observed, respectively, over the uncontaminated control (at FC 100%).

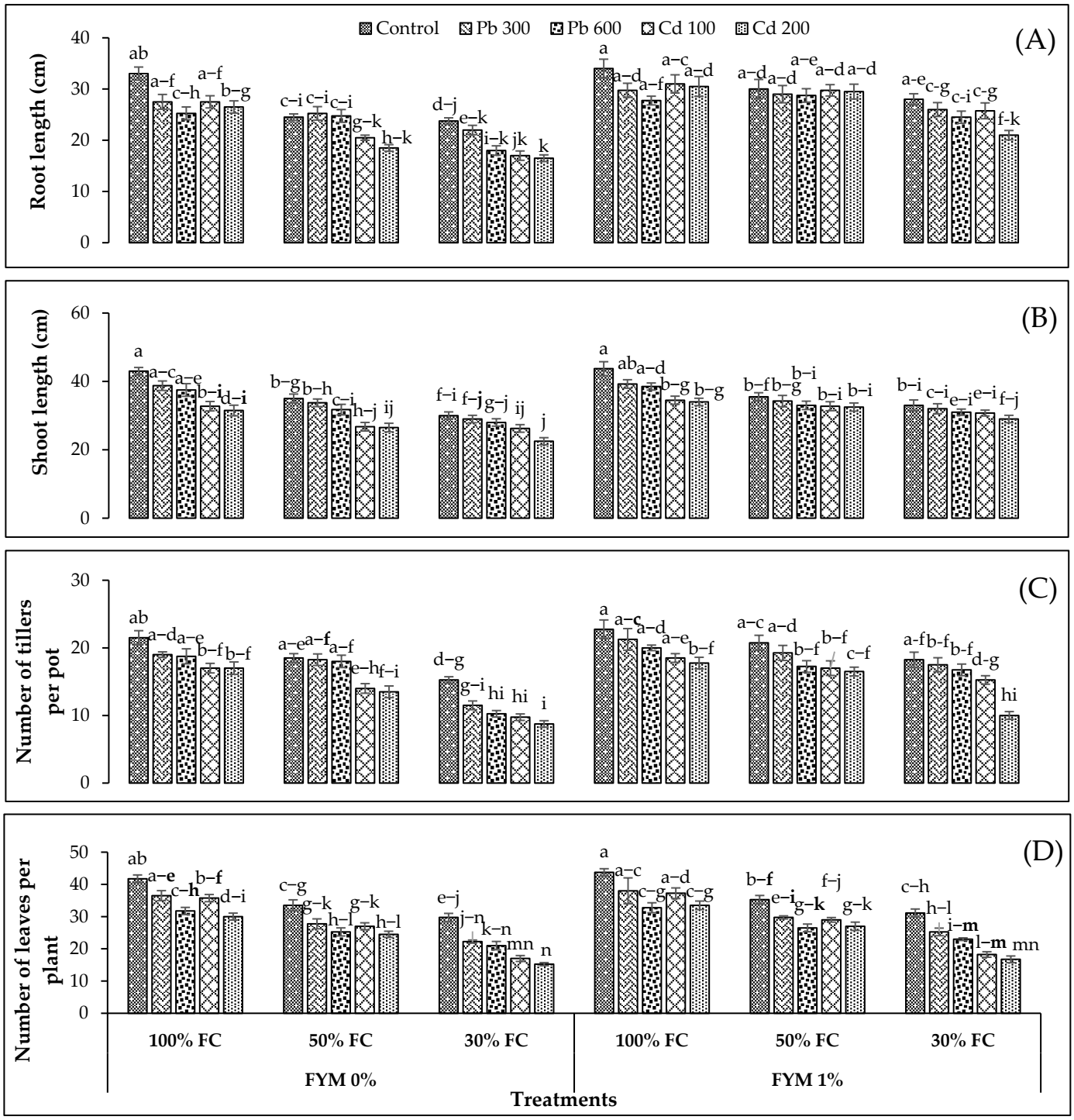

Figure 1. Effects of farmyard manure and drought on root length (**A**), shoot length (**B**), number of tillers per pot (**C**), and number of leaves per plant (**D**) of ryegrass in Pb- and Cd-contaminated soil. The standard errors and means of four replicates are represented by the error bars and columns, respectively, and different small letters on columns show the statistical difference (i.e., significance and/or non-significance) among different means ($p \leq 0.05$). FC, field capacity; Pb, lead; Cd, cadmium.

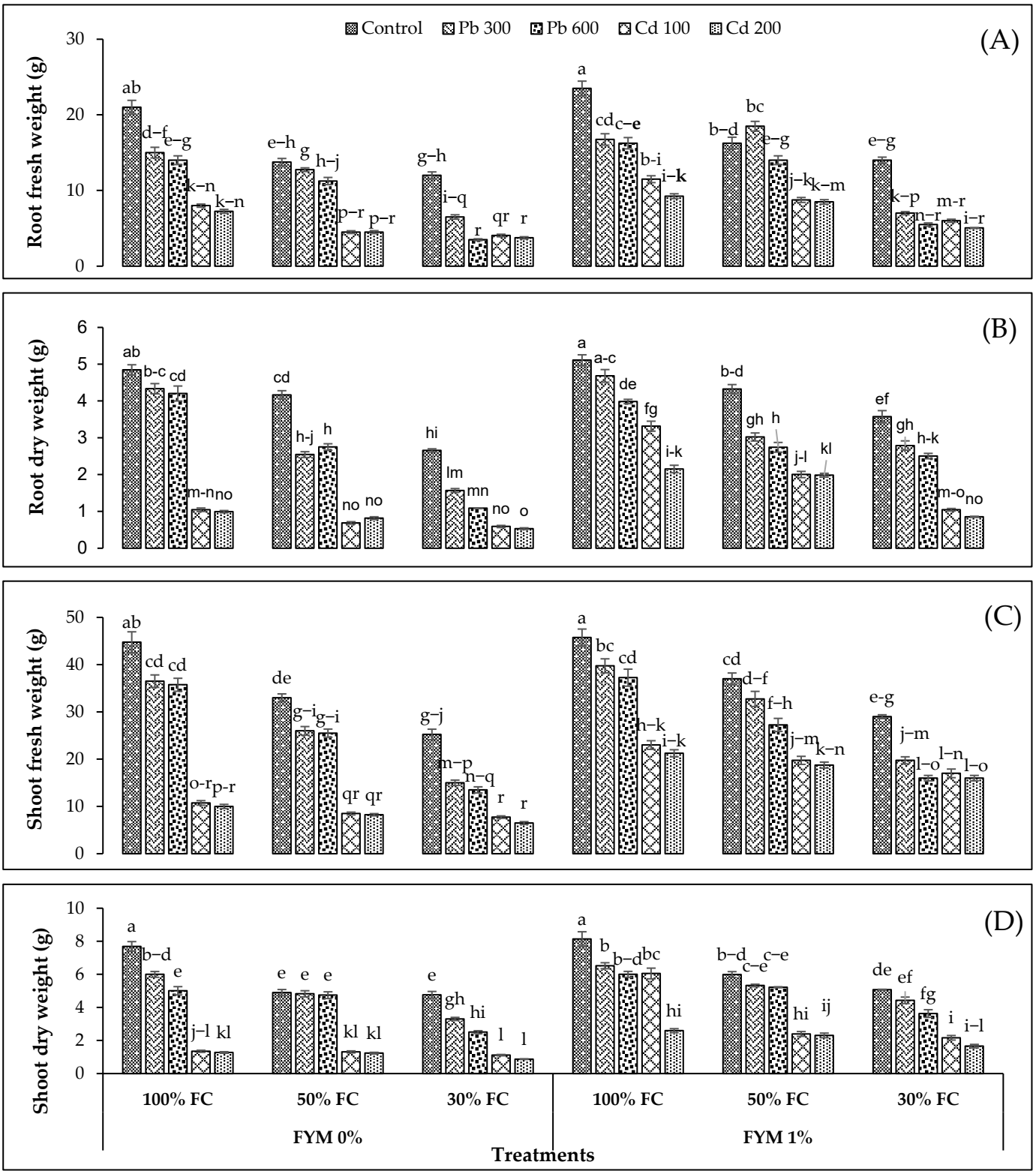

Figure 2. Effects of farmyard manure and drought on root fresh weight (**A**), root dry weight (**B**), shoot fresh weight (**C**) and shoot dry weight (**D**) of ryegrass in Pb- and Cd-contaminated soil. The standard errors and means of four replicates are represented by the error bars and columns, respectively, and different small letters on columns show the statistical difference (i.e., significance and/or non-significance) among different means ($p \leq 0.05$). FC, field capacity; Pb, lead; Cd, cadmium.

However, when these plants were grown at 50% of FC, the root length (25.7%), shoot length (18.6%), root fresh weight (34.5%), root dry weight (14.0%), shoot fresh weight (26.3%), shoot dry weight (36.3%), number of tillers per plant (14.0%), and number of leaves per plant (19.8%) were also significantly reduced compared with the control (FC 100%). Almost similar results were found at 30% of FC, where 28.0–45.1% decreased growth was observed in all biomass attributes, as compared to uncontaminated control (at 100% FC) (Figures 1 and 2). Moreover, at 50% of FC, a decrease of up to 41.3%, 34.6%, 43.7%, and 30.2% was observed in the growth attributes of ryegrass under 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively, as compared to their respective controls (i.e., Pb and Cd contamination at 100% FC). Similarly, at 30% of FC, both HMs caused significant retardation of growth (17.1–75.0%) when compared with their respective controls at 100% FC (Figures 1 and 2).

More importantly, the results from the treatments with the application of 1% FYM showed a moderate to significant increase in all growth attributes as compared to un-amended treatments (without FYM). The application of 1% FYM increased the growth attributes of ryegrass up to 10.6%, 16.6%, 77.7%, and 53.9% in the presence of 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively, as compared to un-amended contaminated controls. Similarly, increases of up to 18.3% (at 50% FC) and 25.7% (at 30% FC) were also observed as compared to their respective un-amended controls (without FYM and HMs). Moreover, at 50% FC and 1% FYM, a significant increase of 75.0% was monitored with 600 mg kg^{-1} Pb over the control. Likewise, the addition of FYM in treatments at 30% FC caused up to a 57.0% increase in growth attributes when subjected to 600 mg kg^{-1} Pb, respectively, over their contaminated controls (without FYM at 30% FC). A similar trend was observed in the case of Cd at 30% FC, causing up to a 59.4% increase in growth attributes with the FYM addition over the respective controls (Figures 1 and 2).

3.3. Physiological Attributes of Ryegrass

In the present study, results indicated a significant reduction ($p \leq 0.05$) in most of the physiological attributes (except MSI) of ryegrass when grown in Pb- and Cd-contaminated soil (Table 2, Figures 3 and 4). The SPAD value (19.3% and 32.5%), RWC (6.40% and 6.70%), MSI (1.80% and 3.60%), Chl a (22.9% and 33.0%), Chl b (26.8% and 44.4%), total Chl (24.3% and 36.9%), carotenoids (9.52% and 20.9%), PAR (9.67% and 15.8%), transpiration rate (8.05% and 19.5%) and WUE (11.1% and 15.6%) were reduced in the presence of Pb contamination (i.e., 300 and 600 mg kg^{-1}), respectively, over the normal control. Similarly, under 100 and 200 mg kg^{-1} of Cd contamination, all the physiological attributes (except RWC, MSI, and transpiration rate) were significantly decreased, by 8.89–32.9% and 13.3–50.4%, respectively.

Nevertheless, when the plants were grown at 50% and 30% FC, the physiological traits of ryegrass were reduced by 4.80–52.6% and 9.40–73.7%, respectively, as compared to the uncontaminated control (100% FC). Moreover, decreases of up to 53.4%, 56.3%, 51.2%, and 60.0% were observed with 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively, as compared to their respective control (i.e., Pb and Cd contamination at 100% FC). Similarly, at 30% of FC, the SPAD value, RWC, MSI, Chl a, Chl b, total Chl, carotenoids, PAR, transpiration rate, and WUE were reduced by 6.20–76.7%, 8.70–81.3%, 13.8–81.3%, and 15.9–86.7% with 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively, compared with those of their respective contaminated controls (i.e., Pb- and Cd-added treatments at 100% FC) (Table 2, Figures 3 and 4).

Table 2. Effects of farmyard manure and drought on photosynthetic assimilation rate, transpiration rate, water use efficiency, membrane stability index, and relative water content of ryegrass in Pb- and Cd-contaminated soil.

Photosynthetic Assimilation Rate (μmol CO^2 m^{-2} s^{-1})							Transpiration Rate (mmol H_2O m^2 s^{-1})					
Treatments	0% FYM			1% FYM			0% FYM			1% FYM		
Drought level	100%	50%	30%	100%	50%	30%	100%	50%	30%	100%	50%	30%
Control	19.1 ± 1.1 b	9.00 ± 0.4 f	5.00 ± 0.1 hj	22.0 ± 1.3 a	12.0 ± 0.5 e	6.00 ± 0.2 g–i	4.35 ± 0.1 ab	2.20 ± 0.1 g–i	1.40 ± 0.04 j–m	5.00 ± 0.21 a	3.00 ± 0.1 ef	1.60 ± 0.06 i–m
Pb2	17.1 ± 0.8 bc	8.00 ± 0.3 fg	4.00 ± 0.8 i–k	18.0 ± 0.9 bc	9.00 ± 1.0 f	5.00 ± 0.1 h–j	4.00 ± 0.1 b–d	1.90± 0.1 g–k	1.20 ± 0.03 k–m	4.50± 0.19 ab	2.57 ± 0.1 fg	1.40 ± 0.2 j–m
Pb3	16.0 ± 0.6 cd	7.00 ± 0.9 fg	3.00 ± 0.1 jk	15.0 ± 0.5 d	7.00 ± 0.2 f–g	3.00 ± 0.1 jk	3.50 ± 0.7 de	1.70 ± 0.1 h–m	1.00 ± 0.05 m	4.00± 0.1 b–d	2.20 ± 0.07 g–i	1.20 ± 0.05 k–m
Cd2	16.0 ± 1.0 cd	7.00 ± 0.2 f–h	3.00 ± 0.13 jk	19.0 ± 1.4 b	7.00 ± 0.5 f–g	4.00 ± 0.8 i–k	4.10 ± 0.2 b–d	2.00 ± 0.1 g–j	1.30 ± 0.04 j–m	4.70 ± 0.2 ab	2.40 ± 0.1 f–h	1.50 ± 0.06 i–m
Cd3	15.0 ± 1.2 d	6.00 ± 0.7 g–i	2.00 ± 0.08 k	14.9 ± 1.3 d	6.00 ± 0.2 g–i	2.00 ± 0.07 k	3.60 ± 0.8 c–e	1.80 ± 0.1 h–l	1.10 ± 0.09 lm	4.30± 0.6 a–c	1.90 ± 0.1 g–k	1.30± 0.03 j–m
Water use efficiency (mmol CO^2 mol^{-1} H_2O)							Membrane stability index (%)					
Treatments	0% FYM			1% FYM			0% FYM			1% FYM		
Drought level	100%	50%	30%	100%	50%	30%	100%	50%	30%	100%	50%	30%
Control	4.50 ± 0.1 bc	2.40 ± 0.1 eg	1.50 ± 0.07 jk	5.00 ± 0.1 a	2.80± 0.14 e	1.70 ± 0.07 ij	82.7 ± 2.76 a	78.7 ± 3.49 a–c	75.0 ± 6.59 a–e	84.2 ± 6.03 a	81.0 ± 4.55 a–c	76.9 ± 7.67 a–e
Pb2	4.00 ± 0.1 d	2.10± 0.09 f–i	1.20± 0.06 k–m	4.50± 0.3 bc	2.50 ± 0.08 ef	1.30 ± 0.07 j–m	81.2 ± 2.31 ab	77.0 ± 2.47 a–c	74.0 ± 4.92 a–e	83.5 ± 6.00 a	79.5 ± 4.89 a–c	74.5 ± 6.54 b–f
Pb3	3.80 ± 0.1 d	1.99 ± 0.08 hi	0.90 ± 0.03 m	4.00± 0.2 d	1.20 ± 0.07 k–m	1.00 ± 0.05 lm	79.7 ± 2.98 ab	76.0 ± 6.29 a–d	67.0 ± 1.28 c–f	83.0 ± 5.68 a	77.0 ± 6.12 a–e	69.7 ± 2.78 d–f
Cd2	4.10 ± 0.1 cd	2.20 ± 0.05 f–h	1.30± 0.05 j–m	4.70 ± 0.3 ab	2.70 ± 0.08 e	1.40 ± 0.07 j–l	80.7 ± 0.99 ab	74.0 ± 4.56 a–e	62.5 ± 4.37 ef	83.5 ± 2.77 a	74.0 ± 6.12 a–c	63.4 ± 1.65 ef
Cd3	3.90 ± 0.1 d	2.00 ± 0.07 g–i	1.00± 0.03 lm	4.10 ± 0.3 cd	1.40± 0.07 j–l	1.00± 0.05 lm	80.0 ± 1.77 ab	77.0 ± 5.05 a–c	61.2 ± 2.34 a	82.9 ± 2.23 ab	78.0 ± 6.85 a–c	62.9 ± 4.35 f–k
Relative water content (%)												
Treatments	0% FYM			1% FYM								
Drought level	100%	50%	30%	100%	50%	30%						
Control	86.0 ± 4.00 ab	80.2 ± 4.10 a–e	77.0 ± 4.22 c–f	88.0 ± 2.14 a	82.7 ± 2.62 a–c	77.7 ± 3.80 b–f						
Pb2	80.5 ± 2.62 a–e	78.5 ± 1.92 b–f	75.5 ± 2.30 c–g	83.0 ± 3.60 a–c	81.7 ± 3.50 a–d	76.0 ± 2.71 c–g						
Pb3	80.2 ± 2.80 a–e	77.2 ± 3.84 b–f	73.2 ± 2.41 d–h	81.0 ± 4.34 a–e	78.1 ± 3.12 b–f	74.5 ± 1.22 c–g						
Cd2	78.7 ± 1.72 b–f	75.7 ± 2.80 c–g	65.5 ± 2.08 hi	79.5 ± 4.23 a–f	74.8 ± 3.84 c–g	68.0 ± 2.94 g–i						
Cd3	77.7 ± 2.80 b–f	71.2 ± 5.60 f–i	62.5 ± 3.10 i	76.2 ± 2.50 c–g	72.2 ± 2.92 e–h	65.5 ± 1.29 hi						

The standard errors and means of four replicates are represented by the error bars and columns, respectively, and different small letters on columns show the statistical difference (i.e., significance and/or non-significance) among different means ($p \leq 0.05$). FC, field capacity; Pb, lead; Cd, cadmium.

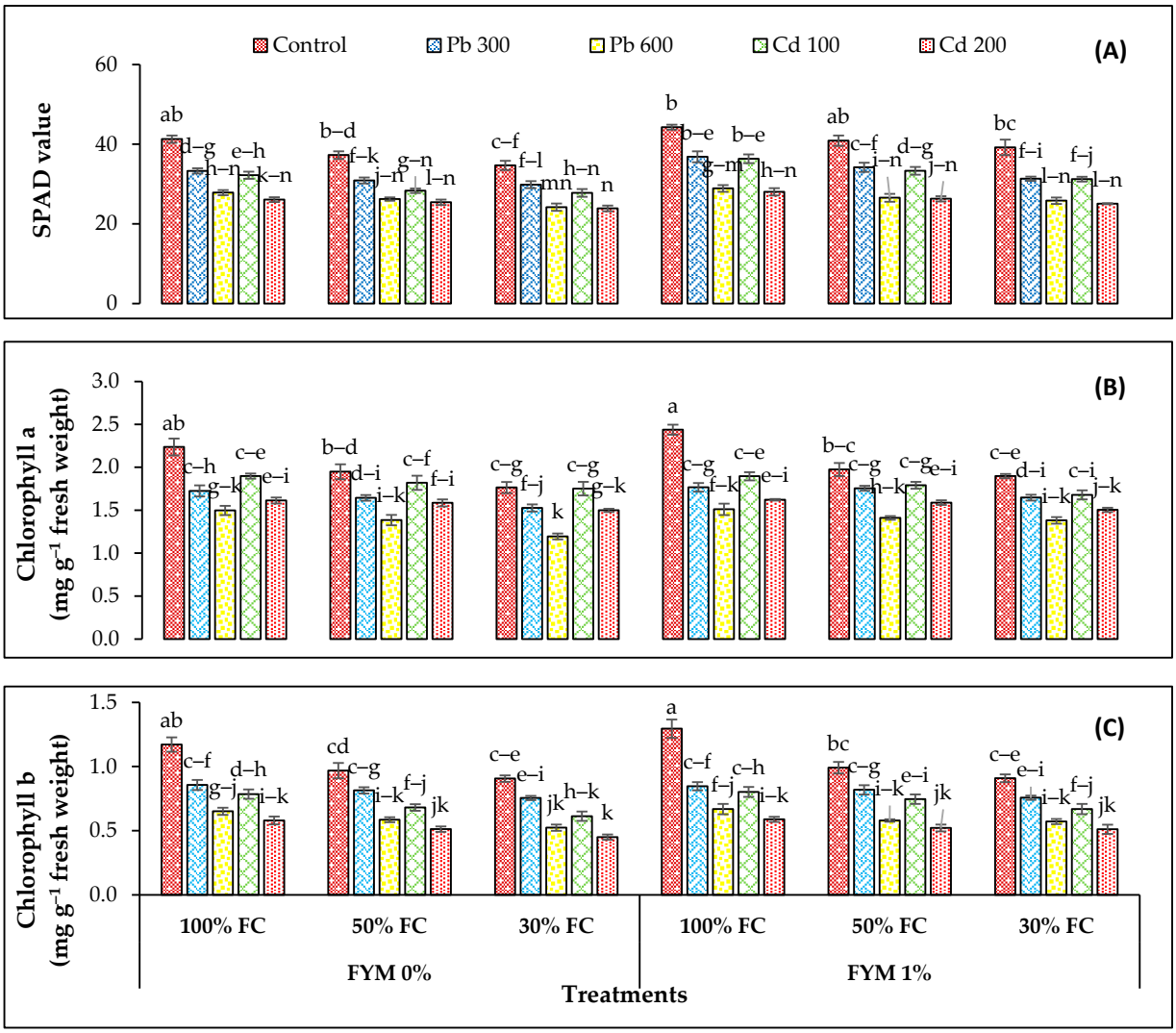

Figure 3. Effects of farmyard manure and drought on SPAD value (**A**), Chl a (**B**), and Chl b (**C**) in Pb- and Cd-contaminated soil. The standard errors and means of four replicates are represented by the error bars and columns, respectively, and different small letters on columns show the statistical difference (i.e., significance and/or non-significance) among different means ($p \leq 0.05$). FC, field capacity; Pb, lead; Cd, cadmium.

Furthermore, the results indicated that a 1% FYM addition caused a significant increase in most of the physiological traits of ryegrass when compared to un-amended treatment (without FYM and HMs) (Table 2, Figures 3 and 4). With the addition of FYM in the uncontaminated treatment, there was an increase of up to 13.6% over the un-amended and uncontaminated controls at 100% FC. The application of FYM (1%) also increased the physiological attributes of ryegrass up to 13.6%, 12.5%, 12.8%, and 16.3% in the presence of 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively, as compared to un-amended contaminated controls. Similarly, increases of up to 26.7% (50% FC) and 16.7% (30% FC) were also observed as compared to their respective un-amended controls.

In addition, at 50% FC and with 1% FYM, significant increases of up to 26.2%, 54.5%, 18.9%, and 11.3% were monitored with 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively. With the addition of FYM in treatments at 30% FC, FYM was revealed to promote increases of up to 15.3% and 22.2% at 300 and 600 mg kg^{-1} Pb, respectively, over their respective controls (without FYM at 30% FC). Similarly, the addition of FYM with Cd contamination of 100 and 200 mg kg^{-1} Cd was shown to increase

up to 13.3% and 15.4%, respectively, physiological attributes, as compared to contaminated controls (without FYM at 30% FC) (Table 2, Figures 3 and 4).

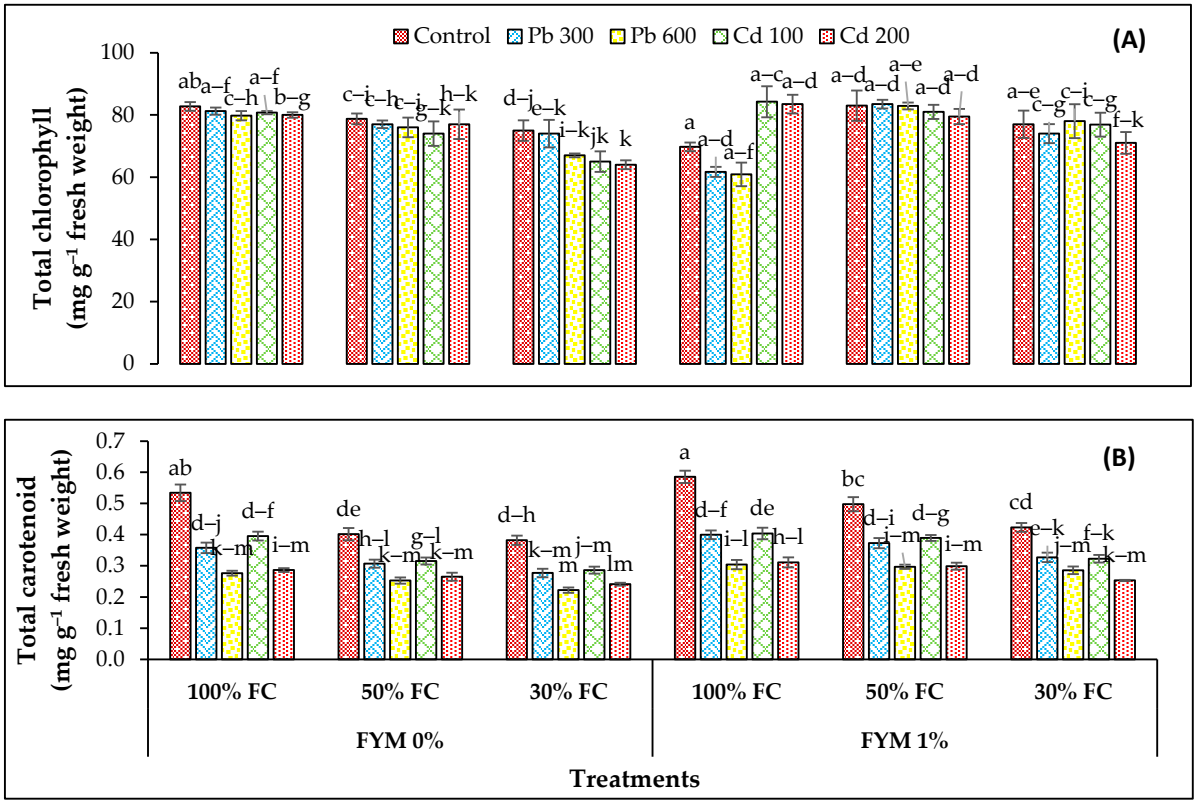

Figure 4. Effects of farmyard manure and drought on total chlorophyll contents (**A**) and total carotenoid contents (**B**) in Pb- and Cd-contaminated soil. The standard errors and means of four replicates are represented by the error bars and columns, respectively, and different small letters on columns show the statistical difference (i.e., significance and/or non-significance) among different means ($p \leq 0.05$). FC, field capacity; Pb, lead; Cd, cadmium.

3.4. Antioxidative Attributes of Ryegrass

Findings from the current study indicated a significant increase in most of the antioxidative attributes of ryegrass Pb and Cd contamination (Figure 5). The antioxidative enzymes were produced more in the presence of H_2O_2 (32.4–94.3%) contents. The SOD (27.7–47.9%), CAT (39.5–90.2%), and POD (67.3–109%) activities were enhanced in the presence of Pb (300 and 600 mg kg^{-1}), respectively. Similarly, under 100 mg kg^{-1} contamination, the H_2O_2, SOD, CAT, and POD were improved by 87.4%, 36.8%, 73.7%, and 73.1%. Likewise, with 200 mg kg^{-1} of Cd contamination, an increase of 64.3–131% was observed in H_2O_2 and antioxidative enzymes compared with their respective controls. However, when plants were grown at 50% and 30% of FC, the H_2O_2 (144% and 285%), SOD (31.5% and 68.5%), CAT (38.3% and 49.1%), and POD (42.4% and 61.6%) contents were increased, respectively, as compared to uncontaminated controls (100% FC). Moreover, 28.5–116%, 29.6–119%, 20.8–95.9%, and 32.3–90.2% increases were observed in antioxidative activities of ryegrass with 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively, as compared to the controls (i.e., Pb and Cd contamination at 100% FC). Similarly, at 30% of FC, H_2O_2 (218%, 159%, 133%, and 158%), SOD (74.5%, 131%, 60.9%, and 101%), CAT (59.7%, 77.5%, 44.6%, and 82.2%) and POD (20.9%, 16.3%, 12.4%, and 39.4%) were increased under 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively, compared with their respective contaminated controls (i.e., Pb- and Cd-added treatments at 100% FC) (Figure 5).

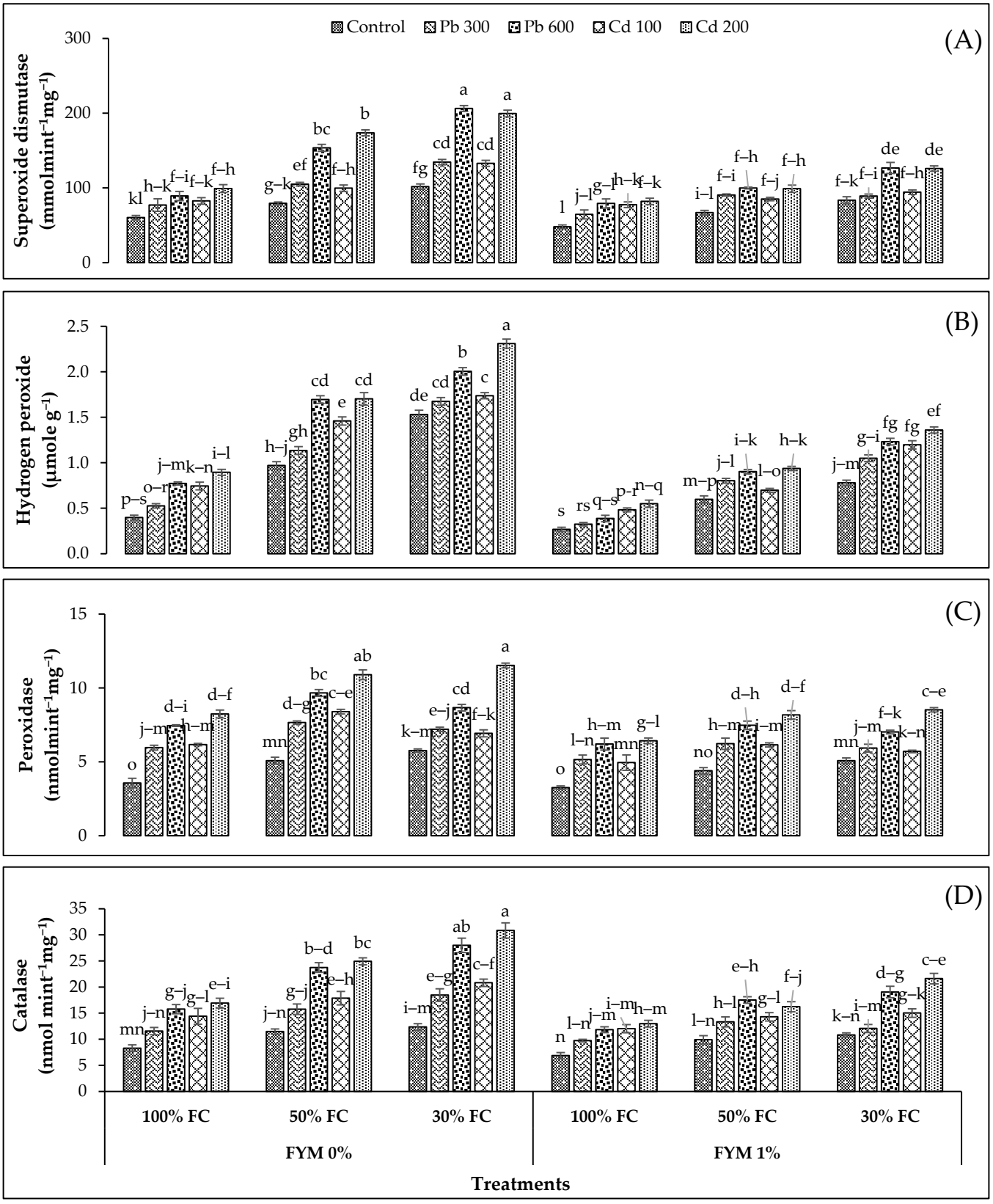

Figure 5. Effects of farmyard manure and drought on SOD (**A**), H_2O_2 (**B**), POD (**C**), and CAT (**D**) of ryegrass in Pb- and Cd-contaminated soil. The standard errors and means of four replicates are represented by the error bars and columns, respectively, and different small letters on columns show the statistical difference (i.e., significance and/or non-significance) among different means ($p \leq 0.05$). FC, field capacity; Pb, lead; Cd, cadmium.

The addition of FYM in the uncontaminated treatment showed a decrease of up to 32.7% in antioxidant activities over the un-amended and uncontaminated controls at 100% FC (Figure 5). The application of FYM (1%) also decreased the antioxidative attributes of ryegrass by up to 29.3%, 46.7%, 35.2%, and 38.5% in the presence of 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively, as compared to un-amended contaminated controls. Similarly, decreases of up to 38.4% (50% FC) and 49.0% (30% FC) in antioxidant attributes were also observed as compared to their respective un-amended controls. Moreover, at 50% FC and 1% FYM, significant decreases of up to 29.3%, 46.7%, 52.2%, and 44.9% were monitored with 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively. Likewise, a similar trend was seen under treatments containing FYM at 30% FC. The addition of FYM in treatments at 30% FC revealed decreases of up to 37.3% and 38.5% at 300 and 600 mg kg^{-1} Pb, respectively, over their respective controls (without FYM at 30% FC). Similarly, the addition of FYM under Cd contamination of 100 and 200 mg kg^{-1} Cd showed decreases of up to 31.1% and 41.2%, respectively, in physiological attributes as compared to contaminated control (without FYM at 30% FC) (Figure 5).

3.5. Heavy Metals Concentration in Plants and Soil

In the present study, the results showed a significant accumulation of Pb and Cd in shoots and roots of ryegrass after 92 days of growth in HMs-contaminated soil (Figure 6). The HMs in shoots and roots were accumulated at 50.4 mg kg^{-1} and 90.2 mg kg^{-1} (in the presence of 300 mg kg^{-1}) and 99.3 mg kg^{-1} and 153 mg kg^{-1} (in the presence of 600 mg kg^{-1} of Pb), respectively. Similarly, under 100 mg kg^{-1} and 200 mg kg^{-1} of Cd contamination, the shoots and roots collectively accumulated 81.3 mg kg^{-1} and 134 mg kg^{-1}, respectively, at 100% FC (Figure 6). Moreover, at 50% of FC and with the presence of 300 mg kg^{-1} Pb, the ryegrass plants accumulated 64.1 mg kg^{-1} (in shoots) and 108 mg kg^{-1} (in roots). Similarly, at 600 mg kg^{-1} Pb, the shoots accumulated 123 mg kg^{-1} and the roots contained 178 mg kg^{-1} at 50% FC. More similar trends were observed in the case of 100 mg kg^{-1} Cd and 200 mg kg^{-1} Cd at 50% of FC, in which shoots contained 47.7 mg kg^{-1} and 65.6 mg kg^{-1}, and roots accumulated 57.8 mg kg^{-1} and 86.1 mg kg^{-1} of Cd, respectively, during the experimental period. Similarly, at 30% of FC, the HMs in the shoots were 70.4 mg kg^{-1}, 131 mg kg^{-1}, 49.3 mg kg^{-1}, and 71.9 mg kg^{-1} with 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively (Figure 6). Likewise, the roots accumulated 112 mg kg^{-1}, 193 mg kg^{-1}, 58.6 mg kg^{-1}, and 92.9 mg kg^{-1} under 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively (Figure 6).

The application of FYM (1%) to soil resulted in accumulations of 36.4 mg kg^{-1}, 79.7 mg kg^{-1}, 27.7 mg kg^{-1}, and 36.8 mg kg^{-1} of Pb in shoots and 75.5 mg kg^{-1}, 125 mg kg^{-1}, 33.3 mg kg^{-1}, and 53.7 mg kg^{-1} of Pb in roots in the presence of 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively. Moreover, in the presence of 300 mg kg^{-1} Pb at 50% FC and with 1% FYM, the plants accumulated 55.6 mg kg^{-1} and 87.1 mg kg^{-1} of Pb in the shoots and roots of ryegrass, respectively. Similarly, at 600 mg kg^{-1} of Pb contamination, the plant shoots and roots accumulated 101 mg kg^{-1} and 147 mg kg^{-1}, respectively. A similar trend was observed in the case of Cd contamination, where plants accumulated around 80 mg kg^{-1} and 112 mg kg^{-1} of Cd when subjected to 100 mg kg^{-1} Cd and 200 mg kg^{-1} Cd, respectively, at 50% FC (Figure 6). With the addition of FYM in treatments at 30% FC, the results revealed 60.2 mg kg^{-1} and 109 mg kg^{-1} (in shoots) and 97.9 mg kg^{-1} and 150 mg kg^{-1} (in roots) at 300 mg kg^{-1} and 600 mg kg^{-1} Pb, respectively, and in the presence of Cd, 37.9 mg kg^{-1} and 49.7 mg kg^{-1} Cd accumulation in shoots and 49.9 mg kg^{-1} and 71.4 mg kg^{-1} Cd in roots at 100 mg kg^{-1} and 200 mg kg^{-1} Cd, respectively, was observed (Figure 6). Overall, higher amounts of both HMs were accumulated in roots than shoots.

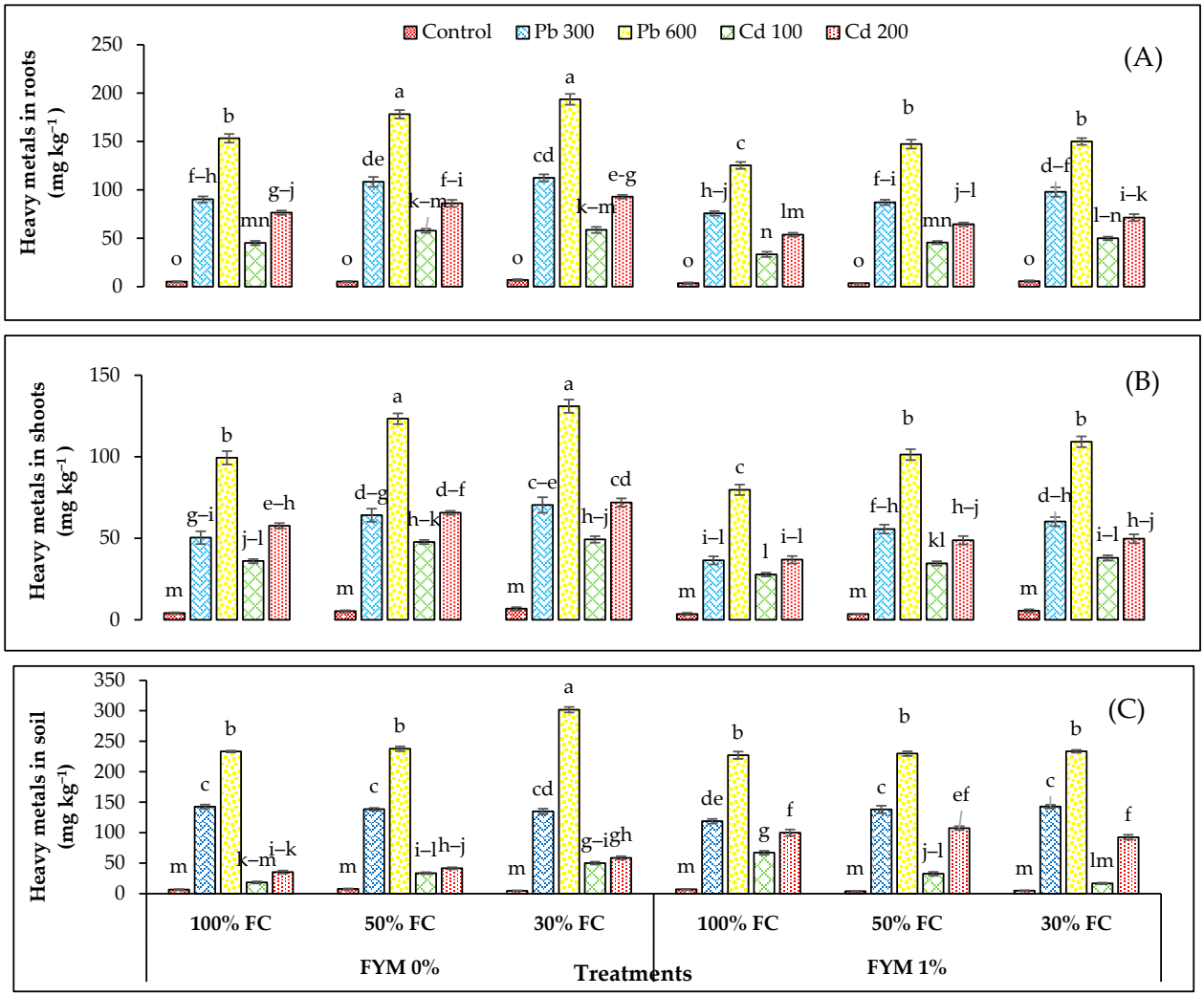

Figure 6. Effects of farmyard manure and drought on HMs uptake in roots (**A**) and shoots (**B**) of ryegrass, and residual HMs contents in soil (**C**). The standard errors and means of four replicates are represented by the error bars and columns, respectively, and different small letters on columns show the statistical difference (i.e., significance and/or non-significance) among different means ($p \leq 0.05$). FC, field capacity; Pb, lead; Cd, cadmium.

The remaining contents of Pb in soils were 47.3% and 38.8% as compared to the initially applied Pb concentration (i.e., 300 mg kg^{-1} and 600 mg kg^{-1}), respectively. Similarly, at 100 mg kg^{-1} and 200 mg kg^{-1} of Cd contamination, the remaining amounts of Cd were 18.4% and 35.4%, respectively (at 100% FC). At 50% FC, the residual amounts of HMs were 46.1%, 39.5%, 33.6%, and 20.5% at 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively. Similarly, at 30% of FC, the remaining HMs in soils were 44.6%, 50.2%, 48.9%, and 29.3% of 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively (Figure 6).

Additionally, in the FYM-applied treatments, residual concentrations of HMs were 39.3%, 37.8%, 67.1%, and 50.0% against the initial concentrations of 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively. Moreover, at 50% FC and 1% FYM, the remaining amounts of HMs in soil were 46.0%, 38.1% 32.5%, and 53.5% in 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd-applied treatments, respectively. With the addition of FYM in treatments at 30% FC, the residual amounts of HMs in soils were 47.3%, 38.8%, 16.8%, and 46.3% at 300 mg kg^{-1} Pb, 600 mg kg^{-1} Pb, 100 mg kg^{-1} Cd, and 200 mg kg^{-1} Cd, respectively (Figure 6).

In the present study, the results showed a significant accumulation of both Pb and Cd in the ryegrass plants (Figure 6). Furthermore, higher amounts of Pb and Cd were accumulated in the roots than the aboveground parts of ryegrass. Although considerable amounts of both HMs were transferred from roots to shoots, values of TF confirmed the higher accumulation of Cd and Pb in roots than shoots of ryegrass plants (Figure 7). Interestingly, in the absence of FYM, the evaluation of the BCF showed a decreasing trend of the BCF for Cd along with increasing drought levels. However, in the absence of FYM, this trend was entirely different, particularly at a low Cd level (i.e., 100 mg kg^{-1}) (Figure 7). Overall, higher values of BCF were obtained for Cd (than for Pb), suggesting a considerable hyper-accumulation ability of ryegrass for Cd.

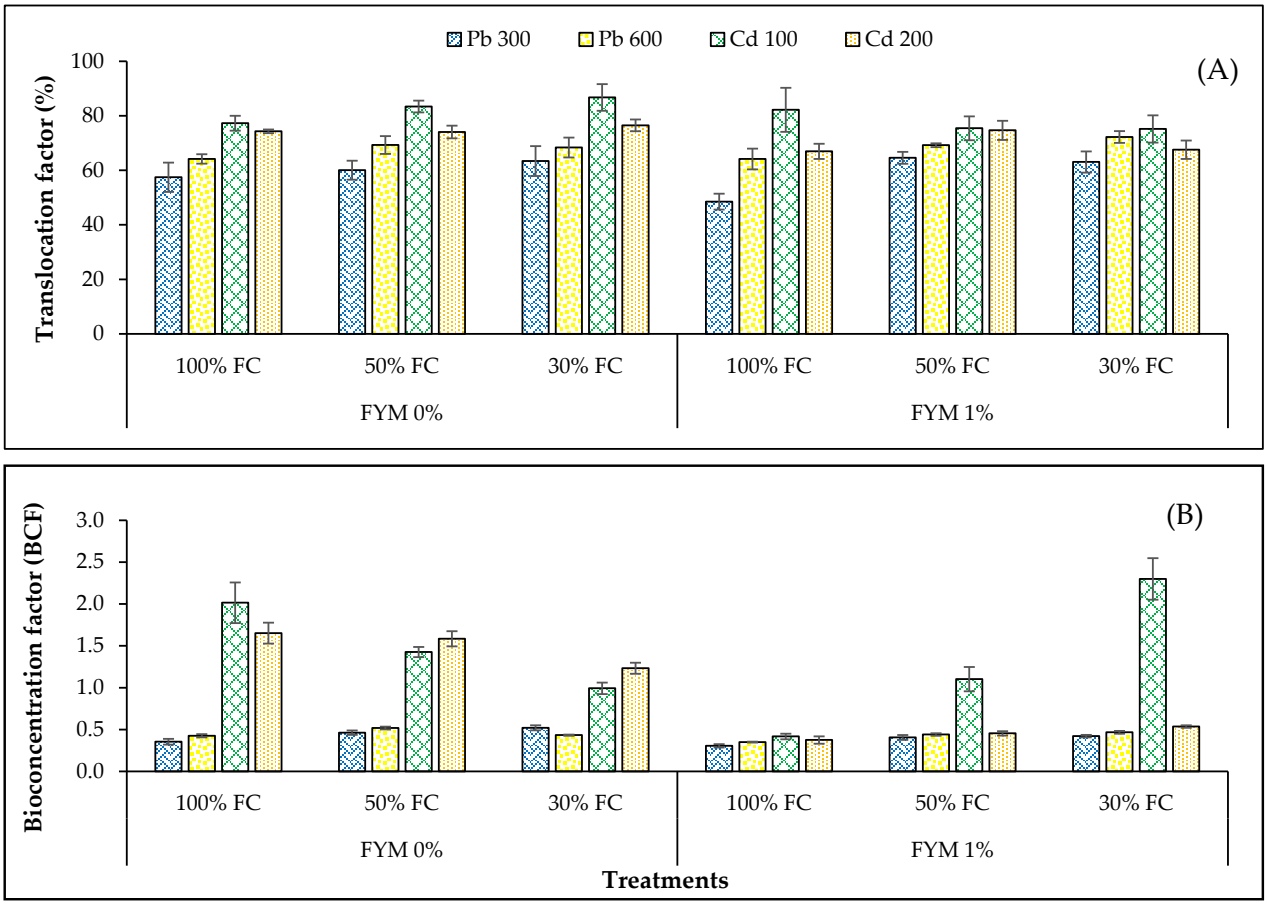

Figure 7. Effects of farmyard manure and drought on translocation (**A**) and bioconcentration (**B**) factors of ryegrass plants at different concentrations of Pb and Cd in soil. The standard errors and means of four replicates are represented by the error bars and columns, respectively. FC, field capacity; Pb, lead; Cd, cadmium; FYM, farmyard manure.

3.6. Correlation Analysis

In the present study, a correlation analysis was performed to evaluate the relationships among different growth and physiological attributes and heavy metals uptake in ryegrass plants (Tables 3 and 4). Regardless of HM types and concentrations, the results showed a strong positive correlation among most of the growth, physiological, and biochemical attributes (i.e., $R^2 \leq 0.99$) of ryegrass. However, negative correlations (R^2: up to −0.99) were observed among the heavy metal contents in the plant tissues and most of the growth, physiological, and biochemical attributes of ryegrass plants confirmed the phytotoxic effects of Cd and Pb on ryegrass plants. Moreover, HMs contents in soil were positively correlated with HMs contents in roots and shoots of ryegrass plants (R^2: 0.44–0.98) (Tables 3 and 4).

Table 3. Correlation between Pb contents in soil and plant tissues and growth and physiological parameters of ryegrass.

	Pb (300 mg kg^{-1})																		
Parameters	RL	SL	RFW	RDW	SFW	SDW	NT	NL	SPAD	Chl. A	Chl. B	TC	PAR	TR	RWC	MSI	WUE	PbR	PbSL	PbS
RL	1.00																			
SL	0.83	1.00																		
RFW	0.85	0.77	1.00																	
RDW	0.85	0.98	0.69	1.00																
SFW	0.90	0.96	0.91	0.92	1.00															
SDW	0.91	0.98	0.83	0.97	0.98	1.00														
NT	0.94	0.86	0.79	0.84	0.87	0.92	1.00													
NL	0.82	0.99	0.77	0.98	0.97	0.97	0.81	1.00												
SPAD	0.93	0.84	0.81	0.87	0.90	0.90	0.81	0.87	1.00											
Chl. A	0.99	0.86	0.89	0.86	0.93	0.93	0.94	0.85	0.90	1.00										
Chl. B	0.74	0.94	0.87	0.85	0.95	0.92	0.77	0.93	0.74	0.81	1.00									
TC	0.98	0.85	0.83	0.89	0.91	0.92	0.88	0.86	0.97	0.96	0.74	1.00								
PAR	0.74	0.98	0.72	0.95	0.93	0.93	0.73	0.99	0.82	0.77	0.92	0.80	1.00							
TR	0.79	0.97	0.76	0.96	0.95	0.94	0.75	0.99	0.88	0.81	0.91	0.85	0.99	1.00						
RWC	0.90	0.86	0.96	0.81	0.96	0.91	0.83	0.88	0.93	0.92	0.89	0.91	0.83	0.88	1.00					
MSI	0.86	0.95	0.87	0.92	0.99	0.96	0.81	0.97	0.92	0.88	0.94	0.89	0.96	0.98	0.96	1.00				
WUE	0.77	0.97	0.76	0.95	0.95	0.94	0.74	0.99	0.86	0.79	0.93	0.83	1.00	1.00	0.87	0.98	1.00			
PbR	−0.95	−0.82	−0.75	−0.89	−0.87	−0.89	−0.82	−0.85	−0.97	−0.92	−0.69	−0.99	−0.79	−0.85	−0.87	−0.87	−0.82	1.00		
PbSL	−0.36	−0.38	−0.33	−0.41	−0.42	−0.42	−0.29	−0.45	−0.66	−0.29	−0.31	−0.47	−0.47	−0.52	−0.52	−0.54	−0.53	0.52	1.00	
PbS	−0.88	−0.91	−0.69	−0.95	−0.90	−0.93	−0.82	−0.93	−0.96	−0.86	−0.76	−0.94	−0.90	−0.93	−0.85	−0.92	−0.91	0.95	0.63	1.00

Table 3. *Cont.*

	Pb (600 mg kg^{-1})																		
Parameters	RL	SL	RFW	RDW	SFW	SDW	NT	NL	SPAD	Chl. A	Chl. B	TC	PAR	TR	RWC	MSI	WUE	PbR	PbSL	PbS
RL	1.00																			
SL	0.70	1.00																		
RFW	0.84	0.88	1.00																	
RDW	0.74	0.97	0.86	1.00																
SFW	0.71	0.96	0.96	0.93	1.00															
SDW	0.91	0.87	0.98	0.86	0.92	1.00														
NT	0.88	0.84	0.84	0.91	0.82	0.91	1.00													
NL	0.67	0.99	0.90	0.95	0.98	0.88	0.81	1.00												
SPAD	0.77	0.99	0.90	0.96	0.95	0.92	0.90	0.97	1.00											
Chl. a	0.85	0.94	0.87	0.98	0.89	0.91	0.97	0.91	0.96	1.00										
Chl. b	0.66	0.99	0.85	0.97	0.94	0.85	0.86	0.98	0.98	0.94	1.00									
TC	0.92	0.71	0.70	0.72	0.62	0.79	0.81	0.65	0.77	0.83	0.68	1.00								
PAR	0.52	0.96	0.84	0.91	0.96	0.78	0.71	0.98	0.91	0.83	0.95	0.49	1.00							
TR	0.62	0.98	0.88	0.91	0.96	0.84	0.74	0.99	0.95	0.86	0.96	0.63	0.97	1.00						
RWC	0.76	0.96	0.97	0.93	1.00	0.94	0.85	0.98	0.96	0.91	0.94	0.67	0.94	0.96	1.00					
MSI	0.79	0.94	0.98	0.91	0.99	0.97	0.87	0.96	0.96	0.91	0.93	0.68	0.90	0.93	0.99	1.00				
WUE	0.43	0.93	0.76	0.89	0.91	0.72	0.69	0.94	0.89	0.79	0.95	0.43	0.97	0.94	0.89	0.86	1.00			
PbR	−0.83	−0.80	−0.70	−0.76	−0.67	−0.78	−0.79	−0.74	−0.84	−0.84	−0.77	−0.97	−0.60	−0.73	−0.71	−0.72	−0.56	1.00		
PbSL	−0.93	−0.70	−0.74	−0.80	−0.66	−0.83	−0.95	−0.65	−0.77	−0.90	−0.71	−0.88	−0.51	−0.57	−0.70	−0.73	−0.47	0.80	1.00	
PbS	−0.79	−0.90	−0.79	−0.83	−0.80	−0.83	−0.77	−0.86	−0.92	−0.87	−0.86	−0.90	−0.75	−0.87	−0.82	−0.82	−0.72	0.97	0.72	1.00

RL, root length; SL, shoot length; RFW, root fresh weight; RDW, root dry weight; SFW, shoot fresh weight; SDW, shoot dry weight; NT, no. of tillers; NL, no. of leaves; SPAD value; Chl. a, chlorophyll a; Chl. b, chlorophyll b; TC, total carotenoids; PAR, photosynthetic assimilation rate; TR, transpiration rate; RWC, relative water content; MSI, membrane stability index; WUE, water use efficiency; PbR, Pb in roots; PbSL, residual Pb in soil; PbS, Pb in shoots.

Table 4. Correlation between Cd contents in soil and plant tissues and growth and physiological attributes of ryegrass.

	Cd (100 mg kg^{-1})																			
Parameters	RL	SL	RFW	RDW	SFW	SDW	NT	NL	SPAD	Chl. A	Chl. B	T. chl	TC	PAR	TR	RWC	MSI	WUE	CdR	CdSL	CdS
RL	1.00																				
SL	0.98	1.00																			
RFW	0.93	0.95	1.00																		
RDW	0.80	0.80	0.93	1.00																	
SFW	0.86	0.82	0.85	0.90	1.00																
SDW	0.67	0.68	0.84	0.95	0.84	1.00															
NT	0.96	0.92	0.88	0.74	0.76	0.64	1.00														
NL	0.71	0.73	0.80	0.65	0.41	0.54	0.81	1.00													
SPAD	0.95	0.96	0.99	0.93	0.90	0.85	0.89	0.73	1.00												
Chl. A	0.42	0.47	0.60	0.47	0.11	0.40	0.53	0.93	0.49	1.00											
Chl. B	0.88	0.90	0.91	0.74	0.61	0.62	0.93	0.95	0.87	0.79	1.00										
T. chl	0.97	0.93	0.89	0.75	0.77	0.64	1.00	0.81	0.90	0.53	0.93	1.00									
TC	0.93	0.94	0.93	0.75	0.67	0.59	0.92	0.89	0.90	0.70	0.98	0.93	1.00								
PAR	0.67	0.74	0.81	0.68	0.42	0.66	0.75	0.94	0.76	0.90	0.91	0.75	0.83	1.00							
TR	0.72	0.78	0.85	0.71	0.47	0.66	0.77	0.95	0.79	0.89	0.93	0.78	0.87	1.00	1.00						
RWC	0.67	0.64	0.71	0.58	0.36	0.48	0.81	0.98	0.65	0.89	0.91	0.81	0.83	0.87	0.87	1.00					
MSI	0.67	0.68	0.77	0.65	0.39	0.56	0.79	1.00	0.70	0.94	0.93	0.79	0.86	0.93	0.94	0.98	1.00				
WUE	0.73	0.78	0.86	0.72	0.47	0.65	0.79	0.97	0.79	0.91	0.95	0.79	0.89	0.99	0.99	0.91	0.96	1.00			
CdR	−0.92	−0.95	−0.99	−0.92	−0.85	−0.86	−0.87	−0.76	−0.99	−0.56	−0.89	−0.88	−0.89	−0.83	−0.85	−0.67	−0.74	−0.84	1.00		
CdSL	−0.42	−0.06	−0.37	−0.60	−0.30	−0.68	−0.03	−0.23	−0.30	−0.35	−0.13	−0.42	−0.09	−0.33	−0.32	−0.14	−0.26	−0.32	0.44	1.00	
CdS	−0.97	−0.99	−0.96	−0.87	−0.89	−0.79	−0.91	−0.70	−0.99	−0.43	−0.87	−0.92	−0.90	−0.73	−0.77	−0.62	−0.66	−0.76	0.98	0.57	1.00

Table 4. *Cont.*

	Cd (200 mg kg^{-1})																			
Parameters	RL	SL	RFW	RDW	SFW	SDW	NT	NL	SPAD	Chl. A	Chl. B	T. chl	TC	PAR	TR	RWC	MSI	WUE	CdR	CdSL	CdS
RL	1.00																				
SL	0.97	1.00																			
RFW	0.96	0.93	1.00																		
RDW	1.00	0.94	0.91	1.00																	
SFW	0.91	0.85	0.94	0.95	1.00																
SDW	0.81	0.83	0.81	0.89	0.84	1.00															
NT	0.86	0.85	0.87	0.96	0.97	0.67	1.00														
NL	0.88	0.86	0.89	0.78	0.53	0.65	0.83	1.00													
SPAD	0.84	0.82	0.86	0.75	0.50	0.63	0.98	0.74	1.00												
Chl. A	0.89	0.91	0.92	0.88	0.77	0.85	0.89	0.92	0.54	1.00											
Chl. B	0.75	0.75	0.77	0.66	0.37	0.52	0.97	0.98	0.84	0.79	1.00										
T. chl	0.80	0.88	0.80	0.62	0.54	0.59	0.87	0.90	0.89	0.85	0.93	1.00									
TC	0.84	0.89	0.85	0.77	0.60	0.70	0.96	0.96	0.93	0.95	0.90	0.92	1.00								
PAR	0.96	0.92	0.97	0.92	0.73	0.83	0.96	0.94	0.95	0.89	0.83	0.93	0.79	1.00							
TR	0.71	0.71	0.73	0.52	0.31	0.41	0.87	0.92	0.80	0.88	0.92	0.82	0.79	0.99	1.00						
RWC	0.75	0.75	0.77	0.60	0.44	0.52	0.84	0.91	0.86	0.84	0.93	0.81	0.81	0.98	0.85	1.00					
MSI	0.78	0.80	0.78	0.60	0.38	0.49	0.96	0.97	0.83	0.97	0.93	0.94	0.87	0.93	0.88	0.97	1.00				
WUE	0.76	0.76	0.78	0.70	0.41	0.56	0.97	0.98	0.86	1.00	0.83	0.96	0.90	0.85	0.81	0.96	0.95	1.00			
CdR	0.62	0.63	0.64	0.44	0.26	0.35	0.79	0.87	0.77	0.83	0.90	0.76	0.71	0.99	0.98	0.88	0.80	−0.79	1.00		
CdSL	−0.90	−0.93	−0.90	−0.91	−0.97	−0.96	−0.69	−0.67	−0.89	−0.55	−0.73	−0.74	−0.84	−0.53	−0.64	−0.58	−0.58	−0.48	0.51	1.00	
CdS	−0.53	−0.50	−0.53	−0.71	−0.88	−0.83	−0.55	−0.09	−0.42	−0.54	−0.49	−0.29	−0.40	−0.12	−0.42	−0.27	−0.18	−0.75	0.75	0.66	1.00

RL, root length; SL, shoot length; RFW, root fresh weight; RDW, root dry weight; SFW, shoot fresh weight; SDW, shoot dry weight; NT, no. of tillers; NL, no. of leaves; SPAD value; Chl. a, chlorophyll a; Chl. b, chlorophyll b; Total chl, total chlorophyll; TC, total carotenoids; PAR, photosynthetic assimilation rate; TR, transpiration rate; RWC, relative water content; MSI, membrane stability index; WUE, water use efficiency; CdR, Cd in roots; CdSL, residual Cd in soil; CdS, Cd in shoots.

4. Discussion

In recent years, various organic sources have been used either individually or in combination to mitigate the adverse effects of HMs on plants, as well as to reduce the bioavailability of metals in soil [41,42]. Typically, the application of organic amendments in soil has been observed to decrease the bioavailability of metals in the soil and inhibit their translocation from roots to shoots [43,44]. Several studies have demonstrated that the presence of drought and/or HMs stresses have a detrimental impact on the growth and physiology of crops [44–46]. In the present study, 1% FYM was used as an organic amendment at different levels of drought stress (100, 50, and 30%) to study the possible reduction in Cd and Pb toxicity, uptake, and accumulation in ryegrass.

Our results showed that Pb and Cd had adverse effects on plant growth and physiology, and caused significant phytotoxicity to the ryegrass plants. Our results are consistent with the findings of Lou et al. [19], who showed that Cd and Pb contaminations cause significant toxicity to ryegrass and impair plant growth. As HMs are not biologically important, they could impose harmful effects on plants by causing chlorosis, photosynthetic inhibition, water imbalance, and lower nutrient assimilation that leads to growth reduction [22,47]. Our research elaborated that HMs stress affected ryegrass plants' morphological, physiological, and biochemical parameters and eventually reduced plant growth. The findings of Hossain et al. [48] demonstrated that Cd hinders several metabolic and physiological processes in plant tissues by the production of ethylene and H_2O_2 that become harmful to plants and suppress plant growth. Our findings support the observations of Mukhtar et al. and Xie et al. [49,50] who also reported that Cd stress reduces the biomass, chlorophyll contents, photosynthesis, transpiration rate, stomatal conductance, and water-using efficiency of plants. A possible reason for stunted growth could be the inhibition of intracellular enzymatic processes due to lower chloroplast pigmentations, membranes damage, vacuoles rupture stomatal blockage, and the activation of ascorbate peroxidase (APX), CAT, SOD, and POD that leads toward the growth reduction [48,51].

Moreover, both drought as well as HMs were the main limiting factors that caused phytotoxic impacts on plant physiological processes, probably through the production of ROS such as hydrogen peroxide (H_2O_2) in this study. We found that drought could disturb grasses' physiology, resulting in destructive mechanisms' activation. It could be due to the rationale provided by Maestri et al. [52], who reported that a large amount of ROS causes severe damage to the cell membrane and enzymes. Proteins and lipids peroxidation, the protection system besides oxidative damage that increases the antioxidants such APX, SOD, CAT, and POD ultimately have adverse effects on the physiological processes of plants. Furthermore, we found that drought enhanced the reduction of the chlorophyll content and RWC of ryegrass. These findings support the observations of Ali et al. [53] showing the deleterious effects of drought on plant physiology. Several other studies also reported the adverse effects of drought and HMs on the physiology and biochemical response of ryegrass [54,55].

In this study, the application of FYM demonstrated significant enhancement in ryegrass growth and development. A possible reason behind this impact could be that the addition of FYM can accelerate the availability of NO^{-3} in soil, which becomes helpful for plant growth [56]. Also, the application of FYM increases the uptake of N, P, and K along with trace elements, with an overall increase in plant biomass, as reported by Mahmood et al., Ur Rehman et al. and Mahanty et al. [57–59]. Furthermore, it has been shown that manures can reduce the availability of hazardous HMs by chelating them with soil colloids. Other possible reasons for higher growth and improved physiology of ryegrass by the addition of FYM could be the production of water-exchangeable organic carbons that bind free metal ions to ligands and decrease the pH of soil [22,60].

In the present study, ryegrass was able to remove substantial amounts of Cd and Pb from the soil. Our results are consistent with the findings of Jadia and Fuleka [61], who reported higher HMs contents in the different tissues of plants. The Pb and Cd uptake in plants increased with increasing concentrations of HMs and was directly linked with the

initial amount of both HMs applied to soil, suggesting the concentration-dependent mode of uptake of Pb and Cd by ryegrass [62,63]. Evaluations of the TF and BCF in ryegrass in the present study affirm that ryegrass plants have a substantial potential to remediate Pb and Cd from contaminated soils. Furthermore, our results showed that the addition of FYM enhanced the HMs stabilization in ryegrass plants, suggesting the important role of roots and FYM in the phytoextraction process. Our results are consistent with those of Ashraf et al. [64], who reported that organic amendments enhance the phytoremediation (stabilization) of HMs in ryegrass. According to Zhang et al. [65], ryegrass could grow in HMs-contaminated soil and show improved phytoremediation ability in the presence of organic amendments. Our findings are also in line with the observations of Ashraf et al. [64], who reported the important role of FYM in the phytoextraction of HMs by ryegrass in contaminated soil. The reduced HMs uptake and vigorous plant growth in the presence of FYM might be due to the acidification of the rhizosphere (due to the release of organic acids by the microbes in FYM) that causes higher nutrient availability to plants and subsequently improved plant growth [66]. Our findings indicate that the addition of FYM could be useful in reducing HMs uptake in grasses and enhancing phytoremediation and sustainable production of grasses under water-deficient conditions. According to Madhavan et al. and Alloway [67,68], the acceptable limits of Pb and Cd in soil are 600 and 8.0 mg kg^{-1}, respectively. The addition of FYM to soil could enhance phytoremediation of plants, and reduce HMs below their critical values in soil.

5. Conclusions

The higher amounts of Pb and Cd in soil decreased the overall growth of ryegrass by altering the physiological and enzymatic activities. Ryegrass plants were able to tolerate the drought stress, but the addition of higher amounts of HMs disturbed the defense system of plants and retarded the overall growth of ryegrass. The application of FYM reduced the phytotoxicity of Pb and Cd to ryegrass and effectively increased plant growth and physiology even under drought stress. Moreover, ryegrass plants have a considerable potential to uptake and remediate HMs from contaminated soil. Accumulation of Pb and Cd was higher in roots than in aerial parts. The FYM reduced HMs uptake by plants and enhanced the overall removal of Pb and Cd from soil (even under drought stress). Our findings suggest that the addition of FYM could be practicable for phytoremediation of Pb and Cd from soil by ryegrass even under drought stress. The developed eco-friendly technology could be used to treat HMs-contaminated soils and is a suitable option for sustainable and safe fodder production on water-limited lands.

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