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ACC-deaminase producing plant growth promoting rhizobacteria and biochar mitigate adverse effects of drought stress on maize growth

Subhan Danish[1], Muhammad Zafar-ul-Hye[1], Fauzia Mohsin[2], Mubshar Hussain[3,4]*

1 Department of Soil Science, Bahauddin Zakariya University, Multan, Pakistan, **2** Department of Biology, Government College for Women Chungi, Multan, Pakistan, **3** Department of Agronomy, Bahauddin Zakariya University, Multan, Pakistan, **4** Agriculture Discipline, College of Science Health, Engineering and Education, Murdoch University, Murdoch, Australia

* mubashiragr@gmail.com

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Abstract

Availability of good quality irrigation water is a big challenge in arid and semi arid regions of the world. Drought stress results in poor plant growth and low yield; however, the rhizobacteria, capable of producing 1-aminocyclopropane-1-carboxylate (ACC)-deaminase are likely to improve crop growth and productivity under drought stress. Similarly, biochar could also ameliorate the negative impacts of drought stress. Therefore, this pot experiment was conducted to evaluate the role of ACC-deaminase producing plant growth promoting rhizobacteria (PGPR) alone and in combinations with timber-waste biochar in improving maize growth under drought stress. The ACC-deaminase producing rhizobacteria, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Achromobacter xylosoxidans* and *Leclercia adecarboxylata* were studied along with two rates (0.75 and 1.50% of the soil weight) of biochar under three moisture levels i.e., normal moisture, mild drought stress and severe drought stress. The *E. cloacae* in conjunction with higher rate of biochar produced a significant improvement i.e., up to 60, 73, 43, 69, 76 and 42% respectively, in grain yield plant^{-1}, photosynthetic rate, stomatal conductance, chlorophyll a, total chlorophyll and carotenoids contents of maize as compared to the control under mild drought stress. Similarly, *A. xylosoxidans* with higher rate of biochar also enhanced grain yield plant^{-1}, photosynthetic rate, stomatal conductance, chlorophyll a, total chlorophyll and carotenoids contents of maize up to 200, 213, 113, 152, 148 and 284%, respectively over control under severe drought stress. In conclusion, combination of ACC-deaminase containing PGPR, *A. xylosoxidans* and biochar (0.75%) proved an effective technique to improve maize growth and productivity under drought stress.

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Introduction

Drought stress has emerged as a big threat to sustainable crop production globally [1]. Climate models reveal that severity of drought stress is expected to increase day by day [2–4]. The demand of irrigation water is expected to raise by 10% till 2050 [4]. Higher evapotranspiration rate and less precipitation would also be the reasons of elevation in drought intensity, if visualized in connection with the present climate change trend [5].

Drought stress results in low nutrient uptake by plants, poor root growth and photosynthesis [1]. Drought stress, like other abiotic stresses, also stimulates ethylene production referred as stress ethylene via raised level of 1-aminocyclopropane-1-carboxylic acid [ACC], an immediate precursor of ethylene biosynthesis in higher plants through methionine pathway [6]. Accumulation of stress ethylene restricts the elongation of roots and consequently growth of shoots [7].

Although water management, traditional breeding and genetic engineering are thought to be quite effective in mitigating drought stress, but high technicalities involved in these approaches make them less adoptable. Inoculation of plant growth promoting rhizobacteria (PGPR) is an effective technology and can be implemented easily [8,9]. Inoculation of PGPR improves root elongation [10], phosphorus (P) and potassium (K) solubilization [11,12] and secretion of growth hormones [13]. ACC-deaminase containing PGPR can regulate ethylene level in plants via its break down into α-ketobutyrate and ammonia [14] under abiotic stresses [15,16]. The plants treated with ACC-deaminase producing PGPR have shown a significant improvement in stomatal conductance and photosynthesis [17].

Activated black carbon biochar is another organic amendment which can reduce the drought stress by increasing soil water holding capacity [18]. It is manufactured by the process of pyrolysis at high temperature and under limited or no supply of oxygen using waste feedstock [19,20]. Low surface area, high porosity and resistance against decomposition [18] make biochar one of most useful amendment for improving physio-chemical characteristics of soil [21] and mitigation of drought stress [22–25].

In recent past, most of the scientists focused on either inoculation of ACC-deaminase containing PGPR or application of biochar separately to mitigate the adverse effects of drought stress. However, the combined effect of both has merely been tested. Therefore, the current study was designed to evaluate the effectiveness of combined application of ACC-deaminase producing PGPR and timber-waste biochar to improve the performance of maize (*Zea mays* L.) under drought stress.

Materials and methods

Experiment site

The experiment was conducted in the research area of the Department of Soil Science, Bahauddin Zakariya University Multan, Pakistan. Experiment was laid out following completely randomized design (CRD) with factorial arrangement and replicated three times

Soil characteristics

The polythene bags having dimensions of 75 cm deep × 45 cm diameter with 15 kg soil capacity were used as pots. A bulk soil sample was collected from the plough layer near the bank of Chenab River, Multan, Punjab, Pakistan (30.306222 N, 71.437861 E). The soil of the selected area was previously characterized as dark yellowish brown, moderately calcareous, weakly structured and well-drained with Cambic subsurface horizon and an Ochric epipedon [21]. Hydrometer method was followed for textural analysis of soil [26]. The available P was

Table 1. Pre-experimental characteristics of soil and timber waste biochar.

Soil	Unit	Value	Biochar	Unit	Value
Sand	%	60	pH	-	7.26
Silt	%	30	EC_e	dS m^{-1}	1.22
Clay	%	10	Volatile Matter	%	8.96
Texture	Sandy Loam		Ash content	%	28.9
pH_s	-	8.11	Fixed Carbon	%	62.1
EC_e	dS m^{-1}	1.69	Total N	%	0.21
Organic Matter	%	0.54	Total P	%	0.62
Extractable P	mg kg^{-1}	9.26	Total K	%	1.61
Extractable K	mg kg^{-1}	229	Total Na	%	0.19

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determined by following Olsen and Sommers [27]. Extractable K was determined by following Nadeem et al. [28] and Walkley [29] protocol was followed for soil organic matter determination. The pre-experimental soil characteristics are provided in Table 1.

Drought-tolerant ACC deaminase containing PGPR strains

Out of 37 PGPRs isolated from maize rhizosphere collected from Multan, Punjab, Pakistan, four most efficient drought-tolerant ACC deaminase containing PGPR identified as *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Achromobacter xylosoxidans* and *Leclercia adecarboxylata* were used in this study. These PGPR strains were able to grow at –0.85 MPa osmotic potential generated through 20% polyethylene Glycol 6000. The Dworkin and Foster minimal salt medium was used to grow the ACC-deaminase producing PGPR. Composition of the medium was 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g, $MgSO_4.7H_2O$, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements: 1mg $FeSO_4.7H_2O$, 10 mg H_3BO_3, 11.19 mg $MnSO_4.H_2O$, 124.6 mg $ZnSO_4.7H_2O$, 78.22 mg $CuSO_4.5H_2O$, 10 mg MoO_3, pH = 7.2 and 0.5M 1-aminocyclopropane-1-carboxylic acid as the only nitrogen source was used [30]. The strains were grown on DF minimal salt medium [30].

Characteristics of PGPR

For confirmation of AcdS gene NCBI gene bank was used which confirmed that *E. cloacae* [https://www.ncbi.nlm.nih.gov/nuccore/KM501058.2], *P. aeruginosa* [https://www.ncbi.nlm.nih.gov/nuccore/CP014948.1/] and *A. xylosoxidans* [https://www.ncbi.nlm.nih.gov/nuccore/AY604540.1],[https://www.ncbi.nlm.nih.gov/nuccore/AY604539.1] have AcdS, while work is yet continued on *L. adecarboxylata*. For determination of ACC-deaminase activity, produced by PGPR (*E. cloacae* = 402.1 ± 27.29, *A. xylosoxidans* = 381.17 ± 11.69, *P. aeruginosa* = 115.2 ± 16.14 and *L. adecarboxylata* = 296.1 ± 21.69 μmol α-ketobutyrate nmol g^{-1} protein h^{-1}) methodologies of El-Tarabily [31] and Honma and Shimomura [32] were followed. Indole acetic acid without (*E. cloacae* = 3.39 ± 0.41, *A. xylosoxidans* = 5.52 ± 0.79, *P. aeruginosa* = 2.94 ± 0.49 and *L. adecarboxylata* = 2.11 ± 0.17μg/ml) and with L-tryptophan (*E. cloacae* = 78.79 ± 0.35, *A. xylosoxidans* = 61.19 ± 0.14, *P. aeruginosa* = 21.3 ± 0.37 and *L. adecarboxylata* = 61.59 ± 0.20μg/ml) was determined by using Salkowski reagent [33]. Pikovskaya's medium was used to examine the phosphorus solubilizing activity of PGPR (*E. cloacae* = 66.3 ± 0.38, *A. xylosoxidans* = 77.4 ± 0.98, *P. aeruginosa* = 29.1 ± 1.19 and *L. adecarboxylata* = 20.1 ± 1.29 μg/ml) [34]. Potassium solubilizing activity of PGPR (*E. cloacae* = 19.1 ± 0.82, *A. xylosoxidans* = 24.5 ± 0.42, *P. aeruginosa* = 12.6 ± 0.92 and *L. adecarboxylata* = 16.4 ± 1.40 μg/ml) was examined according to Setiawati and Mutmainnah [35].

Production of timber-waste biochar

Timber waste was collected from local market for the production of biochar. All the waste material was sun-dried and pyrolyzed at 389 ˚C for 80 min in pyrolyzer [20]. Biochar was then grinded to achieve fine powder ($\leq$2 mm). Finally, timber waste BC was stored in airtight plastic jars in dark at room temperature.

Characterization of timber-waste biochar

Biochar pH and EC were measured in biochar to water ratio of 1:20 w/v [20]. Biochar was wet digested in a di-acid mixture of HNO_3:$HClO_4$ in 2:1 ratio [36]. After digestion, yellow color method was used for determination of P on a spectrophotometer [37]. Potassium (K) and sodium (Na) concentration in the digests were measured using flamephotometer [38]. For the determination of N, H_2SO_4 digestion [37] was done followed by distillation on Kjeldahl's distillation apparatus [39]. The ash content and volatile matter in biochar was analyzed by heating the sample in a muffle furnace at 550˚C and 450˚C respectively [40]. The fixed carbon in BC was calculated using the equation [41]

$$\text{Fixed Carbon } (\%) = 100 - [\%\text{Volatile Matter} + \%\text{Ash Content}]$$

The characteristics of biochar are given in Table 1.

Fertilizers

The pots were filled with 12 kg soil. The N, P and K were applied at the rate of 200, 150 and 100 kg ha^{-1}, respectively using urea, diammonium phospahate (DAP) and muriate of potash (MOP) as source [42]. Three splits of N were applied during the growth period, while whole P and K were applied at the time of sowing.

Seed inoculation

The seeds of maize (cv. Kenzo-123 Hybrid) were purchased from certified seed dealer of the Government of Punjab, Pakistan and sodium hypochlorite (5%) solution was used for seed sterilization. Finally, seeds were washed thrice with ethanol (95%) followed by three washings with sterilized deionized water [43]. Sterilized seeds (100 g) were inoculated with 1 ml of inoculum (0.5 optical density at 535 nm wavelength) along with 10% sugar solution. When inoculum and sugar solution were uniformly stuck on seeds, a final top dressing of peat and clay (3:1 ratio) was done as described by Ahmad et al. [44]. Before inoculation of seeds, peat and clay mixture was sterilized at 121˚C for 20 min in an autoclave [45]. For control treatment seeds were top dressed with peat and clay along with 10% sugar solution without PGPR strain [46].

Seeds sowing and drought stress

Four seeds of maize were sown in each pot and two healthy seedlings were maintained by thinning after completion of germination. In control, normal moisture (NM) was maintained at the level of 70% of field capacity throughout the experiment. Mild (MD) and severe drought stress (SD) were maintained at 50% and 30% of field capacity, respectively as suggested by Boutraa et al. [47]. For determination of soil field capacity, 1 kg soil was taken in a pot having hole at bottom. The soil was saturated with water and left over night. Gravitational water was collected after 24h. Finally, field capacity of soil was calculated by difference method using

equation:

$$\text{Field Capacity (\%)} = \frac{\text{Water added (ml)} - \text{Water leached (ml) in 24hr}}{\text{Soil Weight (g)}} \times 100$$

Application rate of biochar and treatments

The application rates of biochar were as follows; control, having no biochar (BC_0), 0.75% w/w biochar ($BC_{0.75}$) and 1.50% w/w biochar ($BC_{1.50}$) of total soil contained in the pot. The PGPR treatments (T) included: control (No PGPR + No biochar), *P. aeruginosa*, *E. cloacae*, *A. xylosoxidans*, *L. adecarboxylata*, $BC_{0.75}$, *P. aeruginosa* + $BC_{0.75}$, *E. cloacae* + $BC_{0.75}$, *A. xylosoxidans* + $BC_{0.75}$, *L. adecarboxylata* + $BC_{0.75}$, $BC_{1.50}$, *P. aeruginosa* + $BC_{1.50}$, *E. cloacae* + $BC_{1.50}$, *A. xylosoxidans* + $BC_{1.50}$ and *L. adecarboxylata* + $BC_{1.50}$.

Harvesting at vegetative stage

For shoot length, electrolyte leakage, chlorophyll contents, proline contents and nutrients concentration in the shoot, vegetative stage harvesting (one plant from each pot) was done after 50 days of sowing. The shoot length of maize was measured using the measuring tape.

Gas exchange parameters

Infra-Red Gas Analyzer [CI-340 Photosynthesis system, CID, Inc. USA] was used for determination of net photosynthetic rate, net transpiration rate and stomatal conductance. The readings were taken (45 days old plants) on a sunny day, at saturating intensity of light between 10:45 and 11:40 AM [48].

Chlorophyll contents and proline

The chlorophyll a, b, total chlorophyll and carotenoids contents were analyzed in the fresh leaves according to Arnon [49]. For proline assessment, Bates et al. [50] method was followed. The proline was extracted from 0.1 g fresh leaves in 2 ml of methanol (40%). After extraction, the 1 ml mixture of glacial acetic acid and 6M orthophosphoric acid (3:2 v/v) was mixed in 1 ml extract along with 25 mg ninhydrin. The solution was incubated at 100 ^{o}C for 60 min. When the solution cooled down, 5ml toluene was added. For the estimation of proline contents, absorbance was noted on a spectrophotometer at 520 nm wavelength.

Electrolyte leakage

The methodology of Lutts et al. [51] was followed for determination of electrolyte leakage (EL). To remove dust particles from leaf surfaces, leaves were washed with deionized water and then discs were cut with steel cylinder of diameter 1 cm. Uniform size discs having weight about 1g were immersed in a 20 ml deionized water in test tube which was incubated at 25 ^{o}C for 24h. The EC (EC1) was determined using pre-calibrated EC meter. The second EC (EC2) was noted after heating (120 ^{o}C for 20 min) the test tubes in a water bath. The final value of EL was calculated using the equation as follows;

$$\text{Electrolyte Leakage (EL\%)} = \text{EC1/EC2} \times 100$$

Harvesting for yield attributes

The maize plants were harvested at maturity for the determination of shoot dry weight, 100-grains weight and grains yield plant^{-1}.

NPK concentration in grain and shoot

For N, the samples were digested with sulfuric acid [37] followed by distillation on Kjeldahl's distillation apparatus [39]. The yellow colour method was used for the determination of P concentration by taking absorbance at 420 nm on a spectrophotometer [37]. The potassium concentration in maize shoot and grain was measured by running the digested samples on flame photometer as described by Nadeem et al. [38].

Statistical analysis

Statistical analysis of the data was carried out using standard statistical procedures given by Steel et al. [52]. Two-way analysis of variance applied using Statistix 8.1 software for calculation of significance of treatments and various levels of drought stress. All the treatments means were compared using Tukey's test at $p \leq 0.01$.

Results

Gas exchange attributes

Applied treatments (T), drought stress (D) and interaction (T × D) among them had significant effect on photosynthetic rate, transpiration rate and stomatal conductance (S1 Table). The $BC_{0.75}$ proved significantly better for photosynthetic and transpiration rate, but did not differ for stomatal conductance as compared to the control at SD (Table 2). The highest increase of 0.73 and 2.13-fold in photosynthetic rate was noted as compared to the control where *E. cloacae* + $BC_{1.50}$ and *A. xylosoxidans* + $BC_{1.50}$ were applied under MD and SD, respectively. Application of *A. xylosoxidans* + $BC_{1.50}$ gave an increase of 0.83 and 2.46-fold in transpiration rate at MD and SD, respectively. However, *E. cloacae* + $BC_{1.50}$ resulted in an increase of 0.42-fold in stomatal conductance at MD while *A. xylosoxidans* + $BC_{1.50}$ showed 1.10-fold improvement in stomatal conductance at SD as compared to the control (Table 2).

Chlorophyll contents

Applied treatments (T), drought stress (D) and interaction (T × D) among them had significant effect on chlorophyll a and b, and total chlorophyll contents of maize except that interactive (T × D) effect was non-significant on chlorophyll b contents (S1 Table). Application of $BC_{0.75}$ and $BC_{1.50}$ showed significant improvement as compared to the control in chlorophyll a under MD and SD (Table 3). However, $BC_{1.50}$ performed better for total chlorophyll as compared to the control. The highest increase (0.22-fold) in chlorophyll a was noted under NM where *P. aeruginosa* + $BC_{1.50}$ and *E. cloacae* + $BC_{1.50}$ were applied. However, application of *E. cloacae* + $BC_{1.50}$ and *A. xylosoxidans* + $BC_{1.50}$ gave an increase of 0.69 and 1.52-fold in chlorophyll a content under MD and SD, respectively (Table 3). Application of *E. cloacae* + $BC_{1.50}$ and *A. xylosoxidans* + $BC_{1.50}$ resulted in and increase of 0.76 and 1.48 in total chlorophyll under MD and SD, respectively. $BC_{0.75}$ and $BC_{1.50}$ significantly differed than control for chlorophyll b, where $BC_{1.50}$ was better than $BC_{0.75}$ (Table 3). Inoculation of *P. aeruginosa*, *E. cloacae*, *A. xylosoxidans* and *L. adecarboxylata* along with $BC_{1.50}$ proved relatively better than $BC_{0.75}$ for production of chlorophyll b. The application of all PGPR with $BC_{1.50}$ significantly improved chlorophyll b production compared to the rest of treatments. The highest increase of 0.74-fold in chlorophyll b was noted with *L. adecarboxylata* + $BC_{1.50}$ (Table 3).

Carotenoids, proline and electrolyte leakage

Applied treatments (T), drought stress (D) and their interaction (T × D) significantly altered carotenoids, proline and electrolyte leakage (S1 Table). The $BC_{0.75}$ with and without PGPR

Table 2. Interactive effect of ACC-deaminase containing PGPRs and timber waste biochar on gas exchange attributes under various levels of drought stress.

Treatments (T)	Photosynthetic Rate (μmol CO_2 m^{-2} s^{-1})				Transpiration Rate (mmol H_2O m^{-2} s^{-1})				Stomatal Conductance (mol CO_2 m^{-2} s^{-1})			
	Drought levels (D)											
	NM	MD	SD	Means	NM	MD	SD	Means	NM	MD	SD	Means
Control (No PGPR + No BC)	24.6 a-h	13.1 m-q	6.30 q	14.7 G	3.50 a-g	2.13i-m	1.01 n	2.21I	0.186 a-h	0.141 h-l	0.083 m	0.137 G
P. aeruginosa	25.0 a-g	13.3 l-q	7.20 p-q	15.2 FG	3.75 a-e	2.07i-m	1.05 n	2.29HI	0.213 a-d	0.143 h-l	0.085 m	0.147 FG
E. cloacae	27.0 a-c	16.4i-o	9.40 o-q	17.6 D-G	3.87 a-d	2.50 h-l	1.72 l-n	2.70 GH	0.214 a-d	0.171 d-j	0.116 k-m	0.167 D-F
A. xylosoxidans	26.3 a-d	19.0 e-m	10.6 n-q	18.6 C-F	3.58 a-g	2.76 f-k	1.86 k-n	2.73 F-H	0.211 a-d	0.173 c-i	0.119 j-m	0.168 D-F
L. adecarboxylata	27.5 a	13.8 k-p	7.40 p-q	16.2 E-G	3.76 a-e	2.15i-m	1.34mn	2.42 HI	0.216 a-d	0.138 h-l	0.097 lm	0.150 E-G
BC0.75	27.3 ab	18.6 e-m	13.9 k-p	19.9 A-D	4.14 a-c	2.88 e-j	2.04 j-m	3.02 E-G	0.224 ab	0.173 c-i	0.128 i-m	0.175 B-E
***P. aeruginosa* + BC0.75**	26.2 a-d	18.2 f-m	13.5 l-p	19.3 B-E	4.16 a-c	2.94 d-j	2.16i-m	3.09 D-G	0.213 a-d	0.173 c-i	0.127 i-m	0.171 C-E
***E. cloacae* + BC0.75**	25.3 a-e	20.9 a-k	17.7 h-n	21.3 A-C	4.30 ab	3.40 b-h	2.64 g-l	3.45 B-E	0.225 a	0.190 a-h	0.168 d-j	0.194A-C
***A. xylosoxidans* + BC0.75**	24.5 a-h	20.2 b-l	18.1 g-m	20.9 A-D	4.04 a-c	3.29 c-h	2.65 g-l	3.33 C-E	0.214 a-d	0.186 a-h	0.160 e-k	0.187 A-D
***L. adecarboxylata* + BC0.75**	27.5 a	18.0 g-m	14.1 k-p	19.8 A-D	4.42 a	2.98 d-j	2.20 i-m	3.20 C-F	0.220 a-c	0.173 c-i	0.121 j-m	0.171 C-E
BC1.50	26.3 a-d	21.9 a-j	17.1i-n	21.7 A-C	4.40 a	3.68 a-f	2.92 d-j	3.67 A-C	0.218 a-d	0.192 a-g	0.149 f-k	0.186 A-D
***P. aeruginosa* + BC1.50**	27.1 ab	19.9 c-m	15.3 j-o	20.8 A-D	4.40 a	3.43b-h	3.03 d-i	3.62 A-C	0.227 a	0.198 a-e	0.144 g-l	0.190 A-D
***E. cloacae* + BC1.50**	25.2 a-f	22.6 a-i	19.4 d-m	22.4 AB	4.34 ab	3.78 a-e	3.37 b-h	3.83 AB	0.218 a-d	0.200 a-e	0.173 c-i	0.197 AB
***A. xylosoxidans* + BC1.50**	27.2 ab	22.4 a-j	19.7 d-m	23.1 A	4.40 a	3.89 a-d	3.54 a-g	3.94 A	0.226 a	0.197 a-f	0.174 b-i	0.199 A
***L. adecarboxylata* + BC1.50**	27.5 a	20.3 b-l	16.7i-n	21.5 A-C	4.32 ab	3.40 b-h	2.87 e-j	3.53 A-D	0.220 a-c	0.183 a-h	0.140 h-l	0.181 A-D
Means	26.3 A	18.6B	13.7C		4.09A	3.02B	2.29C		0.216 A	0.176B	0.132C	

Means sharing different letters are significantly different ($p \leq 0.01$). $BC_{0.75}$ = 0.75% Biochar, $BC_{1.50}$ = 1.50% Biochar; NM = Normal moisture, MD = Mild drought stress, SD = Severe drought stress

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Table 3. Interactive effect of ACC-deaminase containing PGPRs and timber waste biochar on chlorophyll contents under various levels of drought stress.

Treatments (T)	Chlorophyll a (mg g^{-1} F.W.)				Chlorophyll b (mg g^{-1} F.W.)				Total Chlorophyll (mg g^{-1} F.W.)			
	Drought levels D)											
	NM	MD	SD	Means	NM	MD	SD	Means	NM	MD	SD	Means
Control (No PGPR + No BC)	0.760 b-i	0.490 k-n	0.293 o-p	0.514 G	0.39	0.26	0.15	0.27 F	1.15 d-l	0.75 p-t	0.45 u	0.78 G
P. aeruginosa	0.826 a-e	0.547 j-n	0.253 p	0.542 FG	0.43	0.30	0.15	0.29 EF	1.26 b-h	0.84 n-s	0.41 u	0.84 FG
E. cloacae	0.829 a-e	0.620 g-m	0.390 n-p	0.613 EF	0.40	0.29	0.21	0.30 D-F	1.22 c-i	0.91 l-r	0.60tu	0.91 F
A. xylosoxidans	0.845 a-e	0.610 h-m	0.37 m-p	0.630 D-F	0.43	0.31	0.21	0.32 C-F	1.27 b-h	0.92 k-r	0.65 s-u	0.95 EF
L. adecarboxylata	0.861 a-e	0.553 j-n	0.287 p	0.567 FG	0.42	0.27	0.16	0.28 EF	1.28b-h	0.83 o-t	0.45 u	0.85 FG
BC0.75	0.921 a-c	0.717 d-j	0.487 k-n	0.705 C-E	0.43	0.34	0.26	0.34 C-E	1.34 a-e	1.05 h-o	0.74 q-t	1.05 DE
***P. aeruginosa* + BC0.75**	0.892 a-d	0.723 c-j	0.483 l-o	0.699 C-E	0.43	0.35	0.24	0.34 C-E	1.33 a-f	1.07 h-o	0.73 r-t	1.04 DE
***E. cloacae* + BC0.75**	0.902 a-d	0.800 a-h	0.583 i-m	0.762 A-C	0.42	0.36	0.30	0.36 CD	1.32 a-f	1.16 d-k	0.89 m-s	1.12 CD
***A. xylosoxidans* + BC0.75**	0.892 a-d	0.787 a-h	0.630 f-l	0.770 A-C	0.45	0.38	0.31	0.38 BC	1.34 a-e	1.17 d-j	0.94 j-r	1.15 B-D
***L. adecarboxylata* + BC0.75**	0.889 a-d	0.720 d-j	0.507 k-n	0.705 C-E	0.48	0.35	0.26	0.36 CD	1.37 a-d	1.07 g-n	0.76 p-t	1.07 CD
BC1.50	0.810 a-f	0.737 b-j	0.613 g-m	0.720B-D	0.51	0.43	0.38	0.44 AB	1.32 a-f	1.16 d-k	0.99 i-p	1.16 B-D
***P. aeruginosa* + BC1.50**	0.928 ab	0.730 c-j	0.617 g-m	0.758 A-C	0.51	0.42	0.36	0.43 AB	1.44 a-c	1.15 d-l	0.98 j-q	1.19 A-C
***E. cloacae* + BC1.50**	0.927 ab	0.827 a-e	0.717d-j	0.823 A	0.55	0.49	0.34	0.46 A	1.48 ab	1.32 a-g	1.06 h-o	1.28 A
***A. xylosoxidans* + BC1.50**	0.881 a-d	0.803 a-g	0.727 c-j	0.804 AB	0.52	0.45	0.39	0.45 A	1.40 a-d	1.26 b-h	1.12 e-m	1.26 AB
***L. adecarboxylata* + BC1.50**	0.972a	0.757 b-i	0.677 e-k	0.802 AB	0.56	0.42	0.42	0.47 A	1.53 a	1.18 d-j	1.09 f-m	1.27 AB
Means	0.875 A	0.695 B	0.513 C		0.46 A	0.36 B	0.28 C		1.34 A	1.06 B	0.79 C	

Means sharing different letters are significantly different ($p \leq 0.01$). Non-significant interactive effect (T × D) did not have any letter.

F.W. = Fresh weight; $BC_{0.75}$ = 0.75% Biochar, $BC_{1.50}$ = 1.50% Biochar; NM = Normal moisture; MD = Mild drought stress; SD = Severe drought stress

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Table 4. Interactive effect of ACC-deaminase containing PGPRs and timber waste biochar on chlorophyll contents under various levels of drought stress.

Treatments (T)	Carotenoids (mg g^{-1} F.W.)				Proline (μmol g^{-1} F.W.)				Electrolyte Leakage (%)			
	Drought levels D)											
	NM	MD	SD	Means	NM	MD	SD	Means	NM	MD	SD	Means
Control (No PGPR + No BC)	0.80 a-g	0.55 h-l	0.19 o	0.51 E	5.34 q	12.23 c-e	15.92 a	11.16 A	22.0 l-q	52.0 a-e	64.7 ab	46.2 A
P. aeruginosa	0.81 a-f	0.61 e-k	0.28 no	0.57 DE	5.98 o-q	11.34 c-i	15.97 a	11.10 A	18.7 pq	49.3 b-h	65.7 a	44.6 AB
E. cloacae	0.82 a-e	0.65 d-k	0.30 m-o	0.59 DE	6.00 o-q	9.34 e-m	13.73 a-c	9.69 BC	19.3 o-q	37.7 e-l	55.0 a-d	37.3 B-D
A. xylosoxidans	0.80 a-g	0.58 g-k	0.30 m-o	0.56 C-E	6.41 m-q	9.68 e-k	12.95 b-d	9.68 BC	18.0 q	40.0 d-k	56.3 a-c	38.1 BC
L. adecarboxylata	0.93 ab	0.64 e-k	0.32 l-o	0.63 B-D	6.06 o-q	11.89 c-g	15.73 c-g	11.23 A	19.7 o-q	50.3 a-g	64.0 ab	44.7 AB
$BC_{0.75}$	0.87 a-d	0.74 a-h	0.48 k-n	0.70 A-C	6.43 m-q	9.17 f-n	12.03 c-f	9.21 CD	19.3 o-q	39.0 e-k	50.7 a-g	36.3 C-E
***P. aeruginosa* + $BC_{0.75}$**	0.90 a-c	0.74 a-h	0.50 j-n	0.71 AB	6.07 o-q	9.66 e-k	11.93 c-g	9.22 CD	18.7 pq	40.7 c-k	49.7 b-h	36.3 C-E
***E. cloacae* + $BC_{0.75}$**	0.88 a-c	0.78 a-g	0.51 i-m	0.72 AB	5.69 p	7.48 j-q	9.39 e-l	7.52 E	18.3 pq	32.3 i-q	41.7 c-k	30.8 C-F
***A. xylosoxidans* + $BC_{0.75}$**	0.87 a-d	0.76 a-h	0.50 j-n	0.71 AB	6.38 n-q	7.76 j-q	9.17 f-n	7.77 DE	17.7 q	30.7 j-q	41.7 c-k	30.0 D-F
***L. adecarboxylata* + $BC_{0.75}$**	0.90 a-c	0.77 a-h	0.59 f-k	0.75 A	5.38 q	10.02 d-j	11.61 c-h	9.01 CD	20.0 n-q	41.7 c-k	51.7 a-f	37.8 B-D
$BC_{1.50}$	0.89 a-c	0.77 a-h	0.62 e-k	0.76 A	6.00 o-q	7.94 j-q	9.60 e-l	7.85 DE	18.0 q	36.0 f-m	46.7 c-i	33.6 C-F
***P. aeruginosa* + $BC_{1.50}$**	0.94 a	0.78 a-g	0.65 d-k	0.79 A	5.65 q	7.83 j-q	9.08 g-n	7.52 E	20.7 m-q	34.0 h-p	42.3 c-k	32.3 C-F
***E. cloacae* + $BC_{1.50}$**	0.90 a-c	0.77 a-h	0.69 c-k	0.79 A	6.45 m-q	6.88 k-q	8.57 i-p	7.30 E	20.0 n-q	29.0 j-q	36.7 e-l	28.6 EF
***A. xylosoxidans* + $BC_{1.50}$**	0.92 ab	0.76 a-h	0.73 a-i	0.80 A	6.04 o-q	6.72 l-q	7.88 j-q	6.88 E	18.0 q	28.0 k-q	35.7 g-n	27.2 F
***L. adecarboxylata* + $BC_{1.50}$**	0.89 a-c	0.79 a-g	0.71 b-j	0.80 A	6.04 o-q	7.07 k-q	8.69 h-o	7.27 E	19.7 o-q	35.0 g-o	44.7 c-g	33.1 C-F
Means	0.87 A	0.71 B	0.49 C		5.99 C	9.00 B	11.48 A		19.2 C	38.4 B	49.8 A	

Means sharing different letters are significantly different ($p \leq 0.01$). Non-significant interactive effect (T × D) did not have any letter. $BC_{0.75}$ = 0.75% Biochar, $BC_{1.50}$ = 1.50% Biochar; NM = Normal moisture; MD = Mild drought stress; SD = Severe drought stress

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remained better than control for carotenoids under SD (Table 4). An increase of 0.18, 0.42 and 2.84-fold in carotenoids was noted with *P. aeruginosa* + $BC_{1.50}$, *E .cloacae* + $BC_{1.50}$ and *A. xylosoxidans* + $BC_{1.50}$ under NM, MD and SD, respectively. The *A. xylosoxidans* without BC proved better than *P. aeruginosa*, *E .cloacae*, *L. adecarboxylata* and control under NM (Table 4). However, $BC_{0.75}$ decreased proline as compared to control, while remained statistically at par with $BC_{1.50}$ under NM, MD and SD (Table 4). The highest reduction of 0.42 and 0.50-fold in proline was noted with *A. xylosoxidans* + $BC_{1.50}$ under MD and SD, respectively (Table 4). However, application of *P. aeruginosa* + $BC_{0.75}$ under SD proved more effective than only *P. aeruginosa* for electrolyte leakage. Maximum reduction of 0.22, 0.86 and 0.81-fold in electrolyte leakage was noted with *A. xylosoxidans* + $BC_{1.50}$ as under NM, MD and SD, respectively (Table 4).

Shoot length and shoot dry weight

Treatments (T), drought stress (D) and their interaction (T × D had significant effect on shoot length and dry weight (S1 Table). The PGPRs without biochar remained statistically at par for shoot length under MD and control. However, under SD, *E. cloacae* without biochar showed statistically better results than control (Table 5). Likewise, $BC_{0.75}$ improved shoot length only under SD as compared to control. However, *E. cloacae* + $BC_{0.75}$ and *A. xylosoxidans* + $BC_{0.75}$ remained better than $BC_{0.75}$ alone and control for shoot length under MD and SD (Table 5).

100-grain weight and grain yield $plant^{-1}$

Treatments (T), drought stress (D) and theirinteraction (T × D) significantly influenced 100-grain weight and grain yield $plant^{-1}$ (S1 Table). Application of *E. cloacae* + $BC_{1.50}$ and *A. xylosoxidans* + $BC_{1.50}$ remained better than *E. cloacae* + $BC_{0.75}$ and *A. xylosoxidans* + $BC_{0.75}$

Table 5. Interactive effect of ACC-deaminase containing PGPRs and timber waste biochar application on shoot length and shoot dry weight of maize under various levels of drought stress.

Treatments	Shoot length (cm)				Shoot dry weight (g)			
	Drought levels							
	NM	MD	SD	Means	NM	MD	SD	Means
Control (No PGPR + No BC)	125.2a-h	88.1j-m	55.4o	89.6E	68.8 a-g	48.4 h-m	32.2 m	49.8 G
P. aeruginosa	117.5c-j	97.0h-l	56.5no	90.3E	75.1 a-d	48.7 h-m	36.1lm	53.3 FG
E. cloacae	136.3a-c	115.2d-k	85.7k-n	112.4CD	72.1 a-f	57.3 e-j	38.1 k-m	55.9 E-G
A. xylosoxidans	142.1a-e	116.6c-j	83.6l-o	114.1CD	73.6 a-e	61.3 c-j	44.7 i-m	59.9 C-F
L. adecarboxylata	119.9b-i	93.9i-l	59.2m-o	91.0E	73.3 a-e	48.4 h-m	38.7 k-m	53.5 FG
BC0.75	126.0a-h	114.1d-k	89.8i-l	110.0D	75.1 a-d	57.4 e-j	46.5 i-m	59.6 C-F
***P. aeruginosa* + BC0.75**	138.2a-e	117.5c-j	86.0k-n	113.9CD	73.5 a-e	53.1 g-k	44.5 j-m	57.0 D-G
***E. cloacae* + BC0.75**	153.0a	141.2a-e	127.5a-g	140.6A	74.4 a-d	68.4 a-g	56.3 f-j	66.4 A-C
***A. xylosoxidans* + BC0.75**	151.7a	144.1a-d	119.5b-i	138.4A	75.6 a-d	65.1 b-h	53.7 g-k	64.8 B-D
***L. adecarboxylata* + BC0.75**	155.4a	112.4e-l	89.7i-l	119.2B-D	78.4 ab	56.2 f-j	44.9 i-m	59.9 C-F
BC1.50	153.2a	133.7a-f	103.9f-l	130.3AB	77.6 a-c	61.5 c-i	50.6 h-l	63.2 C-E
***P. aeruginosa* + BC1.50**	155.1a	127.0a-h	97.1h-l	126.4A-C	84.8 a	60.2 d-j	49.2 h-l	64.7 B-D
***E. cloacae* + BC1.50**	153.7a	140.7a-e	128.6a-g	141.0A	84.6 a	73.0 a-f	60.3 d-j	72.6 AB
***A. xylosoxidans* + BC1.50**	143.0a-d	146.8a-c	118.9c-i	136.2A	84.5 a	71.5 a-f	64.9 b-h	73.7 A
***L. adecarboxylata* + BC1.50**	149.7ab	127.6a-g	102.8g-l	126.7A-C	83.7 a	63.8 b-h	50.7 h-l	66.0 A-C
Means	141.3A	121.1B	93.6C		77.0 A	59.6 B	47.4 C	

Means sharing different letters are significantly different ($p \leq 0.01$).

$BC_{0.75}$ = 0.75% Biochar; $BC_{1.50}$ = 1.50% Biochar; NM = Normal moisture; MD = Mild drought stress; SD = Severe drought stress

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under SD for 100-grain weight (Table 6). The application of $BC_{0.75}$ significantly differed than control for 100-grains weight and grain yield plant^{-1} (Table 6). *E. cloacae* + $BC_{0.75}$ gave an increase of 0.45-fold under MD, while *A. xylosoxidans* + $BC_{1.50}$ exhibited 1.07-fold improvement under SD as than control for 100-grain weight.

Discussion

Drought stress decreased growth and yield attributes of maize (Tables 5 & 6). Chlorophyll contents and gas exchange attributes were also reduced under drought stress (Tables 2–4). Low conductance of stomata decreased CO_2 diffusion in leaves [53,54]. Such reduction in CO_2 disturbs the mechanism of carboxylation, photosynthesis and inhibit the regeneration of RuBP [55]. Translocation of N, P and K is also decreased under limited availibility of water, that is one of major cause of reductiuon in growth attributes [56]. However, co-application of PGPRs and timber-waste biochar significantly enhanced maize shoot growth and yield under various levels of drought stress. A significant improvement in shoot growth showed efficacy of co-application of *E. cloacae* and *A. xylosoxidans* along with biochar as compared to sole application of biochar and/or control under drought stress.

The improvement in shoot growth might be attributed to reduction in stress ethylene level by *E. cloacae* and *A. xylosoxidans*. Higher synthesis of ACC-deaminase by *E. cloacae* and *A. xylosoxidans* might be the major cause of their better performance than *P. aeruginosa* and *L. Adecarboxylata* regarding maize growth and yield under drought stress. Mayak et al. [15] stated that elevated level of ACC in plants, especially under limited availability of water and nutrients, increases ethylene concentration in root and shoot. The plant roots and seeds exude ACC into rhizosphere that is converted by PGPR secreted ACC-deaminase into NH_3 and α-ketobutyrate and ultimately ethylene level reduces. Reduction in ethylene results in better

Table 6. Interactive effect of ACC-deaminase containing PGPRs and timber waste biochar on 100-grains weight and grains yield pot^{-1} under various levels of drought stress.

Treatments (T)	100-grains weight (g)				Grains yield pot $^{-1}$ (g)			
	Drought levels							
	NM	MD	SD	Means	NM	MD	SD	Means
Control (No PGPR + No BC)	16.30 a-f	10.68 k-p	6.96 r	11.31 F	125 a-g	79 h-k	34 mn	79 G
P. aeruginosa	16.80 a-e	10.90 i-p	7.42 qr	11.71 EF	137 a-d	77 h-k	35 l-n	83 FG
E. cloacae	16.80 a-e	13.80 e-j	9.02 n-r	13.21 B-E	146 ab	97 f-i	56 k-n	100 D-F
A. xylosoxidans	16.37 a-f	13.83 e-j	8.78 o-r	12.99 C-E	139 a-c	92 f-k	58 j-n	96 E-G
L. adecarboxylata	16.83 a-e	12.12 h-m	8.11 p-r	12.35 D-F	135 a-e	74 h-k	26 n	78 G
BC0.75	17.43 a	13.97 c-h	10.24 l-q	13.88 BC	142 ab	96 f-j	73 h-l	104 B-E
***P. aeruginosa* + BC0.75**	17.13 ab	13.21 g-l	10.02 m-q	13.46 B-D	137 a-d	91 f-k	77 h-k	102 C-F
***E. cloacae* + BC0.75**	17.10 ab	15.51 a-g	10.88 i-p	14.49 AB	138 a-c	120 a-g	95 f-j	118 A-D
***A. xylosoxidans* + BC0.75**	17.30 a	15.47 a-g	10.81 j-p	14.53 AB	141 ab	125 a-g	90 f-k	119 A-C
***L. adecarboxylata* + BC0.75**	16.90 a-d	11.92 h-n	9.85 m-r	12.89C-E	143 ab	88 g-k	69 i-m	100 C-F
BC1.50	17.13 ab	13.90 d-i	12.60 g-m	14.54 AB	137 a-d	108 b-h	77 h-k	107 A-E
***P. aeruginosa* + BC1.50**	17.10 ab	13.80 e-j	12.80 g-m	14.57AB	146 a	99 d-i	72 h-l	106 A-E
***E. cloacae* + BC1.50**	17.00 a-c	15.35 a-g	14.14 b-h	15.50 A	150 a	127 a-f	97 f-i	125 A
***A. xylosoxidans* + BC1.50**	17.27 a	15.29 a-g	14.44 a-h	15.67 A	145 ab	120 a-g	102 c-i	122 AB
***L. adecarboxylata* + BC1.50**	16.80 a-e	13.35 f-k	11.46 h-o	13.87 BC	140 a-c	98 e-i	77 h-k	105 B-E
Means	16.95 A	13.54 B	10.50 C		140 A	99 B	69 C	

Means sharing different letters are significantly different ($p \leq 0.01$). $BC_{0.75}$ = 0.75% Biochar, $BC_{1.50}$ = 1.50% Biochar; NM = Normal moisture, MD = Mild drought stress, SD = Severe drought stress

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roots elongation that facilitates plant to intake water and nutrients by increasing rhizosphere area [57]. The *E. cloacae* and *A. xylosoxidans* counteracted the adverse effects of drought stress in maize in terms of improvement in grain yield, photosynthetic rate, stomatal conductance chlorophyll a, total chlorophyll and carotenoids contents. In addition to ACC-deaminase activity, the improvement in maize growth might also be favoured due to more production of growth hormone i.e., IAA by *E. cloacae* and *A. xylosoxidans* as as compared to *P. aeruginosa* and *L. Adecarboxylata*. Xie et al. [58] also indicated IAA as an allied factor, playing an important role in crops growth improvement. High IAA synthesis by PGPR increases surface area and length of adventitious and lateral roots in plants that play an important role in nutrients uptake [59]. In addition roots exudates (organic acids, phytosiderophores, sugars, vitamins, amino acids, nucleosides and mucilage) attract PGPR for colonization in roots that improves the uptake of water and solubilization of immobilized nutrients [11,12,60,61].

Furthermore, application of biochar might also be one of the probable reasons for the increase in photosynthesis and transpiration rate, stomatal conductance, nutrients' uptake and reduction in electrolyte leakage under drought stress. Better water and nutrients holding capacity of biochar are directly linked with improvement in crops growth parameters [24,62–64]. Chan et al. [64] argued that improvement in soil cation exchange capacity by application of biochar played a vital role in the availability of N. Younis et al. [65] also reported similar results regarding uptake of P by addition of cotton sticks biochar. Better uptake of K in maize at MD and SD through co-application of *E. cloacae* / *A. xylosoxidans* along with BC might be another favourable factor responsible for the mitigation of drought stress.

According to Singh et al. [66,67] higher amount of K in biochar ash contributes in the improvement of K concentration in plants. Better uptake of K might have maintained the

turgor of cells and stomatal conductance by osmoregulation [68]. Similarly, an increase in shoot P (*E. cloacae* and *A. xylosoxidans*) and K (*E. cloacae* + $BC_{0.75}$ and *A. xylosoxidans* + $BC_{0.75}$) concentration was observed through co-application of PGPR and biochar. This increase in P and K might also be due to better proliferation and activity of the PGPR in the presence of biochar under MD and SD. Findings of Singh et al. [67] supported our argument that the presence of organic carbon in biochar increases the growth of PGPR [67].

Conclusion

Drought stress significantly hampered maize growth and productivity due to impairment in gas exchange traits and photosynthetic pigments. Nonetheless, application of biochar along with *E. cloacae* / *A. xylosoxidans* counteracted the damaging effects of drought stress on maize growth due to notable improvement in gas exchange traits and photosynthetic pigments. Therefore, combined application of *E. cloacae* / *A. xylosoxidans* with biochar seemed a viable option in order to get higher maize yield in drought prone areas.

Supporting information

S1 Table. Statistical summary of growth attributes of maize grown with the application of plant growth promoting rhizobacteria along with biochar under drought stress.
(RTF)

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Author Contributions

Conceptualization: Muhammad Zafar-ul-Hye, Mubshar Hussain.

Data curation: Subhan Danish.

Formal analysis: Subhan Danish.

Funding acquisition: Subhan Danish, Muhammad Zafar-ul-Hye.

Investigation: Subhan Danish, Muhammad Zafar-ul-Hye.

Methodology: Subhan Danish, Muhammad Zafar-ul-Hye.

Project administration: Muhammad Zafar-ul-Hye, Mubshar Hussain.

Resources: Subhan Danish, Mubshar Hussain.

Software: Mubshar Hussain.

Supervision: Muhammad Zafar-ul-Hye, Mubshar Hussain.

Validation: Subhan Danish, Fauzia Mohsin, Mubshar Hussain.

Visualization: Subhan Danish, Fauzia Mohsin.

Writing – original draft: Subhan Danish.

Writing – review & editing: Muhammad Zafar-ul-Hye, Fauzia Mohsin, Mubshar Hussain.

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