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Morphological and physio- biochemical changes in response to exogenous application of 24-epibrassinolide and salicylic acid under water stress in chickpea

Sujatha Edupuganti, Lalitha R and S Anuradha

Abstract
The present study shows that Chickpea plants under water stress, unstressed plants treated with exogenous EBL and SA exhibited the significant increase in phsio-biochemical changes over the normal control plants. Co-application of EBL and SA enhanced the plant height in drought stressed plants significantly by 23.76% (0.0345 $p \leq 0.05$) in comparison with stress control than their individual application. Application of EBL and SA to drought stressed plants improved the root length by 11.52% and 15.15% respectively in comparison with stress control. Exogenous application of EBL and SA reversed the drought stress effect on dry mass accumulation. EBL and SA more significantly improved the RWC content (by 57%; 0.0197 $p \leq 0.05$) than their individual applications compared to stress control. No significant effect of EBL and/or SA was observed on H_2O_2 level in chickpea plants in comparison with untreated control. There was no effect on membrane upon exogenous application of EBL and/or SA to untreated plants. About 53.25% (0.0321 $p \leq 0.05$) improvement in soluble protein content was recorded for unstressed plants treated with EBL plus SA when compared with control, indicating the enhanced effect of combined application. Combined EBL+SA alone application was found to be more effectively increased the free proline levels than their individual treatments (58.4%; 0.028 $p \leq 0.05$ vs 26.76%; 0.0462 $p \leq 0.05$ and 17.52%; 0.0561 $p \leq 0.05$ respectively) over the proline levels of unstressed control plants. EBL and SA alone treatments also increased the glycine betaine content considerably in chickpea plants but their combined impact was more on glycine betaine accumulation (23% by EBL, 14.46% by SA and 27.48% by EBL+SA respectively) in comparison with untreated control. Effect of EBL and/or SA on SOD, CAT, POD, APX and GR enzyme activities of chickpea plants under drought stress at reproductive stage and stress free conditions were significantly increased. Co-application of EBL and SA alone exhibited the significant enhancement of AsA levels (39.2%; 0.098 $p \leq 0.05$) than their respective individual treatments compared to the control plants. Combined application of EBL+SA alone accounted for the marked increase in the GSH levels (14.4%) compared to the control plants.

Keywords: Chickpea, 28-epibrassinolide, salicylic acid, water stress, anti-oxidant enzymes

Introduction
Salinity is one of most significant soil related issues representing a few wrecking impacts on plants. Among the various abiotic stresses, salt stress is viewed as one of the genuine dangers to crop production under arid and semiarid areas of the world restricting plant growrh and efficiency (Nazar *et al*., 2011; Kordrostami *et al*., 2016) [11, 7]. Salt stress causes harming impacts on yield profitability by distressing plant metabolism including diminished water potential, particle unevenness and harmfulness consequently prompting harvest disappointment (Krishnamurthy *et al*., 2016) [9]. Salinity disrupts plant morpho-physiological processes due to osmotic disturbance and ionic stress (Vinocur and Altman, 2005) [16]. Resultantly the osmotic disturbance can create a water deficient condition called physiological drought (Munns, 2002). Salt stress can restrict photosynthesis by decreasing green pigments (Sudhir and Murthy, 2004) [15] suppressing rubisco activity (Soussi *et al*., 1998) [14] and reducing stomatal conductance, thus affecting internal CO2 availability (Bethkey and Drew, 1992) [3].

Various agronomic and physiological practices are employed to mitigate adverse effects of salt stress and to induce salt stress tolerance in plants. Application of plant growth regulators is one of promising are evidences, showing exogenously applied growth regulators can improve tolerance in plants to different abiotic stresses such as drought, heavy metal stress as well as salt stress (Krishna, 2003; Anjum *et al*., 2016; Shahzad *et al*., 2018) [8, 13]. Brassinosteroids (BRs) are a new class of phytohormones, play numerous important roles in plant growth and development (Clouse, 1996; Kim *et al*., 2009) [5, 6].

24-epibrassnolide (EBL) an active by-product from brassinolide biosynthesis has ability to stimulate different plant metabolic processes such as photosynthesis (Sairam, 1994) [12], protein and nucleic acid biosynthesis (Bajguz, 2000) [2]. EBL also increases activity of ATPase, and carbon dioxide fixation in maize (Zea mays L.), activities of phosphoenol- pyruvate carboxylase (PEPcase) and ribulose-1,5-bisphosphate carboxylase (RuBPase) and concentration of soluble protein in wheat (Braun and Wild, 1984) [4]. Apart from its role in normal plant growth and development, EBL has anti-stress effects on plants helping to mitigate the adversities of different abiotic stresses including drought, cold, salt and heavy metal stress (Krishna, 2003) [8].

This study evaluates the effect of EBL and SA, individually and in combination, on morphological and physio-biochemical changes 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. Chemical Structure of 24-Epibrassinolide and Salicylic acid.

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* 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-80°C until the analyses were conducted.

Growth parameters

Plant height

Plant height was defined as the shortest distance between the upper boundary of photosynthetic tissues and the soil surface, expressed in cm. This was measured using a retractable measuring tape.

Root length

The plants along with the soil were removed from each pot to get the intact roots and dipped in a bucket filled with tap water. The plants were gently stirred and tapped to remove adhering soil particles. This was followed by washing of roots under running tap water. The length of root was measured by using a meter scale.

Biomass of plant

Total biomass referred to the vegetative above ground tissues as well as root tissues were analysed separately. The washed plants were gently soaked with blotting sheets to remove the

adhering water. The root and shoot of each plant were separated and weighed on an electronic balance to record their respective fresh mass. Plant roots and shoots were subsequently transferred to an oven run at 80 °C and left for 48 h after which they were weighed separately to record their dry mass.

Physiological Indices

Leaf relative water content (RWC)

Leaf relative water content (RWC) was determined in first fully expanded leaves from top in normal and stressed plants. Leaves fresh weight (Fw) was recorded immediately and then incubated in distilled water for at least 4 h at 40 °C in the dark, blotted dried and then turgid weight (Tw) was measured. Finally, dry weight (Dw) was determined after drying at 800C for 48h in the oven. The relative water content (RWC) was calculated with the following formula as described by Jones, 2007.

RWC (%) = [(Fresh weight – Dry weight) / (Turgid weight – Dry weight)] × 100

Stress indices

Hydrogen peroxide: The H_2O_2 content was calculated from a standard curve prepared in similar way (Mukherjee and Choudari 1983).

Lipid peroxidation:

The concentration of malondialdehyde (MDA) was calculated by using extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

Biochemical studies

Extraction and Estimation of Soluble Proteins

To 2.5 ml of ethanol homogenate, 2 ml of 10% (v/v) trichloroacetic acid was added and centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded. The precipitate was dissolved in 5 ml of 1% sodium hydroxide and was centrifuged at 4000 rpm for 10 minutes. The supernatant was used for estimation of proteins by Lowry *et al*., (1951) method.

Free Proline

Proline content was estimated by the method of Bates *et al.* (1973).

Glycine betaine (GB)

Analysis was carried out according to the method of Grieve & Grattan (1983).

Antioxidant enzyme activities

For enzyme extracts, fresh leaf sample (1.0 g, without petiole) was ground with liquid nitrogen and suspended in a potassium phosphate (50 mM, pH 7.5) buffer containing 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 0.2 mM EDTA, 2% (w/v) polyvinyl pyro pyrolidone (PVPP). The homogenate was squeezed through two layers of muslin cloth and centrifuged at 15,000g for 20 min. The resultant supernatant was used for measuring the following enzyme assays (4 mM sodium ascorbate was for ascorbate peroxidase). The amount of protein in the enzyme extract was calculated according to Lowry and others (1951).

Superoxide dismutase: (SOD, E.C 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of NBT (Nitroblue tetrazolium) of Beauchamp and Fridovich (1971). A 3 ml of reaction mixture contained 40 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 0.1 ml of enzyme extract and 2 µM riboflavin. Riboflavin was added at the end. The reaction mixture was exposed to 15 watt fluorescent tubes and the decrease in the absorbance of the reaction mixture was read at 560 nm. Fifty percent inhibition was considered as one enzyme unit.

Catalase: (CAT, E.C.1.11.1.6.) activity was determined following Aebi (1974). The rate of H_2O_2 decomposition at 240 nm was measured spectrophotometrically and calculated using a molar extinction coefficient of 45.2mM^{-1} cm^{-1}. The reaction mixture consisted of 50 mM phosphate buffer, 0.1mM H_2O_2 and enzyme extract. One unit of catalase activity was assumed as the amount of enzyme that decomposed 1 µmol of H_2O_2 per mg of soluble protein per minute at 30 ^{0}C.

Peroxidase: (POD, E.C.1.11.1.7) activity was assayed by employing the procedure of Kar and Mishra (1976). To 0.5 ml of enzyme extract, 2.5 ml of 0.1 M phosphate buffer (pH 7), 1 ml of 0.01 M pyrogallol and 1 ml of 0.005 M H_2O_2 were added. A blank was prepared with 0.5 ml of enzyme extract, 3.5 ml of 0.1 M phosphate buffer and 1 ml of 0.005 M H_2O_2. After 5 minutes of incubation at 25 ^{0}C, the reaction was stopped by adding 1 ml of 2.5 N H_2SO_4. The amount of purpurogallin formed was estimated by measuring the absorbance at 420 nm against a blank. The enzyme activity was expressed as Units mg^{-1} protein.

Ascorbate peroxidase (APX; E.C 1.11.1.11) was assayed by the method of Nakano and Asada (1981). The reaction mixture contained 1.5 ml of 50 mM sodium phosphate buffer (pH 7), 0.2 mM EDTA, 0.5 ml of 0.5 mM ascorbic acid, 0.5 ml 0.5 mM H_2O_2 and 0.5 ml of enzyme sample. The activity was recorded as the decrease in absorbance at 290 nm for 1 minute and the amount of ascorbate oxidized was calculated from the extinction coefficient of 2.6 $mM^{-1}cm^{-1}$.

Glutathione reductase (GR; EC 1.6.4.2) activity was performed according to Jiang and Zhang (2002). The reaction mixture contained 500 µl of sodium phosphate buffer (pH 7.0), 100 µl each of 10 mM GSSG, 1 mM NADPH and 180 µl of distilled water. The reaction was started by addition of enzyme extract and NADPH oxidation was recorded as the decrease in absorbance at 340 nm for 1 min. The activity was calculated using the extinction coefficient of NADPH 6.22 $mM^{-1}cm^{-1}$.

Non-enzymatic antioxidants

Ascorbate (AsA)

Ascorbic acid (AsA) was determined according to Hodges *et al*. (1996).

Estimation of reduced glutathione (GSH)

The levels of GSH (reduced form of glutathione) and GSSG (oxidized form of glutathione) were estimated according to the method of Hissin and Hilf (1976).

Results and Discussion

Plant growth parameters*:* Effect of EBL and/or SA on plant height, root length, plant fresh mass and dry mass of chickpea plants under water limited conditions at reproductive stage are presented in Table 1, 2, 3 Fig 1, 2, 3.

Drought stress at reproductive stage considerable decreased the plant height (17.47%; 0.04837 $p \leq 0.05$) in chickpea plants compared to control. However, exogenous EBL and SA application alleviated the drought stress on height in chickpea plants. Supplementation of EBL to drought stressed plants considerably increased the plant height by 14.3% over the stress control. A marginal improvement in plan height was observed in SA treated droughted plants but not statistically significant. Co-application of EBL and SA enhanced the plant height in drought stressed plants significantly by 23.76% (0.0345 $p \leq 0.05$) in comparison with stress control than their individual application. Exogenous EBL and SA alone application to well-watered plants also improved the plant height by 17.25% (p=0.0486) and 9.83% (p=0.321) respectively compared to the unstressed control. However, application of EBL and SA together significantly improved the plant height by 20.35% (0.0258 $p \leq 0.05$) than their individual treatments when compared with control. Our results showed that no statistical significant effect on plant height when well-watered and drought stressed plants treated with SA application.

Drought stress at reproductive stage increased the root length (17%; 0.0567 $p \leq 0.05$) but not statistical significant as compared to well-watered plants. Application of EBL and SA to drought stressed plants improved the root length by 11.52% and 15.15% respectively in comparison with stress control. However, co-application of EBL and SA increased root length significantly than their individuals by 19.52% (0.0368 $p \leq 0.05$) compared to stress control. Unstressed chickpea plants treated with exogenous EBL and SA alone recorded the enhancement of root length by 15% and 11.12% over the unstressed control. A significant increase in root length (22.42%; 0.0297 $p \leq 0.05$) was observed in normal plants treated with EBL plus SA) than their individual treatments when compared with control.

Total dry matter (shoot+root) was significantly declined (41.7%; 0.0237 $p \leq 0.05$) in water-deficit plants at reproductive stage compared to well-watered plants. However, exogenous application of EBL and SA reversed the drought stress effect on dry mass accumulation. Foliar application of EBL was found to be significantly accumulated the total dry mass (62.7%) in droughted plants over the stress control. Similarly, SA application to drought stressed plants also significantly increased total dry mass by 50.6% as compared to the stress control. Co-application of EBL and SA increased the total dry mass accumulation more significantly than their individuals by 77.67% (0.0406 $p \leq 0.05$) compared to stress control. Unstressed chickpea plants treated with exogenous EBL and SA alone accounted for 15.6% (p=0.0486) and 9.3% (p=0.201) increase in total dry matter over the unstressed control. Combined application of EBL plus SA showed the significant accumulation of total dry matter by 22.2% (0.0367 $p \leq 0.05$) than their individual treatments when compared with control.

Collectively, our results demonstrate that EBL and SA, either alone or together, can improve plant growth under drought stress. However, co-application of EBL and SA could improve growth parameters under drought stress more effectively than individual applications of EBL or SA.

Table 1: Plant Height

	Plant height (cm)	
Control	**45.81**	**2.71**
EBR	53.7	4.28
SA	50.3	5.58
EBR+SA	55.12	2.75
Drought	37.8	3.77
D+EBR	43.2	3.45
D+SA	40.8	4.24
D+EBR+SA	46.78	3.58

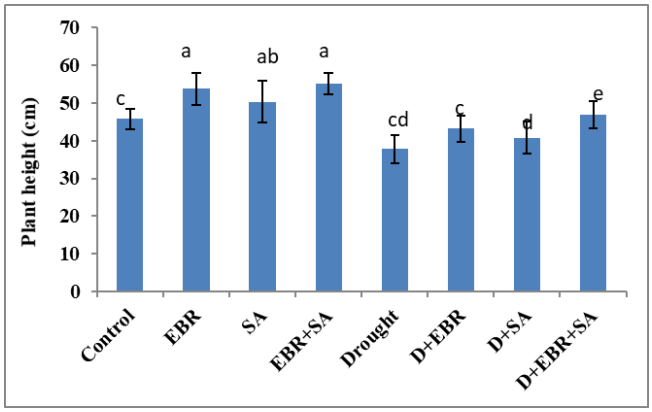

Fig 1: Plant Height

Table 2: Root Length

Root length (cm)		
Control	30.12	2.93
EBR	34.65	1.58
SA	33.47	1.62
EBR+SA	36.87	1.24
Drought	35.25	1.42
D+EBR	39.31	2.21
D+SA	40.59	2.82
D+EBR+SA	42.13	2.11

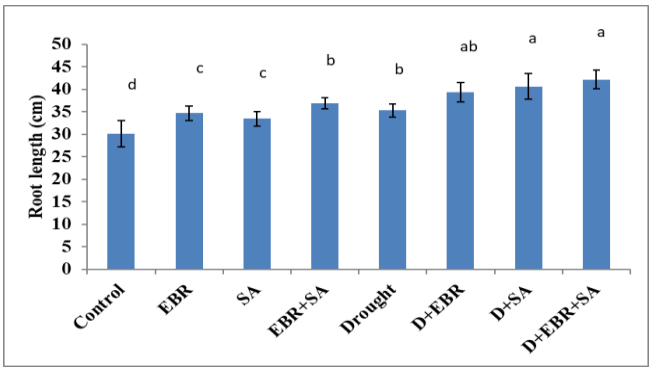

Fig 2: Root length

Table 3: Total dry matter

	Total dry matter (g)	
Control	41.55	2.706
EBR	48.62	3.276
SA	44.87	2.225
EBR+SA	51.22	2.675
Drought	24.22	3.28
D+EBR	39.57	3.445
D+SA	37.53	4.241
D+EBR+SA	42.45	2.577

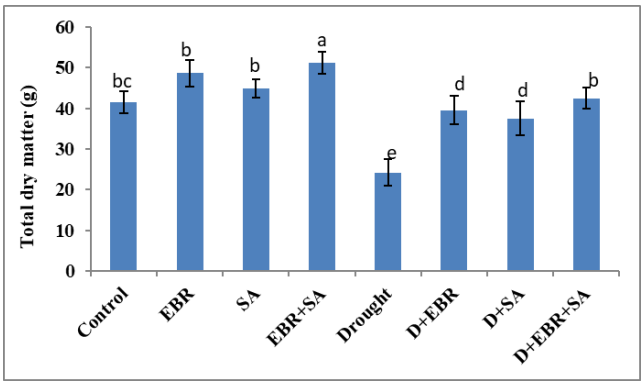

Fig 3: Total dry matter

Physiological Indices

Relative water content (RWC): About 37.42% (0.0358 $p \leq 0.05$) decrease in RWC was recorded in drought stressed plants compared to control. However, exogenous application of EBL and SA individually improved the RWC significantly by 55% and brought near to the control levels. Similarly, exogenous SA application also improved the RWC by 49.4% in chickpea plants under drought stress over stress control. Co-application of EBL and SA more significantly improved the RWC content (by 57%; 0.0197 $p \leq 0.05$) than their individual applications compared to stress control. Exogenous EBL and /or SA application also maintained the RWC content in the control plants Table 4, Fig 4.

Table 4: Relative Water Content

	RWC (%)	
Control	80.91	3.12
EBR	83.57	2.27
SA	82.45	1.27
EBR+SA	87.34	3.68
Drought	50.63	4.81
D+EBR	78.44	5.29
D+SA	75.65	3.77
D+EBR+SA	79.41	2.88

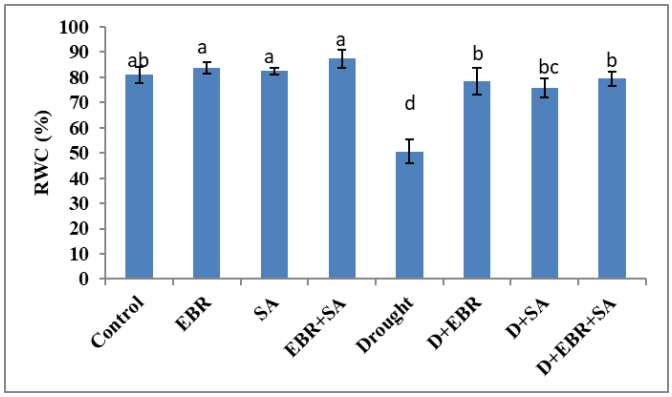

Fig 4: Relative Water Content

Stress Indices

Effect of EBL and/or SA on hydrogen peroxide (H_2O_2), malondialdehyde (MDA) of chickpea plants under drought stress at reproductive stage and stress free conditions are presented in Table. 5, 6 Fig 5, 6.

H_2O_2 levels: H_2O_2 level indicates the severity of oxidative stress in a plant tissue under stress conditions. Data showed that the chickpea plants suffered from oxidative stress as evidenced by steep increase in H_2O_2 content (79.6%, 0.0320 $p \leq 0.05$) compared to the control. Drought stressed plants treated with EBL had lowered the H_2O_2 levels significantly by 25.6% compared to stress control. Similarly, exogenous SA declined the H_2O_2 level by 17.7% in chickpea plants under drought stress over stress control. Moreover, co-application of EBL and SA reduced the H_2O_2 level by 30% (0.0271 $p \leq 0.05$), reflecting that co-application of EBL and SA has a more significant effect than their individual applications on the H_2O_2 level in drought stressed chickpea plants. No significant effect of EBL and/or SA was observed on H_2O_2 level in chickpea plants in comparison with untreated control.

MDA content: Membrane damage is evaluated by measurement of MDA levels showed the significant increase (46.3%; 0.0427 $p \leq 0.05$) in chickpea plants challenged with water-deficit stress at reproductive stage compared to control plants. Exogenous application of EBL and SA to stressed plants was able to reduce the MDA content by 24.6% and 13.2% respectively over the stress control. Drought stressed chickpea plants co-applied with EBL and SA showed significant reduction in MDA content (26.56%; P=0.0281) when compared with stress control suggesting that co-application has a more significant effect than their individual applications. There was no effect on membrane upon exogenous application of EBL and/or SA to untreated plants.

Table 5: H_2O_2 Content

	H_2O_2 (µmol/ gFW)	
Control	12.6	1.26
EBR	14.7	0.84
SA	13.1	2.88
EBR+SA	14.2	1.28
Drought	22.6	2.17
D+EBR	16.8	0.80
D+SA	18.6	1.55
D+EBR+SA	15.8	0.83

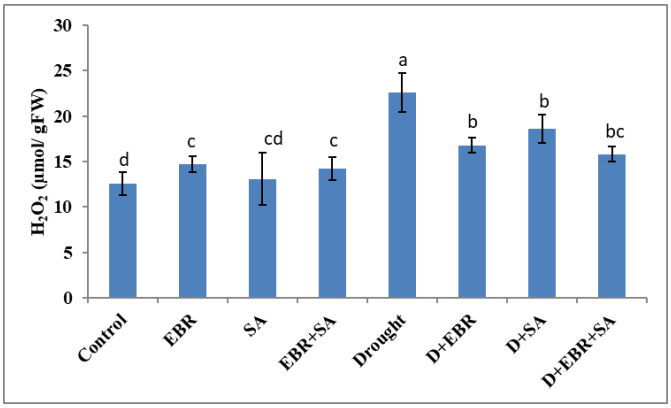

Fig 5: H_2O_2 Content

Table 6: MDA Content

	MDA (nmol/ gFW)
Control	562.8
EBR	498.6
SA	474.8
EBR+SA	552.2
Drought	823.7
D+EBR	621.1
D+SA	715.2
D+EBR+SA	604.9

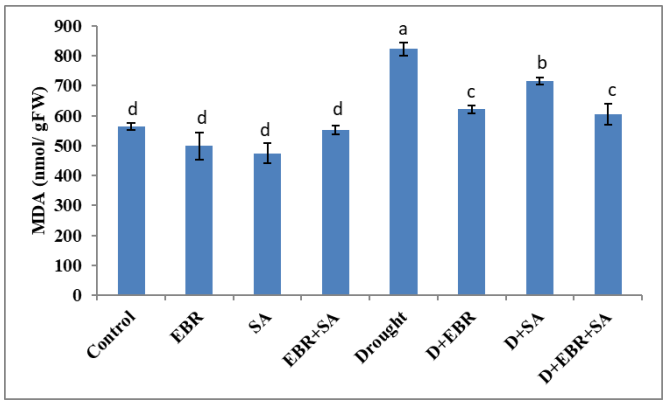

Fig 6: MDA Content

Total soluble protein content: Effect of EBL and/or SA on the soluble protein content in chickpea plants under drought stress at reproductive stage and stress free conditions are presented in Table. 7, Fig 7.

A significant increase in soluble protein content (55.46%; 0.0197 $p \leq 0.05$) was noted in water-deficit plants at reproductive stage as compared to well-watered plants. However, exogenous application of EBL and SA revered the drought stress effect on soluble protein and improved near to control. EBL application significantly increased the soluble protein by 110.25% in droughted plants over the stress control. Similarly, SA application to drought stressed plants also accounted for significant increase in the soluble protein by 85.4% as compared to the stress control. Moreover, co-application of EBL and SA increased the soluble protein more significantly than their individuals by 141.61% (0.0254 $p \leq 0.05$) compared to stress control. Unstressed chickpea plants treated with exogenous EBL and SA alone accounted for 35.96% and 19.09% increase in soluble protein levels over the unstressed control. About 53.25% (0.0321 $p \leq 0.05$) improvement in soluble protein content was recorded for unstressed plants treated with EBL plus SA when compared with control, indicating the enhanced effect of combined application.

Table 7: Total soluble protein content

Control	7.23	0.277
EBR	9.83	0.775
SA	8.61	0.235
EBR+SA	11.08	0.415
Drought	3.22	0.159
D+EBR	6.77	0.524
D+SA	5.97	0.313
D+EBR+SA	7.78	0.632

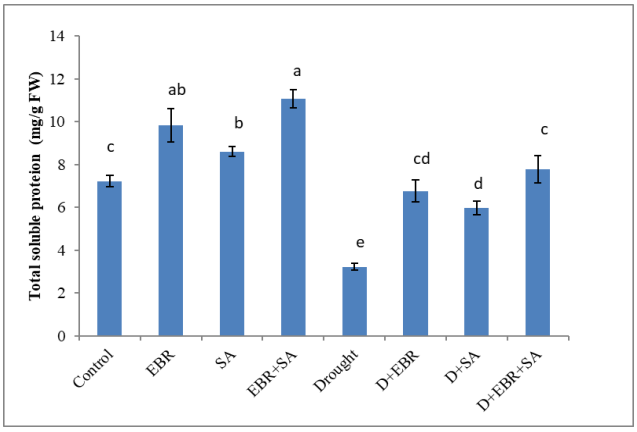

Fig 7: Total soluble protein content

Osmolyte levels: Effect of EBL and/or SA application on the proline and glycine betaine accumulation in leaves of drought stressed chickpea plants is presented in Table 8, 9 Fig 8, 9.

Drought stressed chickpea plants showed a sharp increase in free proline levels by 65% compared to control plants. Fallow up treatment with EBL and SA by foliar spraying led to further enhancement of free proline levels in stressed as well as stress free plants. Drought stressed plants receiving EBL supplementation showed significant enhancement of proline levels by 23.3% over stressed control. Similarly, SA alone supplementation also showed the considerable increase in proline levels (by 17.26%) over the stress control. However, co-application of EBL and SA to drought stressed plants accounted for significant enhancement of free proline levels (37.46%; 0.0311 $p \leq 0.05$) than their individual applications over the stressed control. Plants fed with EBL and SA alone also showed significant elevated free proline levels. Combined EBL+SA alone application was found to be more effectively increased the free proline levels than their individual treatments (58.4%; 0.028 $p \leq 0.05$ vs 26.76%; 0.0462 $p \leq 0.05$ and 17.52%; 0.0561 $p \leq 0.05$ respectively) over the proline levels of unstressed control plants.

A significant accumulation of glycine betaine content (29.32%; 0.0309 $p \leq 0.05$) was noticed in chickpea plants subjected to drought stress at reproductive stage. Exogenous application of EBL significantly increased the glycine betaine content by 18.64% over the stressed control. Individual application of SA also exhibited considerable improvement in glycine betaine content but not significantly (16.41%; p=0.0612) compared to the drought stressed plants. However drought stressed plants treated with EBL+SA exhibited the accumulation of glycine betaine content by 38.63%, reflecting that co-application of EBL and SA has a more significant effect than their individual applications on the improvement of glycine betaine content in drought stressed chickpea plants. EBL and SA alone treatments also increased the glycine betaine content considerably in chickpea plants but their combined impact was more on glycine betaine accumulation (23% by EBL, 14.46% by SA and 27.48% by EBL+SA respectively) in comparison with untreated control.

Table 8: Proline Content

	Proline (µmol/ gFW)	
Control	4.11	0.25
EBR	5.21	0.65
SA	4.83	0.77
EBR+SA	6.51	0.75
Drought	6.78	0.68
D+EBR	8.36	0.38
D+SA	7.95	0.68
D+EBR+SA	9.32	0.47

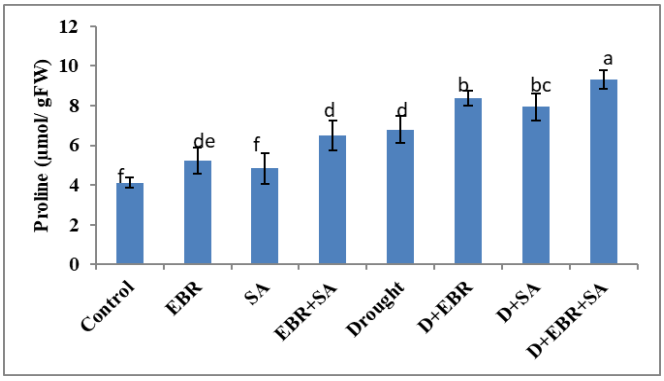

Fig 8: Proline Content

Table 9: Glycine betaine content

	Glycine betaine (µmol/ g FW)	
Control	10.37	1.04
EBR	12.75	0.75
SA	11.87	1.54
EBR+SA	13.22	1.65
Drought	13.41	1.24
D+EBR	15.91	0.85
D+SA	15.61	1.01
D+EBR+SA	18.59	0.97

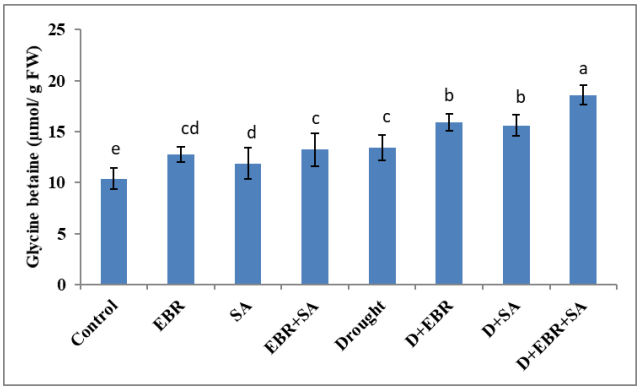

Fig 9: Glycine betaine content

Antioxidative enzyme activities: Effect of EBL and/or SA on SOD, CAT, POD, APX and GR enzyme activities of chickpea plants under drought stress at reproductive stage and stress free conditions are presented in Table 10, 11, 12, 13, 14; Fig 10, 11, 12, 13, 14.

Superoxide dismutase (SOD): Chickpea plants challenged with terminal drought stress showed a significant increase in SOD activity by 47.8% (0.0374 $p \leq 0.05$) in comparison to control. EBL application to drought stressed chickpea plants further significantly enhanced the SOD activity (27.3%) compared to drought stressed plants. Application of SA also caused the significant enhancement of SOD activity by 39% in drought stressed plants over the drought-treatment alone. About 60% (0.0237 $p \leq 0.05$) enhancement of SOD activity was observed with combined treatment of EBL and SA, suggesting that co-application has a more significant effect than their individual applications on the SOD activity in drought stressed chickpea plants compared to drought-treatment. Individual application of EBL and SA as well as their co-application to unstressed plants also exhibited the significant increase in SOD activity by 21.7%, 24% and 28.1% respectively over the control. Between the individual treatments SA alone induced the more SOD activity than the EBL treatment in stressed and unstressed control plants.

Catalase (CAT): Drought stress increased the CAT activity in chickpea plants but not significantly (p=0.0642) over the control plants. EBL application to drought stressed chickpea plants further enhanced the CAT activity by 12.42% compared to drought stressed plants. A significant enhancement in CAT activity (by 29.4%; 0.0176 $p \leq 0.05$) was observed in drought stressed plants upon SA treatment over the stress control. About 32.8% (0.0367 $p \leq 0.05$) enhancement of CAT activity was observed with combined treatment of EBL and SA, suggesting that co-application has a more significant effect than their individual applications on the CAT activity in drought stressed chickpea plants compared to drought-treatment. A small increase in CAT activity was observed in unstressed plants treated with EBL and SA alone over the control. Combination of EBL plus SA treatments to unstressed plants significantly enhanced the CAT activity by 36.74% in comparison with that of un-stressed plants.

Peroxidase (POD): A significant increase in POD activity (33.1%) was noted in plants gown in water-limited conditions at reproductive stage compared to control. Further enhancement of POD activity was observed upon foliar spray of EBL and SA by 22.5% and 17.8% respectively in drought stressed plants over the stress control. Moreover, co-application of EBL and SA increased the POD activity more significantly than their individuals by 55.7% (0.0097 $p \leq 0.05$) compared to stress control. Unstressed chickpea plants treated with exogenous EBL and SA alone accounted for 44.47% and 50.4% increase in POD activity over the unstressed control. About 73.8% (0.0401 $p \leq 0.05$) improvement in POD activity was also recorded for unstressed plants treated with EBL plus SA when compared with control.

Ascorbate peroxidase (APX): Water-deficit stress at reproductive stage reduced the APX activity (16%; 0.0552 $p \leq 0.05$) considerably in chickpea plants over the unstressed control. Foliar application of EBL and SA to drought stressed plants marginally increased the GR activity in comparison with stress control. On the other hand, combination of EBL+SA was found to be significantly enhanced the APX activity by 23.7% (0.0298 $p \leq 0.05$) in drought stressed plants compared to stress control, suggesting the combined effect on the APX activity. Plants fed with EBL and SA alone also showed considerable improvement in APX activity. Combined EBL+SA alone application was found to be more effectively increased the APX activity than their individual treatments (29.7%; 0.0328 $p \leq 0.05$ vs 12.7%; 0.0561 $p \leq 0.05$ and 17.3%; 0.0468 $p \leq 0.05$ respectively) over the unstressed control plants.

Glutathione reductase (GR): When plants challenged with terminal drought stress exhibited a maked increase in GR activity by 20.7% (0.0416 $p \leq 0.05$) in comparison to control. EBL application to drought stressed chickpea plants further significantly enhanced the SOD activity (20.6%) compared to drought stressed plants. Application of SA also caused the significant enhancement of SOD activity by 30.3% in drought stressed plants over the drought-treatment alone. About 41.7% (0.0127 $p \leq 0.05$) enhancement of CAT activity was observed with combined treatment of EBL and SA, indicating that co-application has a more significant effect than their individual applications on the SOD activity in drought stressed chickpea plants compared to drought-treatment. Individual application of EBL and SA as well as their co-application to unstressed plants also exhibited the significant increase in SOD activity by 23.8%, 18% and 28% respectively over the control.

Table 10: SOD Content

	SOD (U/mg protein/min)	
Control	23.51	2.38
EBR	28.618	1.546
SA	29.142	2.506
EBR+SA	30.132	3.276
Drought	34.762	1.225
D+EBR	44.258	2.675
D+SA	48.348	2.28
D+EBR+SA	55.624	3.165

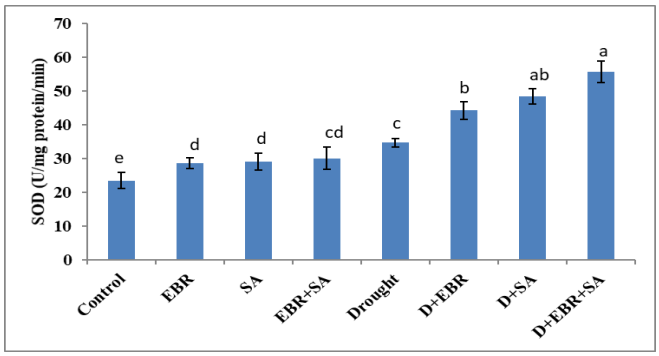

Fig 10: SOD Content

Table 11: CAT Content

	CAT (U/mg protein/min)	
Control	27.49	2.706
EBR	30.38	3.276
SA	32.09	2.225
EBR+SA	37.59	2.675
Drought	31.62	3.28
D+EBR	35.55	3.445
D+SA	40.91	4.241
D+EBR+SA	47.06	2.577

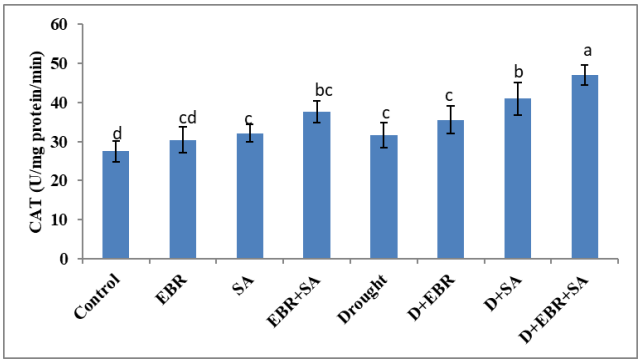

Fig 11: CAT Content

Table 12: POD Content

	POD (U/mg protein/min)	
Control	3.17	0.277
EBR	4.58	0.775
SA	4.77	0.235
EBR+SA	5.51	0.415
Drought	4.22	0.159
D+EBR	5.17	0.524
D+SA	4.97	0.313
D+EBR+SA	6.57	0.632

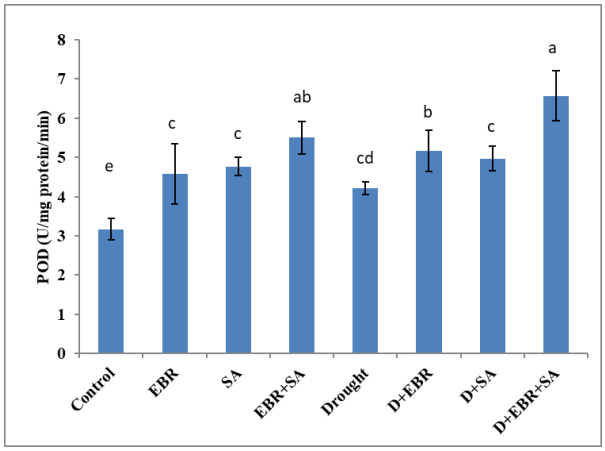

Fig 12: POD Content

Table 13: APX Content

	APX (U/mg protein/min)	
Control	12.82	1.18
EBR	14.39	1.09
SA	15.04	0.73
EBR+SA	16.63	1.21
Drought	14.86	1.7
D+EBR	15.21	1.4
D+SA	16.32	1.58
D+EBR+SA	18.39	0.55

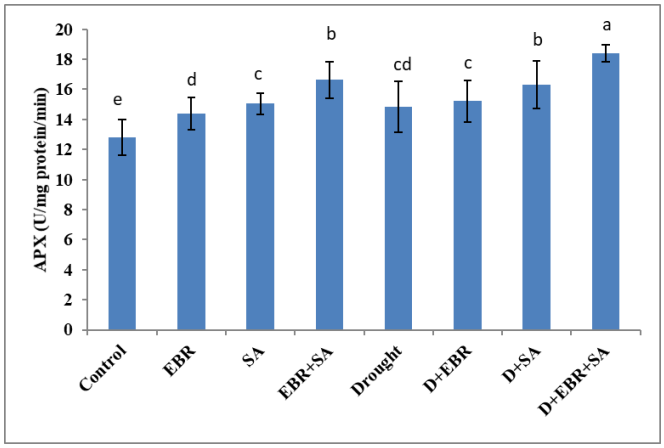

Fig 13: APX Content

Table 14: GR Content

	GR (U/mg protein/min)	
Control	0.478	0.0244
EBR	0.592	0.0825
SA	0.564	0.0285
EBR+SA	0.612	0.0778
Drought	0.577	0.0377
D+EBR	0.696	0.025
D+SA	0.752	0.0157
D+EBR+SA	0.818	0.0909

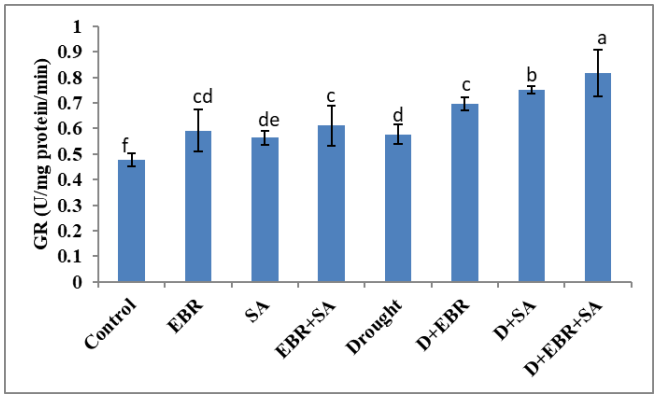

Fig 14: GR Content

Cellular antioxidant profiles of under drought stress. Effect of EBL and/or SA on the cellular AsA and GSH levels of chickpea plants under drought stress at reproductive stage and stress free conditions are presented in Table. 15, 16; Fig 15, 16.

About 32.14% increase in AsA levels was noted in chickpea plants grown under water limited conditions over the control. Application of EBL to drought stressed chickpea plants further significantly enhanced the AsA levels (28.8%) compared to drought stressed plants. Similarly, supplementation of SA to chickpea plants growing under

drought stress was found to raise the AsA levels by 19.8% compared to the control. Drought stressed plants treated with both EBL +SA showed the significant improvement in AsA levels by 47.3% (0.0125 $p \leq 0.05$) suggesting that co-application has a more significant effect than their individual applications on the improvement of AsA content in drought stressed chickpea plants. EBL and SA alone treatments increased the cellular AsA pool by 25% and 17.26% respectively compared to control. However, co-application of EBL and SA alone exhibited the significant enhancement of AsA levels (39.2%; 0.098 $p \leq 0.05$) than their respective individual treatments compared to the control plants.

A significant increase in cellular GSH content (28.5%; 0.0341 $p \leq 0.05$) was observed in chickpea plants under drought stress in comparison with control plants. Exogenous application of EBL and SA alone to drought stressed plants further enhanced the GSH levels considerably by 13.2 and 18.4% over the stressed plants. However, drought stressed plants receiving the both EBL+SA treatments together was exhibited the significant enhancement of GSH levels by 21.8% (0.0437 $p \leq 0.05$) over the stressed control. Our results indicate that EBL and SA co-application can improve the GSH levels more significantly than their independent treatments under drought stress. Supplementation of EBL and SA alone improved the GSH levels but not significantly. Whereas, combined application of EBL+SA alone accounted for the marked increase in the GSH levels (14.4%) compared to the control plants.

Table 15: AsA Content

	AsA	
Control	168	9.012
EBR	210	10.033
SA	197	10.045
EBR+SA	234	13.01
Drought	222	11.221
D+EBR	286	15.079
D+SA	266	18.118
D+EBR+SA	327	15.054

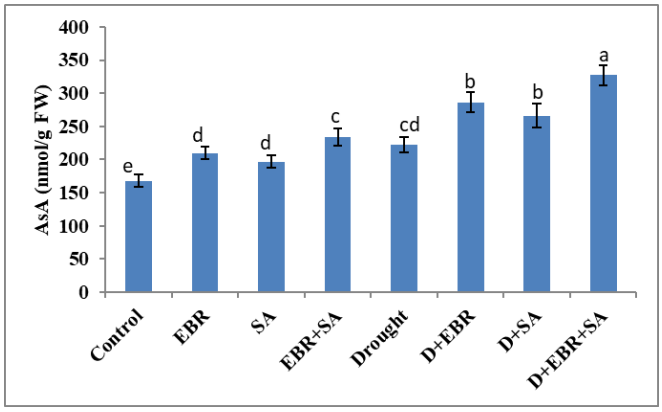

Fig 15: AsA Content

Table 16: GSH Content

	GSH	
Control	431	17.16
EBR	477	20.01
SA	461	12.48
EBR+SA	493	35.57
Drought	554	16.77
D+EBR	627	18.89
D+SA	656	17.98
D+EBR+SA	675	27.17

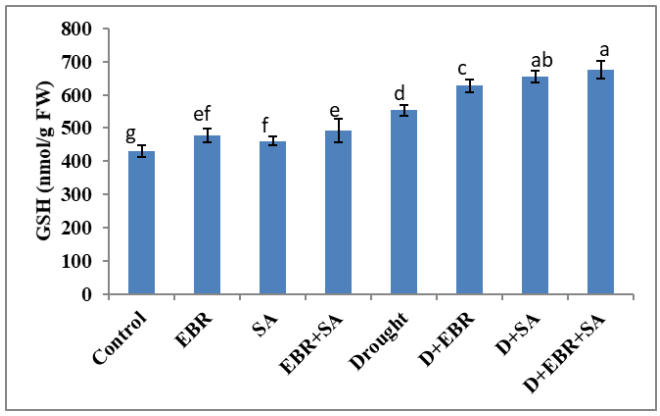

Fig 16: GSH Content

Conclusion

The present study shows that Chickpea plants under water stress, morphological and physio- biochemical changes was reduced by effecting enzymes associated with it. But 28-epibassinolide and salycilic acid application increased morphological and physio- biochemical changes 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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