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# Rhizobacteria Inoculation and Caffeic Acid Alleviated Drought Stress in Lentil Plants

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**Abstract:** Lentil (*Lens culinaris* Medik) is an important component of the human diet due to its high mineral and protein contents. Abiotic stresses, i.e., drought, decreases plant growth and yield. Drought causes the synthesis of reactive oxygen species, which decrease a plant's starch contents and growth. However, ACC-deaminase (1-aminocyclopropane-1-carboxylate deaminase) producing rhizobacteria can alleviate drought stress by decreasing ethylene levels. On the other hand, caffeic acid (CA) can also positively affect cell expansion and turgor pressure maintenance under drought stress. Therefore, the current study was planned with an aim to assess the effect of CA (0, 20, 50 and 100 ppm) and ACC-deaminase rhizobacteria (*Lysinibacillus fusiform*, *Bacillus amyloliquefaciens*) on lentils under drought stress. The combined application of CA and ACC-deaminase containing rhizobacteria significantly improved plant height (55%), number of pods per plant (51%), 1000-grain weight (45%), nitrogen concentration (56%), phosphorus concentration (19%), potassium concentration (21%), chlorophyll (54%), relative water contents RWC (60%) and protein contents (55%). A significant decrease in electrolyte leakage (30%), proline contents (44%), and hydrogen peroxide contents (54%), along with an improvement in cell membrane stability (34% over control) validated the combined use of CA and rhizobacteria. In conclusion, co-application of CA (20 ppm) and ACC-deaminase producing rhizobacteria can significantly improve plant growth and yield for farmers under drought stress. More investigations are suggested at the field level to select the best rhizobacteria and CA level for lentils under drought.

**Keywords:** abiotic stress; plant growth; legume; caffeic acid

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## 1. Introduction

Lentil (*Lens culinaris* Medik) is an important Rabi crop that primarily originated from southwest Asia and the Mediterranean. Lentil is a rich source of proteins, minerals (zinc, iron, cobalt and calcium), carbohydrates, and vitamins (lysine and arginine). Lentil ranks sixth among major pulses in terms of productivity [1]. Asia alone accounts for more than 75% of world lentil production annually [2]. Turkey, China, Canada, India, Armenia and North America are the major producers of lentils globally. Root nodules of lentils play an important role in improving soil fertility through biological nitrogen fixation [3].

Plants usually face two types of stresses, i.e., abiotic and biotic, in their whole life cycle. Drought is a major and important abiotic stress and is a challenge for agricultural scientists under prevailing climate conditions. Water is essentially required for every growth stage, from germination to maturation. Drought affects yield more than any other abiotic stress [4,5]. Generally, productivity losses due to drought stress range from 53–82%, and drought stress alone accounts for more than 54% of yield losses of lentil crops [5,6]. In semi-arid and arid regions, drought is a naturally occurring phenomenon. The result of many factors, i.e., temperature, precipitation, evapotranspiration and wind velocity, drought causes the death of the whole plant. Prolonged drought is the primary source of desertification. Intensity, timing, and drought duration vary from year to year and even from region to region [4]. Drought stress alone induces several changes in the plant's physiological, morphological and biochemical levels [7], and physiological disturbance that decrease photosynthesis. Ribulose bisphosphate carboxylase (Rubisco) activity reduces under drought stress. It is the main enzyme for $CO_2$ fixation and reduces photosynthesis [8].

Against drought stress, plants have adapted two strategies: tolerance and avoidance. Tolerance means the ability to tolerate low water potential in plant tissues. Avoidance is connected with the ability of plants to maintain higher water potential in plant tissues through stomatal control and, as a result, a lowered transpiration rate [9]. Inside the chloroplast mitochondria and peroxisomes, drought stress induces superoxide radicals ($O_2$), reactive oxygen species (ROS), hydroxyl radical (-OH), and alkoxy radical (RO). These ROS induced cell membrane injuries by increasing lipid peroxidation [9].

Plant growth-promoting rhizobacteria (PGPR) are very important bacteria in the rhizosphere and exert beneficial effects on plant growth and soil fertility under conditions such as salinity, drought, heavy metal stress and nutrient unavailability (deficiency/toxicity) stress [10]. The rhizosphere is a zone of high microbial activity, as it is very close to plant roots. Due to high microbial activity, it is also known as a nutrient-rich zone. These rhizobacteria exert beneficial effects on plants directly [11]. The direct role of PGPR includes a reduction in ethylene levels, synthesis of phytohormones, formation of (phosphate, iron) chelates and symbiotic and non-symbiotic nitrogen fixation [12,13]. Furthermore, PGPR also induces systemic disease resistance in plants, using as a biocontrol agent, production of (antibiotics, volatile metabolites, bacteriocins) and degradation of xenobiotics [14].

Ethylene ($C_2H_4$) is a vital phytohormone that plays a role in many physiological processes, i.e., fruit ripening, seed germination, root elongation and flower senescence [10]. Under stress (biotic or abiotic), ethylene levels rise and interfere with the normal ongoing physiological processes of the plant. It is also known as the stress hormone. Premature senescence of flowers might be due to the high concentration of ethylene [15,16]. The ACC (1-aminocyclopropane-1-carboxylic acid) is a precursor of ethylene. ACC-deaminase (1-aminocyclopropane-1-carboxylate deaminase) containing rhizobacteria can reduce ethylene levels under stress conditions through the hydrolysis of ACC into ammonia ($NH_3$) and a-ketobutyrate and promote plant growth under non-favorable climatic conditions [16].

Numerous growth promoter factors have been attributed to protect against cellular and oxidative damage and to regulate enzymatic activities under water stress conditions in crop plants [17]. Phenylpropanoid and flavonoids are two classified categories of phenolic compounds. These phenylpropanoid-containing secondary metabolites can protect plant metabolic pathways through their antioxidant activity. Caffeic acid (CA) and its derivatives

are phenylpropanoid secondary metabolites in nature and can overcome biotic and abiotic stresses (drought, heavy metal stress, salinity and pathogen attack etc.) [5,18,19].

Caffeic acid (3,4-dihydroxycinnamic acid) plays a significant role in many important physiological processes of the plant, such as cell expansion, phototropism, maintaining turgor pressure, and synthesis of lignin, which is considered an important component of the plant cell wall. Caffeic acid is an intermediate product that is produced from L-tyrosine or phenylalanine [5,20,21]. L-tyrosine is converted into p-coumaric acid in some plants and microorganisms through the tyrosine ammonia-lyase (TAL) enzyme present in the cell cytoplasm. P-coumaric acid converts into CA on further hydroxylation [5,22]. Phenylalanine converts into p-coumaric acid through the trans-cinnamic acid pathway by using several enzymes [23]. Caffeic acid converts into ferulic acid by transferring the methyl group to CA by an enzyme O-methyl transferase [24].

Higher accumulation of CA in plants inhibited brown rot disease, which is produced by fungi. It is reported that the exogenous application of CA inhibited several diseases caused by *Fusarium* and *Saccheromyces* species of fungi as a result of its antioxidant activity [19]. Under salinity and drought stress, ROS accumulated in the plant cell. These ROS react with membrane and membrane-bounded organelles of the cell and cause oxidative damage. This ROS causes lipid peroxidation that results in denaturation of cell metabolism and leads to cell death. Caffeic acid and its derivatives can trap ROS through several enzymes controlled by several antioxidant enzymes. Caffeic acid also plays a role in inhibiting lipid peroxidation and shields cells from oxidative damage. Chlorogenic acid, the product of quinic acid and CA, proves to be a strong antioxidant due to its high free radical trapping activity and enhancing tolerance against stress in plants [25,26].

Under drought stress, CA converts to ferulic acid through the O-methyltransferase enzyme. Plant leaves show a higher accumulation of ferulic acid and CA under drought stress. These antioxidants get bound to the cell wall of leaves and protect photosynthetic apparatus from high energy radiations by absorbing them into mesophyll cells [5,24,27]. Caffeic acid and its derivatives are important in enhancing nitrogen fixation and carbohydrates and protein contents in nodules [28]. The present study hypothesized that rhizobacteria and CA would profoundly affect lentil growth in drought conditions.

## 2. Materials and Methods

### *2.1. Experiment Site*

An experimental trial was conducted at the experimental area of the Department of Soil Science, Bahauddin Zakariya University, Multan. The trial consisted of thirteen treatments along with three replications. The experiment was carried out in pots to study the response of lentil crops towards CA and ACC-deaminase-producing rhizobacteria under drought stress. The height and diameter of pots were 30 cm × 25 cm, respectively. Plastic pots were lined with polythene sheets to avoid leaching losses.

### *2.2. ACC-Deaminase Containing Rhizobacterial Strains and Inoculum Preparation*

Two ACC-deaminase-containing rhizobacterial strains obtained from the soil microbiology laboratory, the department of soil science BZU, were used for the experiment. Both strains were capable of producing ACC-deaminase, indole acetic acid, and P and K-solubilization as documented and published previously.

- *Lysinibacillus fusiform* [29]
- *Bacillus amyloliquefaciens* [30,31]

Inoculum was prepared by using a DF minimal salt medium as described by Dworkin and Foster [32]. The composition of the medium was as follows; $MnSO_4$ = 10 µg/L, $KH_2PO_4$ = 4 g/L, $Na_2HPO_4$ = 6 g/L, $ZnSO_4$ = 70 µg/L, $FeSO_4.7H_2O$ = 1 mg/L, $MgSO_4.7H_2O$ = 0.25 g/L, $CuSO_4$ = 50 µg/L, $H_3BO_3$ = 10 µg/L, $MoO_3$ = 10 µg/L, Citric acid = 2 g/L, Glucose = 10 g/L, Gluconic acid = 2 g/L, ACC = 5 mM and distilled water = 1 L. The 7.0 pH of solution was adjusted by using HCl/NaOH = 1 M. Each strain was inoculated and shaken at 100 rpm at 28 ± 1 °C for 72 h.

### 2.3. Seed Dressing

In this trial, Punjab Masoor-2009 was used as a lentil cultivar. Before seed dressing, lentil seeds were surface disinfected using 95% ethanol and then an $HgCl_2$ solution for five minutes [33]. Seeds were dressed by mixing sterilized peat plus clay, 10% (*w*/*v*) sugar solution and bacterial broth culture. Seeds were placed overnight and then sown in pots.

### 2.4. Caffeic Acid (CA)

Three different concentrations of (20, 50 and 100 ppm) solutions were prepared and used in this experiment. The stock solution of 1000 ppm concentration was prepared by dissolving the required amount of CA (3, 4-dihydroxycinnamic acid) in ethanol because the reason is that like dissolves like and final volume was made up to the mark by using deionized (DI) water. The CA stock solution was further diluted to make 20, 50 and 100 ppm concentration solutions. The pH of the solution was maintained at 6.0 by treating with a 0.1 mM phosphate buffer. Caffeic acid of the desired concentration was applied to plant roots on the tenth day after sowing.

### 2.5. Drought Level

In all treatments, drought was imposed and maintained at 50% field capacity, except the control was maintained at 65% field capacity [34,35]. Initially, 100% soil saturation was made by using tap water in 5 kg soil. The weight of 5 kg soil plus tap water was noted on an analytical balance. After that, soil in the pot was left for 24 h for drainage of gravitational water. The soil and water mixture was then weighed again. The difference in weight provides the amount of water needed for 100% FC. Based on this calculation, the unit method was used to calculate water for 65% and 50% FC to maintain normal and drought stress for lentils. Before imposing drought, moisture contents of the soil were determined by weighing soil-filled pots and then saturated. After forty-eight hours of saturation, pots were again weighed and differences in moisture contents were calculated. A measured quantity of water was applied to each pot [10].

### 2.6. Treatments

T1 uninoculated + 100% field capacity
T2 uninoculated + drought
T3 *Bacillus amyloliquefaciens* + drought
T4 *Lysinibacillus fusiform* + drought
T5 CA (20 ppm) + drought
T6 CA (50 ppm) + drought
T7 CA (100 ppm) + drought
T8 CA (20 ppm) + *Bacillus amyloliquefaciens* plus drought
T9 CA (50 ppm) + *Bacillus amyloliquefaciens* plus drought
T10 CA (100 ppm) + *Bacillus amyloliquefaciens* plus drought
T11 CA (20 ppm) + *Lysinibacillus fusiform* plus drought
T12 CA (50 ppm) + *Lysinibacillus fusiform* plus drought
T13 CA (100 ppm) + *Lysinibacillus fusiform* plus drought

In all the treatments, recommended NPK fertilizers were applied at the rate of (13:23:12) kg/ha, respectively, using Di-ammonium phosphate, urea, and sulphate of potash. The whole of P and K fertilizers were applied as a basal dose at sowing, while N fertilizer was applied in two splits. Pots were 30 cm in height and 25 cm in diameter.

### 2.7. Sowing and Harvesting

In the second week of 15 November 2019, sowing was done manually in pots. The lentil crop was harvested with a sickle on 15 March 2020, when 80% of the crop reached maturity.

### 2.8. Pre-Sowing Soil Analysis

The soil was collected from the Department of Soil Science research area, after which each pot was filled with 6 kg of sieved air-dried soil. Different physic-chemical properties of soil were analyzed from the composite soil sample. Using an EC meter and pH meter, electrical conductivity [36] and pH [37] of the soil were determined. Soil textural class was determined by using a hydrometer [38]. Organic matter contents of the soil were found as per the procedure described by Walkley [39]. As described by Jackson [40], total nitrogen in the soil was estimated by the Kjeldahl method. Using a spectrophotometer, available phosphorus in the soil was noted (Biotechnology Medical Services, UV-1602, BMS, Canada). Extractable potassium in the soil was determined from a flame photometer (Jenway PFP-7, England) [41]. The experimental soil was silt loam in nature (Sand 21%, Silt 60%, Clay 19%, pH 8.01, EC 1.03 dS $m^{-1}$, Organic matter 0.63%, total nitrogen 0.0195%, Available phosphorus 4.5 mg $kg^{-1}$ and Extractable potassium 107 mg $kg^{-1}$).

### 2.9. Plant Analyses

Plant height was noted from base to tip with a measuring tape when the plant reached maturity. The number of pods per plant was noted manually by selecting three plants per pot. The 1000-grain weight was determined by multiplying 50 with the weight of 20 seeds per pot randomly. An SPAD chlorophyll meter (SPAD-OSK) was used to measure chlorophyll contents at the flowering stage from the leaves of the lentil plant (as it is an instant method for determining chlorophyll contents) and expressed as an SPAD value [42]. Plant samples after wet digestion were used to determine nitrogen, phosphorus and potassium percentage in plants as described by Wolf [43].

Relative water contents (RWC) of leaves were determined by taking fresh leaf weight. Then the leaves were dipped in distilled water for 24 h, following which their turgid weight was obtained. The leaves were placed in an oven at 80 °C for 48 h to obtain the dry weight of the leaves [44]. The relative water contents were obtained by following the equation given below:

$$\text{RWC (\%)} = [\{\text{fresh weight} - \text{Dry weight}\}]/[\{\text{Turgid weight} - \text{Dry weight}\}] \times 100 \quad (1)$$

Electrolyte leakage (%) was determined by taking leaves of uniform size in test tubes. A 10 mL DI water was added to each test tube and placed for 2 h on the mechanical shaker and EC0 was measured using an EC meter. Next, these leaves were placed in a refrigerator overnight and EC1 was obtained along with test tubes. After that, these test tubes were autoclaved at 121 °C for 20 min and $EC_2$ was obtained after cooling at 25 °C. Electrolyte leakage was obtained by following the equation given below [45].

$$\text{Electrolyte leakage (\%)} = EC_1 - EC_0/EC_2 - EC_0 \times 100 \quad (2)$$

Cell membrane stability (CMS%) was obtained by subtracting electrolyte leakage from 100 [46].

$$\text{CMS (\%)} = 100 - \text{Electrolyte leakage (\%)} \quad (3)$$

Kjeldahl's method was used to determine nitrogen contents in the plant samples. After this, these nitrogen contents were multiplied by a factor of 4.43 to obtain the protein contents in the plant samples [47]. These protein contents were obtained from Kjeldhal's nitrogen and so were expressed on a percentage basis. The protein multiplication factor differs from crop to crop.

Bates et al. [48] has described a common method. It was followed for the determination of proline contents in the plant samples. Leaf samples of 0.5 g were ground and centrifuged for ten minutes with sulfosalicylic acid (5 mL). The final volume of supernatant was made up to 15 mL after obtaining the supernatant. Two mL glacial acetic acid and two mL ninhydrin reagent was added in 2 mL of the supernatant that was obtained from 15 mL of supernatant. This mixture was boiled at 100 °C for an hour in the water bath. Toluene as

5 mL was added into the reaction mixture after cooling. Afterwards, samples of absorbance readings were obtained using a spectrophotometer (520 nm) against the toluene blank. Proline was expressed as (μg/g) of plant samples.

Mondal and Choudhari [49] have also described a method, and it was used for the determination of hydrogen peroxide contents in plant samples. Leaf samples of 0.5 g were ground with 10 mL cold acetone and filtered using Whatman filter paper No.1. Five mL $NH_4OH$ was added into filtrate, then 4 mL titanyl sulphate was added to precipitate the titanium-peroxide complex. The contents were again centrifuged. Ten mL of $H_2SO_4$ was added to dissolve precipitate after discarding the supernatant. Afterwards, the final volume was raised to 20 mL by using DI water. The absorbance of hydrogen peroxide was measured at 415 nm. Hydrogen peroxide standards of 10, 20, 30, 40, 60, 80 and 100 μM were prepared from hydrogen peroxide stock solution. Hydrogen peroxide was expressed as nmol of hydrogen peroxide per gram of plant sample.

### *2.10. Statistical Analysis*

The current pot trial was laid out with three replications and a completely randomized design (CRD). Treatment means were compared at 5% probability level ($p \leq 0.05$) using Tukey's Honest test. Statistix 8.1®″ software was used to analyze the data statistically. Origin2021 software was used for the Pearson correlation and principal component analysis of data.

## 3. Results

### *3.1. Plant Height, Number of Pods Per Plant, 1000-Grain Weight and Chlorophyll Contents*

The data revealed that ACC-deaminase containing rhizobacteria (*Lysinibacillus fusiform* and *Bacillus amyloliquefaciens*) significantly improved growth parameters, plant height, number of pods per plant, 1000-grain weight, and chlorophyll contents of the lentil crop under drought stress as compared to the control. ACC-deaminase containing rhizobacteria was found to perform better under stress (drought, salinity, etc.) because of its ACC-deaminase activity. Bacterial strains were isolated on the medium containing ACC as a sole source of nitrogen, so their main attribute was to produce ACC-deaminase activity. This is why we used ACC-deaminase containing rhizobacteria in the experiment. Further significant improvement in growth parameters and chlorophyll contents were observed when rhizobacterial strains were applied in combination with exogenous CA under drought stress, as shown in Table 1. A combined CA application at 20 ppm and *Bacillus amyloliquefaciens* significantly improved plant height up to 55% compared to the control under drought conditions. Plant height was improved by as much as 53% when CA at 20 ppm was used in combination with *Lysinibacillus fusiform*. The sole application of *Lysinibacillus fusiform* and *Bacillus amyloliquefaciens* significantly improved the number of pods per plant by 15% and 18%, respectively, when compared to control. The combined use of CA at 20 ppm and *Lysinibacillus fusiform* and combined use of CA at 20 ppm plus *Bacillus amyloliquefaciens* significantly improved the number of pods per plant up to 40% and 51%, respectively, in comparison with the control. Conjoint usage of different concentrations of CA and rhizobacterial strains, either *Lysinibacillus fusiform* or *Bacillus amyloliquefaciens,* significantly improved 1000-grain weight up to 53%, 42%, 34%, 55%, 44% and 36%, respectively, over the control in drought stress.

The combined application of CA and ACC-deaminase containing rhizobacteria significantly improved chlorophyll contents up to 55%, not only over control but also over the application of ACC-deaminase containing rhizobacteria and CA.

### *3.2. Nutrients Concentration, Protein and Relative Water Contents*

Similarly, the data about NPK concentration, protein and relative water contents also revealed that these parameters were significantly improved by the combined use of CA and ACC-deaminase containing rhizobacterial strains compared to the control under drought stress. The combined application of CA at 20 ppm along with *Bacillus amyloliquefaciens*

produced a maximum increase in nitrogen contents of 56% compared to the other treatments and control. The combined application CA at 20 ppm and *Bacillus amyloliquefaciens* significantly improved phosphorus concentration up to 19% when compared to the other treatments and control. Potassium concentration was significantly improved by CA when used either with or without ACC-deaminase containing rhizobacterial strains compared to the control. Combined CA application at 20 ppm either with *Lysinibacillus fusiform* or *Bacillus amyloliquefaciens* significantly improved potassium concentration up to 18% and 21%, respectively, compared to the control. The *Bacillus amyloliquefaciens* produced a maximum increase in potassium concentration as compared to the other treatments. The combined application of CA and *Bacillus amyloliquefaciens* produced a maximum increase in RWC up to 60% compared to other treatments and the control. Compared to the control, protein contents were significantly improved by single or combined application of CA and rhizobacterial strains. Protein contents were directly related to nitrogen concentrations, which were determined from Kjeldahl's method. As nitrogen concentration was significantly improved by the combined application of CA and *Bacillus amyloliquefaciens*, protein contents were also increased by as much as 55% compared to other treatments.

**Table 1.** Physico-chemical properties of soil used in the trial.

| Characteristics | Units | Soil |
|---|---|---|
| Textural class | - | Silt loam |
| Sand | % | 21 |
| Silt | % | 60 |
| Clay | % | 19 |
| $pH_s$ | - | 8.01 |
| $EC_e$ | dS $m^{-1}$ | 1.03 |
| Organic matter | % | 0.63 |
| Total nitrogen | % | 0.02 |
| Available phosphorus | mg $kg^{-1}$ | 4.5 |
| Extractable potassium | mg $kg^{-1}$ | 107 |

### *3.3. Electrolyte Leakage, Proline Contents, and Hydrogen Peroxide Contents Decreased, while Membrane Stability Increased*

The results revealed that electrolyte leakage, proline contents, hydrogen peroxide contents decreased, and membrane stability was significantly increased by the sole or combined application of CA and ACC-deaminase containing PGPR compared to control under drought conditions (Tables 1–4). The *Lysinibacillus fusiform* and *Bacillus amyloliquefaciens* significantly decreased electrolyte leakage up to 13% and 16%, respectively, when compared to control. The CA at 100, 50 and 20 ppm decreased electrolyte leakage up to 15%, 17%, and 18%, respectively, compared to the control. CA observed a maximum decrease at 20 ppm treatment. The combined application of CA at 20 ppm and rhizobacterial strains (*Lysinibacillus fusiform*, *Bacillus amyloliquefaciens*) significantly decreased electrolyte leakage by 25% and 30%, respectively, in comparison with the control.

A maximum decrease was noticed with the *Bacillus amyloliquefaciens* strain as compared to other treatments. Proline and $H_2O_2$ contents were increased in plant leaves, indicating the extent of oxidative damage caused by severe dehydration. The ACC-deaminase containing rhizobacteria and exogenous CA application significantly decreased the proline and $H_2O_2$ contents when compared to the control. The combined application of CA and rhizobacterial strains (*Lysinibacillus fusiform*, *Bacillus amyloliquefaciens*) significantly reduced proline contents up to 41%, 44% and $H_2O_2$ contents up to 52%, 54%, respectively, as compared to the control. Cell membrane stability is inversely related to electrolyte leakage. Cell membrane stability increased as electrolyte leakage decreased. Under drought and salinity stress, electrolyte leakage was increased. Exogenous application of CA and ACC-deaminase containing rhizobacterial strains significantly improved membrane stability due to reduced electrolyte leakage, as observed in the trial. The combined application of exogenously applied CA and rhizobacterial strains significantly improved membrane

stability up to 34% and 28%, respectively, over the control. This study found that ACC-deaminase containing rhizobacterial strains in the presence of CA could be an effective approach for improving plant health, growth and soil nutrient contents over the control in drought stress.

**Table 2.** Co-application of CA and ACC-deaminase producing rhizobacteria on growth parameters of lentil crop under drought stress.

| Treatment | Plant Height (cm) | Number of Pods Plant$^{-1}$ | 1000 Grains Weight (g) | Chlorophyll (SPAD Value) |
|---|---|---|---|---|
| Control | 14.32 ± 0.11 $^{i}$ | 22.27 ± 0.12 $^{l}$ | 27.15 ± 0.07 $^{l}$ | 17.42 ± 0.15 $^{j}$ |
| Drought | 12.14 ± 0.11 $^{j}$ | 20.37 ± 0.07 $^{m}$ | 25.21 ± 0.11 $^{m}$ | 18.32 ± 0.10 $^{i}$ |
| Strain1 + Drought | 16.03 ± 0.07 $^{g}$ | 24.19 ± 0.09 $^{i}$ | 29.31 ± 0.08 $^{j}$ | 21.13 ± 0.06 $^{h}$ |
| Strain2 + Drought | 15.25 ± 0.14 $^{h}$ | 23.56 ± 0.10 $^{k}$ | 28.22 ± 0.13 $^{k}$ | 20.61 ± 0.07 $^{h}$ |
| CA (20 ppm) + Drought | 17.08 ± 0.07 $^{c}$ | 25.86 ± 0.05 $^{g}$ | 33.52 ± 0.05 $^{e}$ | 24.75 ± 0.07 $^{e}$ |
| CA (50 ppm) + Drought | 16.81 ± 0.07 $^{d}$ | 24.89 ± 0.08 $^{h}$ | 32.57 ± 0.05 $^{g}$ | 23.68 ± 0.06 $^{f}$ |
| CA (100 ppm) + Drought | 15.43 ± 0.10 $^{h}$ | 23.82 ± 0.08 $^{j}$ | 31.44 ± 0.06 $^{i}$ | 22.92 ± 0.07 $^{g}$ |
| Strain1 + CA (20 ppm) + Drought | 18.84 ± 0.07 $^{a}$ | 30.86 ± 0.07 $^{a}$ | 36.73 ± 0.06 $^{a}$ | 28.24 ± 0.08 $^{a}$ |
| Strain1 + CA (50 ppm) + Drought | 17.48 ± 0.05 $^{b}$ | 28.69 ± 0.06 $^{c}$ | 34.83 ± 0.06 $^{b}$ | 27.79 ± 0.09 $^{ab}$ |
| Strain1 + CA (100 ppm) + Drought | 16.56 ± 0.05 $^{e}$ | 26.41 ± 0.08 $^{e}$ | 33.81 ± 0.08 $^{d}$ | 26.80 ± 0.16 $^{c}$ |
| Strain2 + CA (20 ppm) + Drought | 18.62 ± 0.05 $^{a}$ | 29.65 ± 0.08 $^{b}$ | 34.21 ± 0.09 $^{c}$ | 27.48 ± 0.15 $^{b}$ |
| Strain2 + CA (50 ppm) + Drought | 17.30 ± 0.05 $^{bc}$ | 27.70 ± 0.11 $^{d}$ | 32.96 ± 0.10 $^{f}$ | 26.56 ± 0.22 $^{c}$ |
| Strain2 + CA (100 ppm) + Drought | 16.33 ± 0.08 $^{f}$ | 26.16 ± 0.08 $^{f}$ | 31.87 ± 0.09 $^{h}$ | 25.58 ± 0.20 $^{d}$ |

Within each column, small English letters showed a significant difference at $p \leq 0.05$. Strain1 = *Bacillus amyloliquefaciens*, Strain2 = *Lysinibacillus fusiform*, Drought maintained at 50% of field capacity, Caffeic acid = CA.

**Table 3.** Co-application of CA and ACC-deaminase producing rhizobacteria on nitrogen, phosphorus, potassium and relative water contents of lentil crop under drought stress.

| Treatment | Nitrogen | Phosphorus | Potassium | Protein | RWC |
|---|---|---|---|---|---|
| | (%) | | | (mg/g) | (%) |
| Control | 2.73 ± 0.06 $^{h}$ | 0.02136 ± 0.0050 $^{j}$ | 0.72 ± 0.024 $^{fg}$ | 12.11 ± 0.041 $^{h}$ | 22.39 ± 0.14 $^{i}$ |
| Drought | 2.50 ± 0.05 $^{i}$ | 0.02207 ± 0.0043 $^{i}$ | 0.69 ± 0.024 $^{g}$ | 11.11 ± 0.047 $^{i}$ | 34.51 ± 0.54 $^{h}$ |
| Strain1 + Drought | 3.15 ± 0.05 $^{fg}$ | 0.02329 ± 0.0027 $^{fg}$ | 0.76 ± 0.024 $^{de}$ | 13.99 ± 0.058 $^{fg}$ | 42.08 ± 0.04 $^{f}$ |
| Strain2 + Drought | 3.01 ± 0.06 $^{g}$ | 0.02259 ± 0.0035 $^{h}$ | 0.74 ± 0.024 $^{ef}$ | 13.36 ± 0.041 $^{g}$ | 39.93 ± 0.14 $^{g}$ |
| CA (20 ppm) + Drought | 3.43 ± 0.05 $^{de}$ | 0.02370 ± 0.0035 $^{e}$ | 0.78 ± 0.029 $^{cd}$ | 15.24 ± 0.025 $^{de}$ | 47.49 ± 0.12 $^{d}$ |
| CA (50 ppm) + Drought | 3.39 ± 0.07 $^{e}$ | 0.02299 ± 0.0038 $^{gh}$ | 0.76 ± 0.024 $^{de}$ | 15.03 ± 0.033 $^{e}$ | 44.26 ± 0.17 $^{e}$ |
| CA (100 ppm) + Drought | 3.29 ± 0.08 $^{ef}$ | 0.02162 ± 0.0043 $^{j}$ | 0.72 ± 0.033 $^{f}$ | 14.61 ± 0.025 $^{ef}$ | 42.16 ± 0.09 $^{f}$ |
| Strain1 + CA (20 ppm) + Drought | 3.90 ± 0.10 $^{a}$ | 0.02626 ± 0.0035 $^{a}$ | 0.84 ± 0.047 $^{a}$ | 17.32 ± 0.033 $^{a}$ | 54.95 ± 0.50 $^{a}$ |
| Strain1 + CA (50 ppm) + Drought | 3.80 ± 0.08 $^{ab}$ | 0.02514 ± 0.0043 $^{b}$ | 0.82 ± 0.033 $^{ab}$ | 16.86 ± 0.025 $^{ab}$ | 51.60 ± 0.10 $^{b}$ |
| Strain1 + CA (100 ppm) + Drought | 3.74 ± 0.08 $^{b}$ | 0.02504 ± 0.0035 $^{bc}$ | 0.80 ± 0.033 $^{bc}$ | 16.61 ± 0.041 $^{b}$ | 50.50 ± 0.30 $^{bc}$ |
| Strain2 + CA (20 ppm) + Drought | 3.66 ± 0.08 $^{bc}$ | 0.02470 ± 0.0035 $^{cd}$ | 0.82 ± 0.041 $^{ab}$ | 16.24 ± 0.033 $^{bc}$ | 51.84 ± 0.11 $^{b}$ |
| Strain2 + CA (50 ppm) + Drought | 3.57 ± 0.08 $^{cd}$ | 0.02433 ± 0.0043 $^{d}$ | 0.80 ± 0.024 $^{bc}$ | 15.86 ± 0.025 $^{cd}$ | 48.84 ± 0.13 $^{cd}$ |
| Strain2 + CA (100 ppm) + Drought | 3.54 ± 0.08 $^{cd}$ | 0.02348 ± 0.0035 $^{ef}$ | 0.78 ± 0.024 $^{cd}$ | 15.74 ± 0.041 $^{cd}$ | 47.27 ± 0.13 $^{d}$ |

Within each column, small English letters showed a significant difference at $p \leq 0.05$. Strain1 = *Bacillus amyloliquefaciens*, Strain2 = *Lysinibacillus fusiform, Drought* was maintained at 50% of field capacity, caffeic acid = CA.

### *3.4. Pearson Correlation and Principal Component Analysis*

Pearson correlation showed that proline and hydrogen peroxide were significantly negative in correlation with plant height, number of pod plant$^{-1}$, 1000 seeds weight, SPAD chlorophyll, N, P, K, relative water contents, and protein lentil under drought stress. A significant positive correlation existed between plant height, number of pods plant$^{-1}$, 1000 seeds weight, SPAD chlorophyll, N, P, K, relative water contents and protein of lentil under drought stress. It was also observed that electrolyte leakage was non-significant negative in correlation with plant height, number of pods plant$^{-1}$, 1000 seeds

weight, SPAD chlorophyll, N, P, K, and protein of lentil under drought stress (Figure 1). Principal component analysis showed that proline and hydrogen peroxide were in close relationship with electrolyte leakage. However, the cell membrane was oppositive and far apart from electrolyte leakage, proline and hydrogen peroxide. Similarly, plant height, number of plant pods$^{-1}$, 1000 seeds weight, SPAD chlorophyll, N, P, K, and protein of the lentil were closely associated with each other but were opposite and far apart from electrolyte leakage, proline and hydrogen peroxide (Figure 2).

**Table 4.** Co-application of CA and ACC-deaminase producing rhizobacteria on electrolyte leakage, cell membrane stability, proline contents and hydrogen peroxide contents of lentil crop under drought stress.

| Treatment | Electrolyte Leakage | Cell Membrane Stability | Proline Contents | Hydrogen Peroxide Contents |
|---|---|---|---|---|
| | (%) | | µg g$^{-1}$ | nmol g$^{-1}$ |
| Control | 21.77 ± 0.57 $^{i}$ | 78.22 ± 0.57 $^{a}$ | 0.53 ± 0.04 $^{ij}$ | 1.66 ± 0.04 $^{gh}$ |
| Drought | 52.53 ± 0.20 $^{a}$ | 47.47 ± 0.20 $^{i}$ | 0.91 ± 0.05 $^{a}$ | 2.41 ± 0.06 $^{a}$ |
| Strain + Drought | 44.42 ± 0.17 $^{bc}$ | 55.58 ± 0.17 $^{gh}$ | 0.73 ± 0.06 $^{c}$ | 1.35 ± 0.07 $^{e}$ |
| Strain2 + Drought | 46.18 ± 0.04 $^{b}$ | 53.81 ± 0.04 $^{h}$ | 0.79 ± 0.04 $^{b}$ | 1.50 ± 0.04 $^{d}$ |
| CA (20 ppm) + Drought | 43.39 ± 0.05 $^{cd}$ | 56.60 ± 0.05 $^{fg}$ | 0.63 ± 0.03 $^{ef}$ | 1.56 ± 0.04 $^{c}$ |
| CA (50 ppm) + Drought | 44.00 ± 0.10 $^{bc}$ | 55.99 ± 0.10 $^{gh}$ | 0.67 ± 0.03 $^{de}$ | 1.64 ± 0.04 $^{b}$ |
| CA (100 ppm) + Drought | 45.15 ± 0.07 $^{bc}$ | 54.85 ± 0.07 $^{gh}$ | 0.71 ± 0.03 $^{cd}$ | 1.69 ± 0.03 $^{b}$ |
| Strain1 + CA (20 ppm) + Drought | 36.74 ± 0.06 $^{h}$ | 63.26 ± 0.06 $^{b}$ | 0.51 ± 0.03 $^{j}$ | 1.11 ± 0.04 $^{h}$ |
| Strain1 + CA (50 ppm) + Drought | 37.54 ± 0.05 $^{gh}$ | 62.45 ± 0.05 $^{bc}$ | 0.55 ± 0.03 $^{hi}$ | 1.19 ± 0.05 $^{g}$ |
| Strain1 + CA (100 ppm) + Drought | 38.21 ± 0.08 $^{fgh}$ | 61.79 ± 0.08 $^{bcd}$ | 0.58 ± 0.04 $^{gh}$ | 1.28 ± 0.04 $^{f}$ |
| Strain2 + CA (20 ppm) + Drought | 39.39 ± 0.05 $^{efg}$ | 60.60 ± 0.05 $^{cde}$ | 0.54 ± 0.03 $^{ij}$ | 1.18 ± 0.05 $^{g}$ |
| Strain2 + CA (50 ppm) + Drought | 40.16 ± 0.05 $^{ef}$ | 59.83 ± 0.05 $^{de}$ | 0.57 ± 0.03 $^{ghi}$ | 1.22 ± 0.04 $^{g}$ |
| Strain2 + CA (100 ppm) + Drought | 41.04 ± 0.09 $^{de}$ | 58.95 ± 0.09 $^{ef}$ | 0.60 ± 0.04 $^{fg}$ | 1.31 ± 0.04 $^{ef}$ |

Within each column, small English letters showed a significant difference at $p \leq 0.05$. Strain1 = *Bacillus amyloliquefaciens*, Strain2 = *Lysinibacillus fusiform*, *Drought* was maintained at 50% of field capacity, caffeic acid = CA.

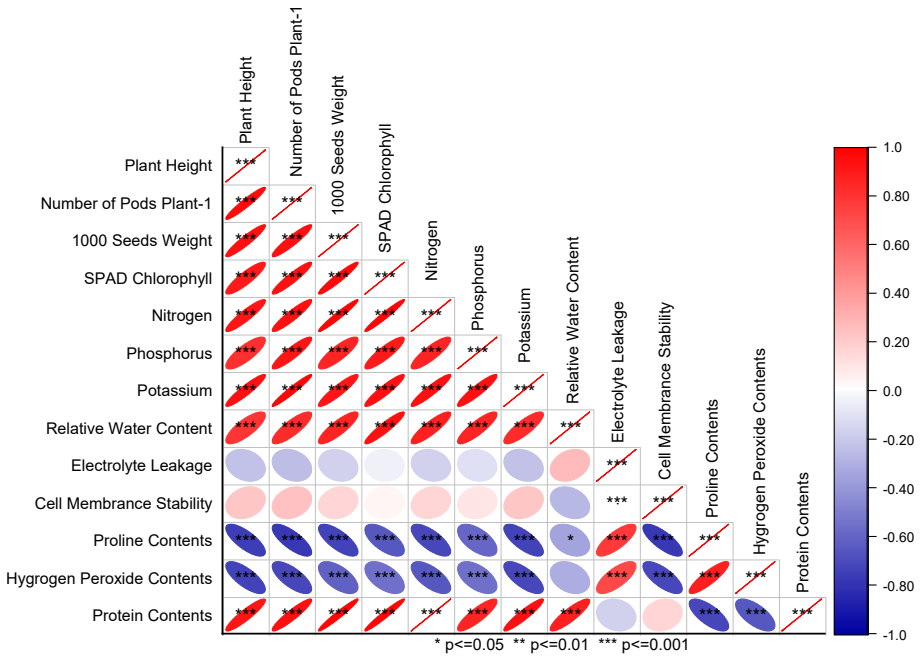


**Figure 1.** Pearson correlation for different growth attributes of lentil. Different stars showed significant change at * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Intensity of blue color is showing negative correlation while intensity of red color is showing positive correlation. Ellipse having no stars are non-significant.

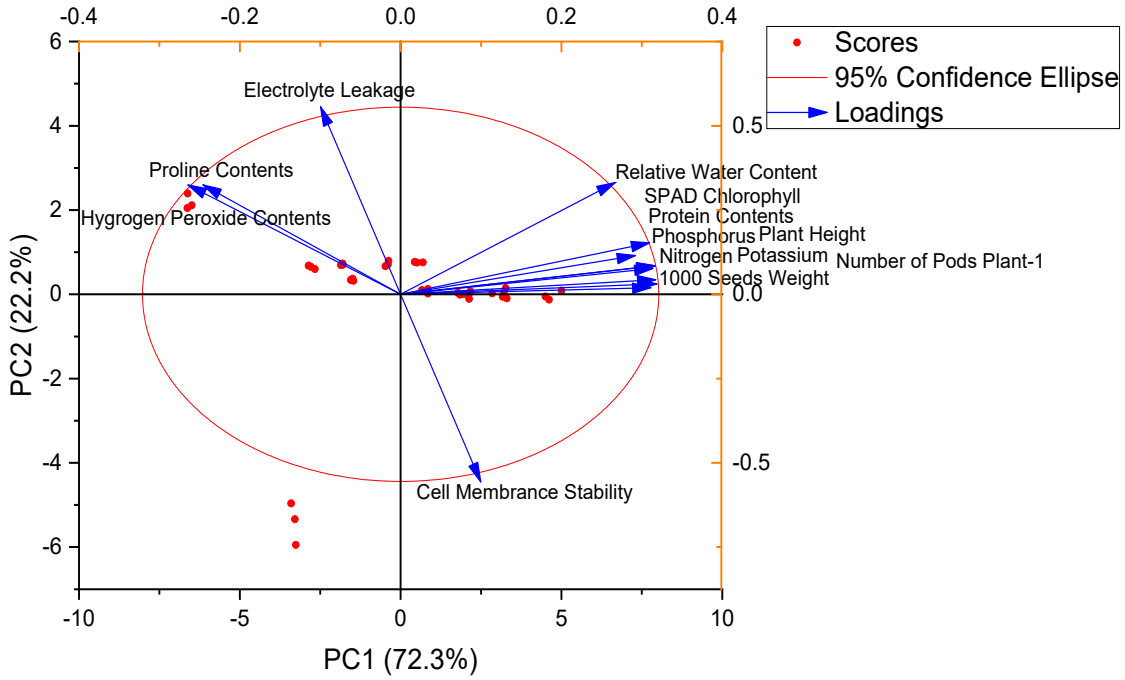


**Figure 2.** Principal component analysis relating relative water content, SPAD chlorophyll, protein content, phosphorus, plant height, nitrogen, potassium, number of pods plant$^{-1}$, 1000 grains weight, electrolyte leakage, proline contents, cell membrane stability and hydrogen peroxide contents using applied treatments as a group. Arrows indicate the loadings for each trait along the first two components which comprised 94.5% of the total variation for 10 traits. The percentages of total variance represented by principal component 1 as PC1 and principal component 2 as PC2.

## 4. Discussion

The data revealed that sole or conjoint application of PGPR (*Bacillus amyloliquefaciens*, *Lysinibacillus fusiform*) and CA significantly improved plant height, number of pods per plant and 1000-grain weight as compared to the control. Plant growth-promoting rhizobacteria secrete phytohormones. These phytohormones mineralized the immobilized P and K of soil, thus enhancing their uptake in the plants [50,51]. The results of the current study are in line with the above findings. Both rhizobacteria, i.e., *Lysinibacillus fusiform* [29] and *Bacillus amyloliquefaciens* [30,31] were capable of solubilizing P and K. Therefore, the improvement in growth attributes was due to better uptake of nutrients by inoculation of rhizobacteria, i.e., N, P and K. The presence of rhizobacteria population also increase the rhizosphere area of crops [52]. Better elongation of roots by PGPR inoculation facilitates the uptake of water in plants under drought stress [53,54]. Improvement in relative water contents (RWC) by PGPR of the current study validated the argument. Furthermore, caffeic acid (CA) also played a key role in regulating nutrient uptake under drought conditions [5]. Results showed that sole application of CA significantly enhanced the uptake of N, P and K compared to drought stress treatment. The application of CA decreased the harmful activities of reactive oxygen species (ROS). Better scavenging of ROS and free radicals by CA protects the plants from oxidative damage [55]. In the current study, less electrolyte leakage by CA treatment justified the above argument.

Leaf chlorophyll contents were also significantly decreased under drought conditions as compared to non-stressed conditions. Conditions where water is not sufficient leads to a reduction in the photosynthetic rate due to stomatal closure, low chlorophyll contents in leaves and ethylene production under drought conditions [56]. This ethylene activates chylase, which, when it came into contact with chlorophyll, initiated chlorophyll degradation. Inoculation of PGPR (*Bacillus amyloliquefaciens*, *Lysinibacillus fusiform*) and the exogenous application of CA significantly improved chlorophyll contents compared to the control. The reason behind improved chlorophyll contents might be the functioning

of ACC-deaminase activity, which lowered ethylene production by its conversion into ammonia and α-ketobutyrate under drought stress [57]. The better uptake of nutrients, especially N, P and K, by rhizobacteria significantly improved chlorophyll contents under drought stress [30,54,58,59]. Caffeic acid and its derivatives convert into ferulic acid and aid in the absorption of high energy radiation in mesophyll cells under drought conditions and, in turn, chlorophyll contents might be improved. Under drought conditions, plant leaves contain high amounts of CA (ferulic acid) derivatives bound with the wall of the leaf and protect photosynthetic machinery from oxidative damage [5].

Under drought stress, plants have adapted the mechanism of increasing proline contents in leaves to maintain osmotic potential in leaves and to protect them from severe dehydration [56]. Our data revealed that sole or conjoint application of PGPR (*Bacillus amyloliquefaciens*, *Lysinibacillus fusiform*) and exogenous application of CA significantly reduced proline contents as compared to the control. The proline contents might be decreased because of increased ACC-deaminase activity, which helped reduce the ethylene level, producing α-ketobutyrate and ammonia, and maintained osmotic potential in the plant body under drought conditions [57]. The CA derivatives also have played a role in decreasing proline contents due to their secondary metabolites, antioxidant, and reactive oxygen species (ROS) capturing activities under water-deficient conditions [5,20,60].

Plants accumulate higher levels of $H_2O_2$ contents to combat oxidative damage caused by drought stress. The PGPR (*Bacillus amyloliquefaciens*, *Lysinibacillus fusiform*) and CA application significantly reduced $H_2O_2$ contents compared to the control. This high level of $H_2O_2$ caused lipid peroxidation under drought stress, which is detrimental to plant growth [61]. The PGPR inoculation lowered $H_2O_2$ contents and prevented lipid peroxidation. This may be because of the enhancing of antioxidant activity and total phenolic contents under drought stress. The CA and its derivatives also played a role in lowering $H_2O_2$ contents by improving antioxidant activity and reducing malondialdehyde (MDA) contents correlated with $H_2O_2$ contents [20,62,63].

## 5. Conclusions

It is concluded that the growth and yield parameters of lentils can be significantly improved through the co-application of ACC-deaminase-producing rhizobacteria (*Bacillus amyloliquefaciens*, *Lysinibacillus fusiform*) and CA under drought stress. However, the effect of *Bacillus amyloliquefaciens* was more prominent than *Lysinibacillus fusiform* for most of the growth attributes of lentils when applied with CA under drought stress. Farming communities can get maximum benefits through the combined application of 20 ppm CA with *Bacillus amyloliquefaciens* in lentil cultivation under drought stress. More investigations are suggested at the field level under variable agro-climatic zones to declare the best application rate of CA with *Bacillus amyloliquefaciens* under drought.

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