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24-Epibrassinolide and salicylic acid alleviate the photosynthesis and yield inhibition under water stress in chick Pea

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Abstract
This study was aimed to find the effects of 28-epibrassinolide (28-EBL) and salicylic acid on chickpea seedlings subjected to water stress, either alone and supplemented with 28-EBL and Salicylic acid treatments. Combined supplementation of EBL+SA alone also exhibited the significant improvement on chlorophyll content (34.4% of Chl a and 35.5 % of Chl b) than EBL and SA alone treatments compared to the control plants. Control plants receiving the EBL+SA alone treatment showed the significant effect (by 32.8%) than the EBL and SA alone treatment (20.4 % and 11.4% respectively) compared to the control for carotenoid levels. combined supplementation of EBL+SA exhibited the significant improvement of P_N (21%; 0.0411, p≤0.05) over their individuals in comparison to control. Co-application of EBL and SA was more effective (44%; 0.0341, p≤0.05) over unstressed control than their individual applications in enhancing g_S in control plants. Plants treated with EBL+SA alone showed a marked increase in Ci (by 20%) compared to control plants. Co-application of EBL and SA was more effective (41.2%; 0.0265, p≤0.05) over unstressed control than their individual applications in enhancing *E* in control plants. Co-application of EBL and SA considerably increased the Fv/Fm and ΦPSII in comparison to unstressed control. Control plants treated with EBL+SA exhibited the more impact on Ru BP case activity (33.1%; 0.0411, p≤0.05) over unstressed control than their individual applications over the control plants. Co-application of EBL and SA was more effective (32.4%; 0.0237, p≤0.05) over unstressed control than their individual applications in enhancing FB Pase activity in control plants. Control plants treated with EBL+SA was found to be more effective on the PGK activity by 63.3% (0.0112, p≤0.05) than their individuals (EBL by 52.1% and SA by 36.1%) compared to control. EBL and SA has a more significant effect than their individual applications on the improvement of leaf starch and sucrose concentrations in drought stressed and well-watered plants.

Keywords: Chickpea, 28-epibrassinolide, photosynthetic pigments, calvin cycle enzymes

Introduction
Water-deficit stress is a noteworthy natural factor restricting in excess of 33% of the arable land the world over. Drought is a typical abiotic stress during the chickpea developing season, which causes a progression of negative impacts on chickpea development, yield (Saeed *et al* 2011 and Loka *et al* 2012) [22, 12]. chickpea is awfully drought touchy harvest causing impetus decrease in yield, since drought stress is a mind boggling marvel that influences the physiology chickpea plant (Feng and Stewart, 2003) [5] (Alishaha and Ahmadikhah, 2009) [1], (Lian *et al* 2009) [14].

During the most recent decade, the foliar utilization of plant development controllers and biomolecules, for example, brassinosteroids and polyamine has turned into a set up system in harvest creation to build yield and nature of the yield under abiotic stresses as drought (Shallan *et al* 2012) [24]. Brassinosteroids (BRs) are a class of steroidal plant hormones (class on new plant hormones) that assume assorted jobs in plant development and formative procedures (Saini *et al* 2015) [23]. Brassinosteroids that were found over thirty years back yet their physiological capacity presently can't seem to be completely clarified (Janeczko *et al* 2016) [9] however BRs assume significant jobs in a wide scope of formative marvels and as of late they turned into a lightening specialist for stress tolerance in plants (Marakli and Gozukirmizi, 2016) [15]. Then again, it is additionally obvious that BRs collaborate with different phyto hormones, for example, auxin, cytokinin, ethylene, gibberellin, jasmonic acid, abscisic acid, salicylic acid and polyamine in managing wide scope of physiological and formative procedures in plants (Xia *et al* 2010) [26]. Moreover, brassinosteroids assume significant jobs in the mind boggling system of plant signal transduction that manages plant development and advancement.

Field and nursery preliminaries have appeared exogenous BRs can likewise improve plant tolerance to abiotic and biotic stress. Likewise, brassinosteroids have been proposed to expand the obstruction of plants to drought stress. In this regard, 24-epibrassinolide (EBL) as a kind of brassinosteroids has a defensive job of on chlorophyll content, the photochemical action of photosystem, film lipids and proteins. The ameliorative impacts by 24-epibrassinolide were intently connected with EBL-incited changes in hostile to oxidative protein exercises and cancer prevention agent substance and they recommended that EBR could improve plant development under drought stress (Zhang, 2012) [10].
The objective of this study was to investigate the impact of 24-epibrassinolide and salicyclic acid on nitrogen metabolism in chickpea subjected to water stress

Materials and Methods
24-Epibrassinolide (EBL) and Salicylic acid (SA) employed in the present study were purchased from Sigma chemicals.

Hormone preparation and concentration selection
The stock solution of EBL was prepared by dissolving the required quantity of BRs in 5 ml of ethanol, in a 100-ml volumetric flask and the final volume was made up to the mark by using double-distilled water. Salicylic acid was dissolved in absolute ethanol then added drop wise to water (ethanol/water: 1/1000 v/v).
The working concentration of EBL and SA i.e. 2.0 µM and 0.5mM respectively were prepared by diluting stock with double distilled water. To choose working concentration for the experiments, a dose response experiment was performed using a wide range of concentrations of EBL (0.1, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 µM) and SA (0.1, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 mm). The concentrations of EBL and SA i.e., 2 µM and 0.5 mm respectively were selected based on the growth response test where significant growth promotion was observed.

Plant material and Rhizobium cultures
The seeds of chickpea (*Cicer arietinum* L.) were procured from National Seed Corporation, Hyderabad, India. Specific strains of *Rhizobium* cultures were obtained from Microbiology Division, IARI, and New Delhi.

Pot experiments
Chickpea seeds were surface-sterilized with 2% sodium hypochlorite (NaOCl) solution for 20 min and washed with double distilled water for 5 times followed by tap water to remove any remaining sodium hypochlorite. *Rhizobium. SS* inoculants were mixed together with sterilized seeds in plastic bag with sticking material. Seeds were placed in a cool place until dried. After drying, 10 uniformly coated seeds were sown at ~25 mm depth in earthen pots (diameter of 35 cm and height of 30 cm) filled with 12 kg of pot mixture containing garden soil and farmyard manure (3:1) up to 5cm from the top. Each pot was watered after sowing to ensure the germination and seedling establishment. After 15 days after sowing (DAS), seedlings were thinned to three plants per pot and maintained in a greenhouse under controlled conditions at Department of Botany, Osmania University, Hyderabad, India. The average day and night temperatures were 30 ± 5°C and 20 ± 2°C, respectively and photoperiod of 16/8 hours day/night regime with light supplemented with 400 W high-pressure sodium lights having photon flux density of 600 µmol m^{-2} s^{-1} and the relative humidity was 55 ± 5% by day and 80 ± 5% at night.

Drought imposition and hormone treatments:
Two days prior to sowing, the pots were irrigated to saturation level and allowed to drain 24 hours to determine the weight of saturated pot. After emergence, plants were maintained at 80% FC of the pot until the start of stress treatments. A custom-made weighing machine was used to weigh the pots to monitor soil water content on alternate days. The control treatment was kept at 80% of the cylinder saturated weight (FC= 80%). At early flowering stage i.e. 60 DAS, drought stress was initiated by withholding the irrigation (when 50% of the plants in the experiment were at the first flower stage). The drought stress was created by withholding irrigation to 25 % of FC of pot (FC = 25 %). The water requirements of the plants were determined as the difference between the weight of a fully irrigated pot and the weight of the pot 24 hours later, after the day's evapotranspiration. This determination was conducted on alternate days to take care of changing water demands of the plants with age. Pots were placed in the greenhouse within a randomized complete block in five replications of each treatment. Tests were done as a factorial experiment in a randomized plot design with three replications under greenhouse conditions. Plants were dived into the following groups:

1. 80% of field (i.e. pot) capacity (FC)-Control
2. 24-epibrassinolide (2 µM) and/or SA (0.5 mM)
3. 25 % of FC -Drought stress
4. 25 % FC + 24-epibrassinolide (2 µM) and/or SA (0.5 mM)Before inducing the drought stress plants were foliar sprayed with 200 ml of EBL (2 µM) and/or SA (0.5 mM) or distilled water with 0.02% Tween 20 (as a control). Salicylic acid and EBL were sprayed at 10 days interval from 60 DAS to till podding stage. Handheld sprayer was used for spraying the plants until runoff in the morning. Morphological and physiological indices were measured in the plants at early podding stages in order to find reproducible. At each sampling, the three youngest fully-expanded leaves of two similar branches of two plants each were harvested just prior to the commencement of the photoperiod, and leaf water relations were measured. Samples for enzyme assays and chemical analyses were frozen in liquid N_2 and stored at-80ºC until the analyses were conducted.

Determination of photosynthetic pigments
Chlorophyll pigments were extracted and estimated according to the method of Arnon (1949) [2]. Fully expanded and mature leaves were randomly selected and homogenized with 80% (v/v) acetone. The green slurry was centrifuged at 5000 rpm for 10 minutes. The supernatant was transferred to a 25 ml volumetric flask. The residual pigments were re-extracted using small amounts of 80% acetone and centrifuged. The supernatant was transferred to the volumetric flask. The extraction was repeated till complete white residue was obtained. The combined chlorophyll extracts were made up to 25 ml with 80% acetone. The optical density was recorded at λ = 645 nm, 663 nm and 480 nm against 80% (v/v) acetone as blank in UV-Visible Spectrometer.
The amount of pigments present in the pigment extract was determined employing the following formulae:

$$\text{Chlorophyll 'a'} = [(\text{OD } 663 \times 12.7) - (\text{OD } 645 \times 2.69)] \times V / (1000 \times W)$$

Chlorophyll 'b' = [(OD 663 × 22.9) – (OD 645 × 4.68)] × V / (1000 × W)

Chlorophyll 'a' = [(OD 663 ×12.7) – (OD 645 × 2.69)] × V / (1000 × W)

Total chlorophylls = [(OD 663 × 20.2) – (OD 645 × 8.02)] × V/ (1000× W)

Carotenoids = (1000 OD 480 – 3.27 (Chl a) – 104 (Chl b)) / 227

Where,
V-volume of the pigment extract; W -weight of the leaf material in grams.
Chlorophyll and carotenoid contents were expressed in mg g/fresh weight.

Gas exchange measurements
Gas exchange parameters were measured in mature, fully expanded leaves from the upper crown of plants. Gas exchange and chlorophyll fluorescence were measured in the same leaf. Gas exchange parameters such as net photosynthetic rate (Pn), transpiration rate (E), stomatal conductance (Gs) and internal CO_2 concentration (Ci) were measured with a Li-Cor model 6400 system (Lincoln, NE, USA). These measurements were carried out on the middle part of the youngest (fully opened second leaf), which avoided the leaf vein. The measurements were conducted from during 8:30 to 10 am., during this time the curtain of the greenhouse was shut down to avoid effects of different light conditions. The saturating photosynthetic photon flux density was between 1000 and 1500 µmol m^{-2} s^{-1} in the leaf chamber during the measurement periods, and the temperature, CO_2 concentration and relative humidity inside the leaf cuvette were always close to ambient air values.

Chlorophyll fluorescence
Chlorophyll fluorescence parameters were determined using a PAM-2500 chlorophyll fluorescence analyser (WALZ, Germany) between 9:00 and 11:00. After a 20 min dark adaptation period, the maximal photochemical efficiency of PSII (Fv/Fm), quantum efficiency of PSII (ϕ_{PSII}) and Photochemical quenching (q_P) were determined. The cuvette of the gas exchange system was modified to accept the fibre optic of the fluorimeter at a 60° angle without significantly interfering with PPFD distribution at the leaf surface. Minimal fluorescence (F_0) was measured under a weak pulse of modulating light over a 0.8 s period, and maximal fluorescence (F_m) was induced by a saturating pulse of light (8000 µmol $m^{-2}s^{-1}$) applied over 0.8 s. The maximal quantum efficiency of PSII was determined as F_v/F_m, where F_v is the difference between F_0 and F_m An actinic light source (600 µmol m^{-2} s^{-1}) was then applied to achieve steady-state photosynthesis and to obtain F_s (steady-state fluorescence yield), after which a second saturation pulse was applied for 0.7 s to obtain F'_m (light-adapted maximum fluorescence). Fluorescence parameters were calculated by the FMS-2, based on the dark-adapted and light adapted fluorescence measurements. The quantum efficiency of PSII (ϕ_{PSII}) and the efficiency of excitation capture by open PSII centres were calculated as $(F'_m–F_s)/F'_m$ and F'_v/F'_m, respectively. Photochemical quenching (q_P) was calculated as $(F'_m–F_s)/(F'_m–F_0)$.

Calvin Cycle Enzymes
Fully expanded trifoliate leaves without petioles were homogenized in ice cold 5 ml of 100 mM Tris-HCl (pH 8) buffer consisting of 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM ß-mercaptoethanol, 2 mM DTT, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 100 mg insoluble PVP and 1 mM PMSF. The extracts were centrifuged at16,000 *g*, for 20 min, (4 °C) and the supernatant was used for the enzyme assays, all of which were based on NADH oxidation at 340 nm, at 25 °C, in 1 mL final volume in the cuvette.

Ribulose-1, 5-bisphosphate carboxylase (RuBPcase: EC.4.1.1.39) Extraction was done as described by Makino *et al*. (1988). RuBPcase was activated for 20 min at 0°C after preparation of the supernatant in the activation medium that contained 75 mM Hepes–KOH at pH 7.5, 10 mM $MgCl_2$ and 10 mM $NaHCO_3$. To determine the Rubisco activity, a 50 µl of extract was added to 900 µl of reaction mixture consisting of 100 mM bicine at pH 8.2, 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM creatine phosphate, 1 mM ATP-2 Na, 0.1 mM NADH, 0.3 mM RuBP, 10 units of phosphocreatine kinase, 10 units of glyceraldehydes-3-phosphate dehydrogenase and 10 units of phosphoglycerate kinase, as described by Sawada *et al*. (1990). The change in absorbance at 340 nm was immediately recorded for every 5 s for 5min. The enzymatic activities were corrected for the decrease in absorbance in a control assay medium prepared without ribulose bisphosphate at 25 ºC.

3- phosphor-glycerate kinase (PGK; EC) PGK activity was determined according to Hatch and Kagawa (1973). The reaction mixture consisted of 100 mM HEPES-KOH **(**pH 7.8), 10 mM $MgCl_2$, 1 mM NaF, 1 mM KH2PO4, 1 mM phosphoglyceric acid, 4 units of triosephophate isomerase and 4 units of glyceraldehydes-3-phosphate dehydrogenase. The reaction was started by the addition of 2 mM ATP and 0.1 1 mM NADH. NADH oxidation was determined spectrophotometrically at 340 nm at 25 ºC.

Fructose 1, 6-bisphosphatase (FBPase; EC 3.1.3.11) FBPase activity was determined by monitoring the absorption at 340 nm, using an extinction coefficient of 6.2 mM-1 cm-1 (Scheibe *et al*., 1986). Total activity was assayed after the crude extract had been activated in a 0.1 ml activation mixture containing 100 mM DTT, 2 mM fructose-1,6- bisphosphate (FBP), 10 mM MgCl2, and 0.1 M Tris–HCl (pH 8.0). The initial activity was assayed immediately after homogenization. The assay mixture consisted of 0.1 M HEPES (pH 8.0), 0.5 mM Na2EDTA, 10 mM MgCl2, 0.3 mM NADP+, 0.6 mM FBP, 0.6 units of glucose- 6-phosphate dehydrogenase from baker's yeast, 1.2 units of glucose phosphate isomerase from baker's yeast, and 100 µl of enzyme extract in a final volume of 1 ml. The reaction was initiated by the addition of enzyme extract.

Ribulose 5-phosphate kinase (RuB5PK: EC 2.7.1.19) RuB5PK activity was performed according to Kagawa (1982). Twenty (20) µl of supernatant was added to the reaction mixture containing 100 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 40 mM KCl, 20 mM phosphoenolpyruvate, 5 mM ATP, 1 mM NADH, 20 mM DTT, 8 units of pyruvate kinase, 10 units of lactate dehydrogenase and 5 units of phosphoriboisomerase. After an incubation period of 15 min, the reaction was initiated by adding 10 µl of 500 mM ribose-5-phosphate and change in absorbance was monitored at 340 nm for every 5 s for 5 min.

Carbohydrate Fractions
Recent fully expanded leaves were harvested and homogenized in 70% (v/v) ethanol and used for soluble sugar

analysis. The residue left after extracting soluble sugars was used for determination of starch content. Ethanol homogenate (2.5 ml) was taken into centrifuge tubes. The tubes were kept in a boiling water bath for 5 minutes. After cooling, the contents were centrifuged at 4,000 rpm for 10 minutes. The supernatant was collected. The residue was re-extracted with 5 ml of 70% (v/v) ethanol and was centrifuged again. This procedure was repeated 3 times. The ethanol supernatants were pooled and made up to 10 ml. This was used for the estimation of total sugars and reducing sugars.

Estimation of total sugars

Total sugars were estimated according to the method of Yoshida *et al*., (1976) [27]. 5 ml of alcohol extract was evaporated to dryness in a clean beaker in a water bath at 60 ºC. The lipids and pigments were removed by washing the evaporated residue repeatedly with diethylether. Then the residue was dissolved in 5 ml of 40 % (v/v) ethanol. This was used for the estimation of total sugars by anthrone reagent.

Anthrone reagent: 200 mg of anthrone dissolved in 100 ml of concentrated sulphuric acid.

One ml of extract was taken and to it 5 ml of anthrone reagent was added. The tubes were heated for 7½ minutes in a boiling water bath. The tubes were cooled and the intensity of brown colour developed was recorded at 630 nm in UV Visible Spectrometer (SCHIMADZU UV-1800, Japan) using blank. The blank consisted of 1 ml of 40 % (v/v) ethanol and 5 ml of anthrone reagent. The total sugars were estimated as D-glucose equivalents. The amount of glucose was found out from a glucose standard curve. The amount of total sugars was expressed as mg g^{-1}fr.wt.

Estimation of reducing sugars

Reducing sugars were determined according to Nelson (1944) method. Nelson reagent was used for the estimation of reducing sugars (Glucose and Fructose, using standard graphs).

Nelson reagent: Nelson reagent was prepared by mixing reagents A and B prior to their use as follows:

Reagent A**:** 2.5 g of sodium carbonate, 2.5 g of sodium potassium tartarate, 2 g of sodium bicarbonate and 400 mg of copper sulphate were dissolved in distilled water and then the volume was made up to 100 ml in a volumetric flask with distilled water.

Reagent B**:** Reagent B contains solution 1 and 2.

Solution 1**:** 2.5g of ammonium molybdate was dissolved in 90 ml of distilled water and to this 2.1 ml of concentrated sulphuric acid was added.

Solution 2**:** 300 mg of sodium arsenate was dissolved in 7.9 ml of distilled water.

Just before use, solution 1 and solution 2 were mixed and heated gently to obtain light yellowish Reagent B.

One ml of Nelson reagent A was added to 1 ml of the sample. A blank was prepared with 1 ml of 70% ethanol instead of sample and 1 ml of reagent A. The colour of the mixture turns to light green. The contents were heated in a water bath for 15 minutes till the green color disappears. It was cooled to room temperature and to this 1 ml of Nelson reagent B was added. Soon after the addition of Nelson reagent B, the mixture turned to thick blue color. The contents were diluted by adding 5 ml of distilled water. The absorbance was recorded in at 550 nm against the blank in UV Visible Spectrometer (SCHIMADZU UV-1800, Japan).

Estimation of Non-reducing sugars

The amount of non-reducing sugars was calculated by the following formulae as given by Loomis and Shull (1937)**:**

$$\text{Non reducing sugars} = (\text{total sugars} - \text{free reducing sugars}) \times 0.95$$

The amount of non-reducing sugars was expressed as glucose equivalents in terms of mg g^{-1} fresh weight.

Estimation of Starch

Starch was estimated from the residue left after alcohol extraction of the sugar by employing the method of Mc. Cready *et al*, (1950) [16]. The starch was solubilized from the residue for 1 hour with 5 ml of 52% perchloric acid. The contents were centrifuged at 3000 rpm for 15 minutes. The supernatant was collected. 1 ml of perchloric acid extract was diluted to 3 ml with distilled water. To this 5 ml of freshly prepared anthrone reagent was added. The mixture was heated in a water bath for 7 ½ minutes at 100 ºC. The contents were cooled and were thoroughly shaken. The absorbance of the contents was measured at 630 nm in a UV Visible Spectrometer (SCHIMADZU UV-1800, Japan) against blank, which was made without the starch extract. The amount of glucose was calculated from a standard curve prepared by using known amount of glucose. The starch content was calculated by multiplying the glucose equivalents present in the sample with 0.9. The content of starch was expressed as mg g^{-1} fresh weight.

Yield parameters

No. of pods/plant

Number of pods per plant was counted from three tagged plants in experimental trial.

No. of seeds/pod

Number of seeds per pod was counted from three tagged plants in experimental trial.

Hundred (100) seed weight (g)

The 100 seeds obtained from selected plant separately were weighed and mean weight of seed per plant was calculated.

Statistical analysis

The results presented are the mean values of 5 replicates. The data analyses were carried out using one-way analysis of variance (ANOVA) followed by Post Hoc Test (Multiple Comparisons) using SPSS (SPSS Inc., Chicago, IL, USA). The differences were considered significant if p was ≤ 0.05. The mean values were compared, and lower-case letters are used in figures/table to highlight the significant differences between the treatments.

Results and Discussion

Photosynthetic pigments. In higher plants abiotic stresses like drought, salinity, heavy metal toxicity, and high light, confer serious damage on the photosynthetic machinery (Lu *et al.*, 2000; Tanyolac *et al*, 2007 [25]; Akther *et al*, 2015; Gururani *et al.*, 2015) [7]. Effect of EBL and SA on the chlorophyll (Chl) a, b and carotenoid contents of chickpea plants under drought stress at reproductive stage and stress-free conditions are presented in Table 1, 2 and 3.

Drought stress at reproductive stage significantly reduced the Chl a (70.5%) and Chl b (61.7%) content in chickpea plants compared to the control. Supplementation of EBL and SA negated the drought effect on Chl a and Chl b content and brought near to control level. EBL treatment accounted for the enhancement of Chl a and Chl b by 152 and 110%

respectively over the stressed control. Similarly, about 97 and 81.2% of increase in Chl a and Chl b content was noticed for SA application in drought stressed plants. However, co-application of EBL+SA showed more significant effect on Chl a and Chl b content (204%; p=0.012 and 137.6%; p=0.0254 respectively) than their respective individual compared to the stressed control. Combined supplementation of EBL+SA alone also exhibited the significant improvement on chlorophyll content (34.4% of Chl a and 35.5 % of Chl b) than EBL and SA alone treatments compared to the control plants.

A significant increase in carotenoid levels (45.7%; 0.029 p≤0.05) were noted in drought stressed plants compared with control. There was no significant effect on carotenoid content was noted in drought stressed plants treated with EBL and SA. In contrast, simultaneous application of EBL and SA to stressed plants was significantly elevated the carotenoid levels by 20% (0.0371, p≤0.05) in comparison with stressed control. Similarly, control plants receiving the EBL+SA alone treatment showed the significant effect (by 32.8%) than the EBL and SA alone treatment (20.4 % and 11.4% respectively) compared to the control.

Leaf gas exchange responses: Effect of EBL and/or SA on net photosynthetic rate (Pn), stomatal conductance (GS), inter cellular CO2 concentration (Ci) and transpiration rate (E) under drought stress at reproductive stage are presented in Table 1, 2 and 3.

Photosynthetic rate (PN): Chickpea plants showed a significant decrease in PN (~50%; p=0.028) subjected to drought stress at reproductive stage compared to the control plants. However, exogenous application of EBL and SA alone improved the PN by 80 % and 61.1% in drought stressed plants over the stressed control. It was further noted that co-application of EBL and SA significantly increased the PN by 89.3% (p=0.018) compared to the drought stress alone. Control plants receiving EBL and SA alone tretments marginally increased the PN in comparison with control. However, combined supplementation of EBL+SA exhibited the significant improvement of PN (21%; 0.0411, p≤0.05) over their individuals in comparison to control.

Stomatal conductance (gS): Under terminal drought stress gS decreased by 48% (0.0271, p≤0.05) over the control. Individual supplementation of EBL and SA to drought stressed plants increased the gS by 67.2 % and 36.2 % over the stressed control. However, application of EBL+SA further significantly increased the gS by 88.8% compared to the stressed control, suggesting the complex nature of interaction between EBL and SA in modulation of gS. Individual EBL and SA application also accounted for considerable increase in gS by 27.3% and 20% respectively over the control. Co-application of EBL and SA was more effective (44%; 0.0341, p≤0.05) over unstressed control than their individual applications in enhancing gS in control plants.

Intracellular CO2 concentration (Ci): A significant reduction (47%; 0.0287, p≤0.05) in Ci was noted for drought stressed plants when compared with controls. Foliar application of EBL to drought stressed plants significantly improved the Ci (41.7%) under water limited conditions. Foliar spray of SA to drought stressed plants also accounted for significant increase in Ci levels by 30.3% in comparison to stressed control. However, EBL and SA applied together enhanced Ci more efficiently (80%; 0.0175, p≤0.05) than their individual applications under drought stress. No significant increase in Ci was recorded for individual applications of EBL and SA with control when compared with only unstressed control. However, plants treated with EBL+SA alone showed a marked increase in Ci (by 20%) compared to control plants.

Transpiration rate (E): Compared to control, transpiration rate (E) was significantly decreased by 41.8% in drought stressed chickpea plants. Individual application of EBL and SA to drought stressed plants enhanced the E significantly (p≤0.05) by 53.4 % and 37.1% over the stressed control. However, application of EBL+SA further significantly increased the E by 65 % (0.0357, p≤0.05) compared to the stressed control, indicating the complex nature of interaction between EBL and SA in modulation of E. EBL and SA alone treatments also accounted for considerable increase in E by 25.1% and 11% respectively over the control. However, co-application of EBL and SA was more effective (41.2%; 0.0265, p≤0.05) over unstressed control than their individual applications in enhancing E in control plants.

Chlorophyll fluorescence responses: Effect of EBL and/or SA on the changes of chlorophyll fluorescence parameters in chickpea leaves under drought stress are shown in Table 1, 2 and 3.

Drought stress at reproductive stage significantly decreased the Fv/Fm and quantum efficiency of PSII (ΦPSII) by 31% and 24.5% respectively in comparison to control. On the other hand, ΦPSII and Fv/Fm were significantly increased by foliar spray of EBL (by 36.7% and 29.6% respectively) and SA (by 34.6% and 27% respectively) in drought stressed plants overt the stress control. The improvement Fv/Fm and ΦPSII was more significant (38.6%; 0.0332 p≤0.05 and 32.2%; 0.0274 p≤0.05) in drought stressed plants treated with EBL+SA than their individual application compared to stressed control. A small increase in Fv/Fm for EBL and SA alone treatment was observed but there was no effect on ΦPSII for EBL and SA alone treatment compared to control. However, co-application of EBL and SA considerably increased the Fv/Fm and ΦPSII in comparison to unstressed control.

A sharp decrease in photochemical quenching coefficient (qP) by 51.4 % and electron transport rate (ETR) by 42.4% was observed in drought stressed plants compared to the control. No significant increase in qP was noticed upon drought stressed plants treated with EBL (p=0.124) and SA (p=0.0847) over the stressed control plants. Whereas, application of both EBL and SA individually to stressed plants significantly increased the ETR by 40.4% and 30.3%. Simultaneous application of EBL and SA to drought stressed plants was able to induce qP and ETR level by 20% and 65% in comparison with drought treatment alone, indicating that co-application of EBL and SA has a more significant effect than their individual applications on the improvement of qP and ETR in drought stressed plants. A small increase in qP and ETR for EBL and SA alone treatments was observed when compared with control. However, co-application of EBL and SA significantly increased the qP (22%; 0.0347 *p*≤0.05) and ETR (22%; 0.0262 *p*≤0.05) in comparison to control. Findings of present study are in coherence with the observations of (Wani *et al.,* 2017; Jiang *et al.,* 2017). Our results are consistent with Liu *et al.,* (2014) who observed that significant variations of chlorophyll fluorescence values upon Cd treatment. Fv/Fm, qP and ΦPSII were significantly

decreased by Cd treatments in comparison with the control suggesting induction of photo inhibition.

Table 1: Chlorophyll 'a' content in chickpea

	Chl a (mg/g FW)	
Control	3.46	0.107
EBR	4.21	0.158
SA	3.86	0.146
EBR+SA	4.65	0.315
Drought	1.02	0.204
D+EBR	2.57	0.248
D+SA	2.01	0.214
D+EBR+SA	3.11	0.321

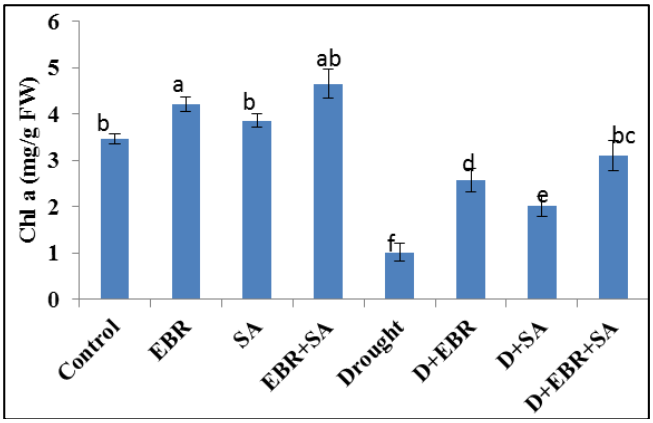

Fig 1: Chlorophyll 'a' content in chickpea

Table 2: Chlorophyll 'b' content in chickpea

	Chl b (mg/g FW)	
Control	2.22	0.167
EBR	2.95	0.198
SA	2.41	0.181
EBR+SA	3.01	0.125
Drought	0.85	0.099
D+EBR	1.79	0.158
D+SA	1.54	0.184
D+EBR+SA	2.02	0.108

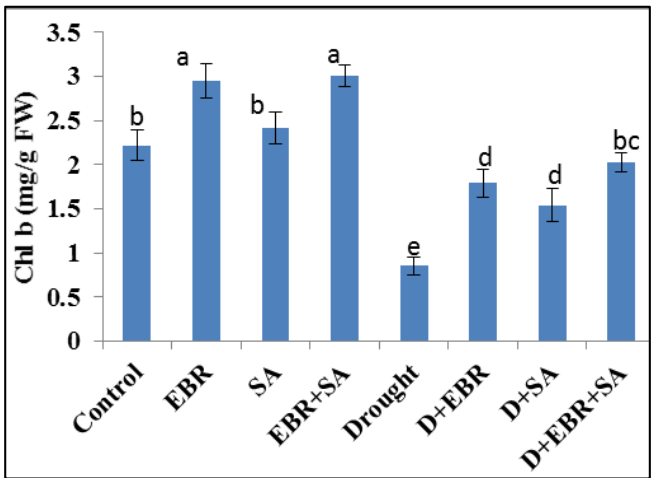

Fig 2: Chlorophyll 'b' content in chickpea

Table 3: Carotenoids content in chickpea

	Carotenoids (mg/g FW)	
Control	0.558	0.094
EBR	0.672	0.046
SA	0.622	0.031
EBR+SA	0.741	0.018
Drought	0.813	0.059
D+EBR	0.866	0.042
D+SA	0.889	0.033
D+EBR+SA	0.959	0.059

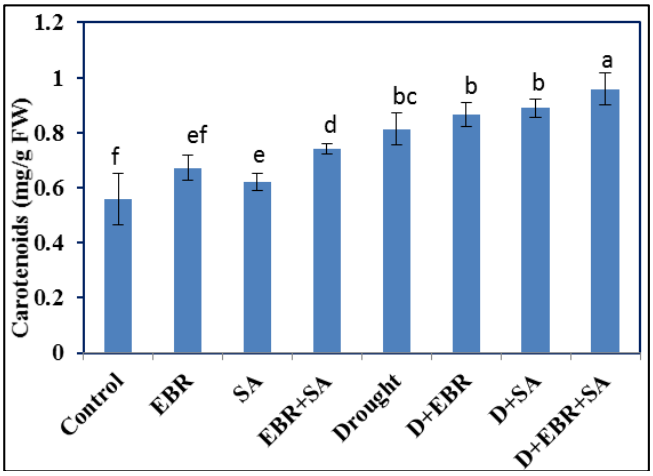

Fig 3: Carotenoids content in chickpea

Calvin cycle enzyme activities: Abiotic stresses such as mineral toxicity, salinity, water deficiency and heat stress adversely affect the carbohydrate metabolism in plants (Devi *et al.*, 2007) [4]. In many plant species, accumulation of soluble sugars occurs to counteract stressful environment through osmotic alterations (Rosa *et al.*, 2009 [20]; Neeta and Shitole, 2010) [17]. Sucrose is primary end product of photosynthesis and is major form of translocated carbon whereas starch comprises the temporary reserve form of carbon which gets finally stored in the grains (Ruan, 2014) [21]. The enzyme sucrose phosphate synthase (SPS) catalyses sucrose biosynthesis in the plant tissues whereas sucrose synthase (SS) and acid invertase (AI) involved in sucrose-cleavage in vivo and translocating the assimilates to diverse pathways in plant storage cells (Rosa *et al.*, 2009 [20]; Ruan, 2014) [21].

Effect of EBLand/or SA on the Ribulose-1,5-bisphosphate carboxylase (RuBPcase), 3- phosphor-glycerate kinase (PGK), Fructose 1,6-bisphosphatase (FBPase) and Ribulose 5-phosphate kinase (RuB5PK) enzyme activities in chickpea leaves under drought stress are shown in Table 4, 5, 6, 7and 8.

Ribulose-1, 5-bisphosphate carboxylase (RuBPcase): About 52% (0.0357, $p \leq 0.05$) in RuBPcase activity was observed in drought stressed chickpea plants compared to control. Exogenous EBL and SA individual treatments significantly increased the RuBPcase activity in drought stressed plants by 54 % and 31.8 % respectively over the stress control. Moreover, co-application of EBL and SA enhanced the RuBPcase activity most significantly (77%; 0.0226, $p \leq 0.05$) over stress control, almost brought near to the

untreated control level, in drought stressed plants. Exogenous EBL and SA alone application also accounted for considerable increase in FBPase activity by 21 % (p=0.0368) and 9.8 % (p=0.0758) respectively over the control. However, when control plants treated with EBL+SA exhibited the more impact on RuBPcase activity (33.1%; 0.0411, p≤0.05) over unstressed control than their individual applications over the control plants.

Fructose 1, 6-bisphosphatase (FB Pase): Drought stress at reproductive stage significantly decreased the FB Pase activity by 32.7% (0.0411, p≤0.05) in comparison to control. Individual supplementation of EBL and SA to drought stressed plants increased the FB Pase activity by 40 % and 42.7 % respectively over the stress control. However, co-application of EBL and SA was enhanced the FB Pase activity by 73.6% (0.0304, p≤0.05) compared to the stress control, suggesting that co-application of EBL and SA has a more significant effect than their individual applications in comparison to stress control. Exogenous EBL and SA alone application also accounted for considerable increase in FBPase activity by 18.5 % and 29.8 % respectively over the control. Our data showed that SA application induced the FBPase activity more than the EBL alone treatment. Co-application of EBL and SA was more effective (32.4%; 0.0237, p≤0.05) over unstressed control than their individual applications in enhancing FBPase activity in control plants.

3- phosphor-glycerate kinase (PGK): A significant decrease in PGK activity (47.8%) was noticed in chickpea plants under drought stress at reproductive stage. Exogenous EBL and SA treatments to drought stressed plants eased the inhibitory effect and significantly improved the PGK activity by 77.5 % and 102% compared to stress control. However, combined application of EBL and SA further enhanced PGK activity more significantly (128%; 0.0205, p≤0.05) than their individual applications under drought stress. Control plants treated with EBL+SA was found to be more effective on the PGK activity by 63.3% (0.0112, p≤0.05) than their individuals (EBL by 52.1% and SA by 36.1%) compared to control.

Ribulose 5-phosphate kinase (RuB5PK): RuB5PK activity was sharply reduced (28.4%; 0.0423, p≤0.05) in drought stressed plants compared with control. Foliar application of EBL to drought stressed plants significantly increased the RuB5PK activity by ~30% over the stress control. Similarly, foliar spray of SA also accounted for the considerable increase in RuB5PK activity by 17.3% but not significantly in drought stressed plants compared to stress control. However, EBL and SA applied together enhanced RuB5PK activity more efficiently (31.8%; 0.0324, p≤0.05) than their individual applications under drought stress. No significant increase in RuB5PK activity was observed for individual applications of EBL and SA compared with only unstressed control. However, combined application of EBL and SA significantly increased the RuB5PK activity (16.3%) compared to control plants.

Carbohydrate fractions: Effect of EBL and/or SA on the levels of carbohydrate fractions in chickpea plants under drought stress at reproductive stage and stress free conditions are presented in Table 4, 5, 6 and 7.

Compared to the well-watered plants, leaf starch and sucrose concentrations were significantly lowered in droughted plants by 35.14% and 42.8% respectively. Supplementation of EBL and SA negated the drought effect on leaf starch and sucrose concentrations and brought near to control level. EBL treatment accounted for the enhancement of leaf starch and sucrose concentrations by 39.19% and 54.5% respectively over the stressed control. Similarly, about 24.56 and 39.57% of increase in leaf starch and sucrose concentrations were noticed for SA application in drought stressed plants. However, co-application of EBL+SA showed more significant effect on leaf starch and sucrose concentrations (52%; 0.0278 *p*≤0.05 and 75.4%; 0.0340 p≤0.05 respectively) than their respective individual compared to the stressed control. Combined supplementation of EBL+SA alone also exhibited the more significant improvement on leaf starch and sucrose concentrations (28.75% of starch and 39.41 % of sucrose) than EBL and SA alone treatments compared to the control plants, indicating that co-application of EBL and SA has a more significant effect than their individual applications on the improvement of leaf starch and sucrose concentrations in drought stressed and well-watered plants.

Drought stress at reproductive stage showed a marked increase in glucose content (22.7%) relative to control plants. Application of EBL to drought stressed plants further significantly enhanced the cellular glucose levels by 19.7% compared to stress control. Exogenous SA application also improved the leaf cellular glucose content but not significant as compared to the stress control. However, about 34.03% (0.0137 p≤0.05) of leaf glucose content was increased with combined treatment of EBL and SA, suggesting that co-application has a more significant effect than their individual applications on the glucose content in drought stressed chickpea plants compared to stress control. Individual application of EBL and SA as well as their co-application to unstressed plants also exhibited the considerable increase in glucose level by 28.31%, 18.24% and 39.41% respectively over the control. Our data demonstrating that co-application of EBL+SA has more effect on leaf glucose content under well-watered and water-deficit conditions.

Compared to the well-watered plants, leaf fructose concentration was significantly lower (47.85%; 0.0137 p≤0.05) in drought stressed plants. Foliar spray of EBL and SA was found to be improved the fructose concentrations statistically insignificant as compared to the stress control. However, combined treatment of EBL+SA exhibited the significant enhancement of leaf fructose concentration by 32.41% (0.0381 p≤0.05) than their individual treatments as compared to drought-treatment. Similarly, well-watered plants treated with EBL+SA improved the leaf fructose concentrations significantly (32.41%; 0.0421 p≤0.05) than the exogenous EBL alone (13.23%; p=0.0687) and SA alone (15.44%; p=0.0845) reflecting that the co-application has a more significant effect than their individual applications on leaf fructose concentration in well-watered plants.

Findings of present study are in coherence with the observations of Gengmao *et al.,* (2014) [6], where carbohydrates were reported to increase in *Salvia miltiorrhiza* plants under NaCl toxicity. Similarly, elevated levels of glucose, fructose and sucrose were observed in *Brassica juncea* plants under Cd toxicity. Glucose and fructose are involved in maintaining osmotic potential and scavenging free radicals in *Oryza sativa* (Pattanagul and Thitisaksakul, 2008) [19]. Furthermore, soluble sugars are also involved in ROS anabolism and catabolism, such as the oxidative pentose phosphate pathway associated with ROS scavenging (Couée *et al.,* 2006) [3].

Table 4: Sucrose content in chickpea

Sucrose (mg/g DW)		
Control	27.18	2.82
EBR	31.25	1.46
SA	30.53	2.06
EBR+SA	33.84	3.76
Drought	17.63	1.35
D+EBR	24.54	2.75
D+SA	21.96	2.28
D+EBR+SA	26.81	3.65

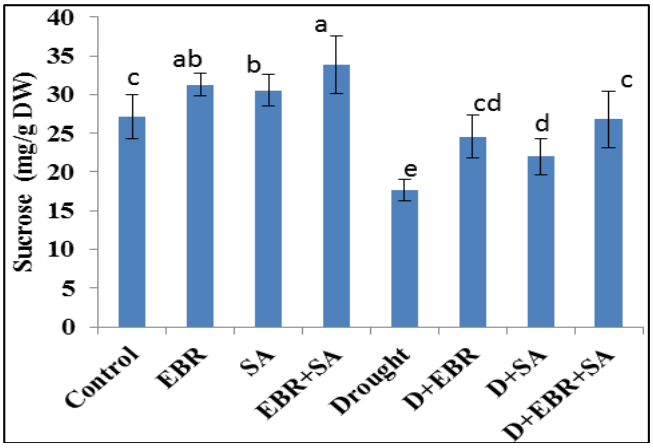

Fig 4: Sucrose content in chickpea

Table 5: Starch content in chickpea

Starch (mg/g DW)		
Control	32.7	2.51
EBR	38.8	1.61
SA	37.7	2.89
EBR+SA	42.1	1.62
Drought	18.7	1.78
D+EBR	28.9	3.91
D+SA	26.1	2.05
D+EBR+SA	32.8	2.81

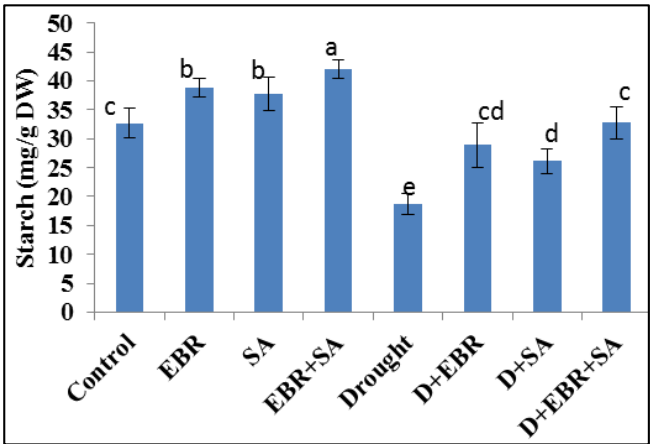

Fig 5: Starch content in chickpea

Table 6: Glucose content in chickpea

	Glucose (mg/g DW)	
Control	18.37	1.81
EBR	23.57	1.25
SA	21.72	0.97
EBR+SA	25.61	2.34
Drought	22.54	1.78
D+EBR	26.98	1.72
D+SA	24.54	1.66
D+EBR+SA	30.21	2.98

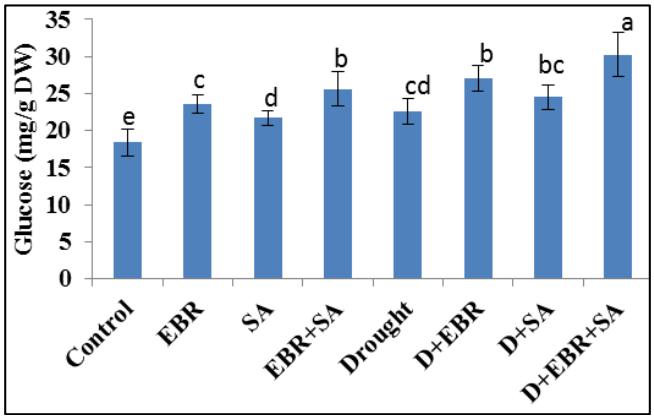

Fig 6: Glucose content in chickpea

Table 7: Fructose content in chickpea

	Fructose (mg/g DW)	
Control	23.51	2.38
EBR	26.62	1.55
SA	27.14	2.51
EBR+SA	31.13	3.28
Drought	34.76	1.23
D+EBR	42.26	2.68
D+SA	40.35	2.28
D+EBR+SA	43.24	3.17

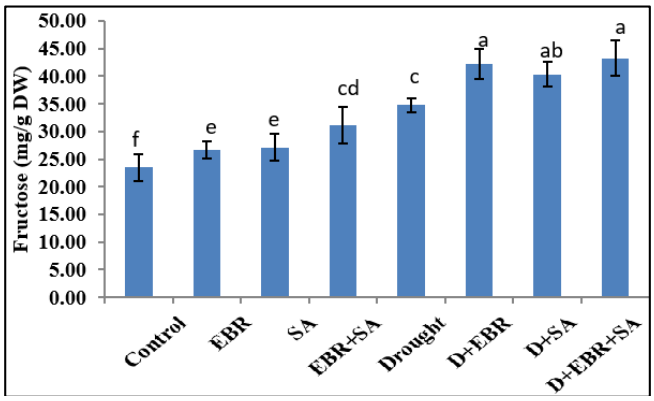

Fig 7: Fructose content in chickpea

Yield components: Effect of EBL and/or SA on yield components of chickpea plants under drought stress and stress free conditions are presented in Table8, 9 and 10.

Number of pods/plant: Drought stress at reproductive stage significantly reduced the number of pods per plant by 41.4% compared to control plants. Exogenous application of EBL and SA alleviated the drought stress effect and improved the number of pods/plant by 62.7% and 50.6% in drought stressed plants in comparison to stress control. Supplementation of EBL and SA together was able to improve the number of pods/plant more significantly (77.67%; 0.0249, $p \leq 0.05$) than their individuals. Unstressed plants treated with EBL and SA alone showed the small improvement in number of pods/plant by 15.6% and 9.3% respectively but not significant. However, combined addition of EBL and SA was significantly enhanced the number of pods/plant (22.2%; 0.0243, $p \leq 0.05$) than their individual applications over unstressed plants, indicating the better effect of combined application.

Number of seeds/pod: A significant reduction (40%; 0.0327, $p \leq 0.05$) in number of seeds/plant was observed in chickpea

plants challenged with drought stress at reproductive stage as compared to control. Foliar application of EBL mitigated the drought stress and significantly improved the number of seeds/plant by 50% in drought stressed plants as compared to stress control. Similarly, about 36.6% of improvement in number of seeds/plant was observed in stressed plants treated with SA over the stress control. However, combined treatment of EBL and SA showed the best effect by increasing the number of seeds/plant (71.8%; 0.0401, $p \leq 0.05$) relative to the plants exposed to drought stress alone. The combined treatment of EBL+SA has more pronounced effect on number of seeds/plant (23.7%; 0.0321, $p \leq 0.05$) in untreated-control plants than their individual treatments reflecting the synergistic effect of number of seeds/plant, relative to the control plants.

100 seed weight: Consequently drought stress significantly decreased the 100 seed weight by 31% (0.0347, $p \leq 0.05$) relative to the control. Application of EBL to stressed plants significantly increased the 100 seed weight by 32% as compared to the stress control. Exogenous SA application also significantly increased the 100 seed weight by 20.7% in drought stressed plants over the stress control plants. However, EBL+SA together synergistically enhanced the 100 seed weight more significantly (43.3%; 0.0228, $p \leq 0.05$) than their individual treatments relative to stress control. Unstressed plants treated with EBL and SA alone application also considerably increased the 100 seed weight by16.7% and 11% relative to the control. Combined treatment of EBL+SA alone recorded the significant enhancement in 100 seed weight by 23.34% (0.0158, $p \leq 0.05$) over the control.

Table 8: Number of pods in chickpea

	Number of pods/plant	
Control	36.41	4.06
EBR	42.1	3.28
SA	39.8	2.23
EBR+SA	44.51	4.75
Drought	21.32	2.78
D+EBR	34.7	3.45
D+SA	32.11	4.24
D+EBR+SA	37.88	5.77

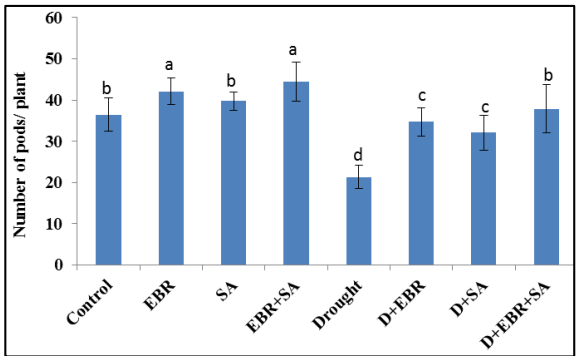

Fig 8: Number of pods in chickpea

Table 9: Number of seeds/pod in chickpea

	No. of seeds/pod	
Control	1.18	0.131
EBR	1.41	0.127
SA	1.33	0.149
EBR+SA	1.56	0.071
Drought	0.71	0.142
D+EBR	1.07	0.178
D+SA	0.97	0.0727
D+EBR+SA	1.22	0.084

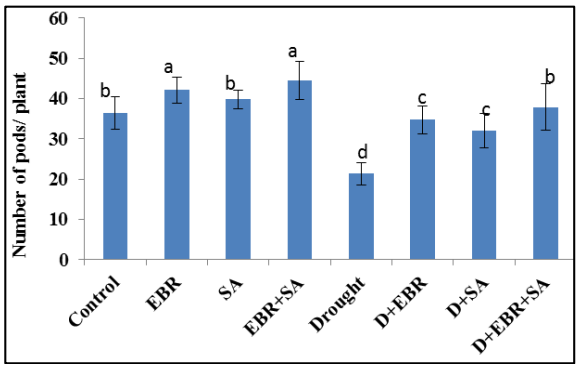

Fig 9: Number of seeds/pod in chickpea

Table 10: 100 seed weight in chickpea

	100 seed weight (g)	
Control	21.12	0.93
EBR	24.65	1.54
SA	23.45	2.81
EBR+SA	28.84	1.24
Drought	15.27	0.89
D+EBR	19.23	1.71
D+SA	17.59	2.61
D+EBR+SA	20.88	1.56

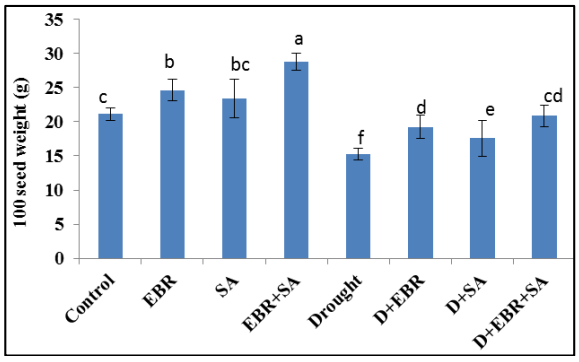

Fig 10: 100 seed weight in chickpea

Conclusion

The present study shows that Chickpea plants under water stress, photosynthetic activity was reduced by effecting enzymes associated with it. But 28-epibassinolide and salycilic acid application increased photosynthetic activity and carbohydrate content even under stress condition. Exogenous application of EBL and SA promotes the growth and development of chickpea plants under different stress conditions. Further research is required for the detailed analysis

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