Impact of 24-Epibrassinolide and Salicylic Acid on Nitrogen Metabolism in Chickpea Subjected to Water Stress

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ABSTRACT

The present study shows that Chickpea plants under water stress, unstressed plants treated with exogenous EBL and SA exhibited the significant increase in nodule fresh mass and dry mass by over the normal control plants. Whereas co-application of EBL and SA was more significantly increased the nodule fresh mass and dry mass by 23% (0.0281, $p \leq 0.05$) and 33.3% (0.0391, $p \leq 0.05$) compared with control than their respective individuals. Co-application of EBL and SA was showed the considerable effect on N_2ase activity (16%; $p=0.0412$) than their individual applications over unstressed plants. Co-application of EBL and SA was more effective (28.2%; 0.0265, $p \leq 0.05$) over unstressed control than their individual applications in enhancing NR activity in control plants. About 48.3% (0.0187, $p \leq 0.05$) of root NiR activity was recorded in plants supplemented with EBL+ SA, suggesting that co-application of EBL and SA has a more significant effect than their individual applications in comparison to control. Plants treated with EBL+ SA showed a marked increase in GS activity (18%; 0.0471 $p \leq 0.05$) over unstressed control reflecting the combined effect on modulation of GS activity than their individual applications. Unstressed plants treated with exogenous EBL and EBL+SA exhibited the significant increase in GOGAT activity by 17% and 24.6% respectively over the normal control plants. SA application was able induce the GOGAT activity marginally over the control plants. Exogenous EBL and SA alone and their combination application to unstressed plants exhibited small enhancement in NADH-GDH activity but not statistically significant over the control. Exogenous SA alone application was found to be marginally enhanced the NO_3^- and NO_2^- accumulation over the control plants. On the other hand, when plants treated with EBL+SA showed a significant accumulation of NO_3^- (28.4; 0.0256 $p \leq 0.05$) and NO_2^- (36%; 0.0189 $p \leq 0.05$) levels than their individual application compared to normal control. Co-application of EBL and SA was more effective (28.2%; 0.0265, $p \leq 0.05$) over unstressed control than their individual applications in enhancing NR activity in control plants. Nitrogen metabolism was reduced by effecting enzymes associated with it. But 28-epibassinolide and salycilic acid application increased nitrogen metabolism 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.

KEYWORDS: Chickpea, 28-Epibrassinolide, Nitrogen Metabolism, Water stress

INTRODUCTION

In the present period, water shortage is one of the principle natural difficulties for plants, which impactsly affects their development and improvement Reddy et al (2004), Tanveer et al (2019). The decrease of water accessibility to plants causes physiological imbalances which eventually diminishes plant productivity Fahad et al (2017). The effect of drought stress on the physiological reactions of plants is reliant upon the dimension of drought, its introduction time and the development phase of plants Okçu et al (2005). Drought stress initiates phytotoxicity by improving collection of reactive oxygen species (ROS) in the plant cells, which is fundamentally because of the awkwardness between ROS age and their searching Cruz de Carvalho (2008). Expanded centralizations of ROS during drought conditions contrarily influence the photosynthetic responses by disturbing the photosynthetic device, including response focuses and chloroplast structures Meng et al (2014), Shao et al (2016), Chen et al (2017), Ying et al (2013). Moreover, upgraded ROS collection supports the debasement of chlorophyll molecules lastly decays the photosynthetic presentation of plants submerged deficiency conditions Cao et al (2015), Zhang et al (2018)

As plants are exposed to various biotic and abiotic factors, they possess an inbuilt system, known as antioxidant system, to regulate the biological processes under adverse environmental conditions. This antioxidant system is comprised of enzymatic and non-enzymatic antioxidants, which work in a systematic manner to control the levels of ROS in plant cells Gill and Tuteja (2010). However, under severe stress conditions, such as high drought levels, this antioxidant system is disrupted, leading to an imbalance in redox homeostasis in plant cells Talaat et al (2015), Murshed et al (2013), Noctor et al (2014).

Plant growth regulators are multifunctional molecules which are well known for their physiological functions in plants Bari and Jones (2009), Kachroo and Kachroo (2007), Fan et al (2018), Guo et al (2017) These molecules also play an important role in providing resistance to plants growing under abiotic stresses, such as heavy metals, temperature, pesticides and drought Tanveer et al (2018).

This study evaluates the effect of EBL and Si, individually and in combination, on nitrogen metabolism in chickpea subjected to water stress.

MATERIALS AND METHODS

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^{o}C until the analyses were conducted.

Nitrogen Metabolism

Nodule number, nodule fresh weight and nodule dry weight

The plant roots were washed with tap water to remove the adhering soil. Nodules formed were visually observed for their size and colour. Symbiotic development by *Rhizobium* was estimated by counting total nodule number per root system.The number of nodules per plant was counted in three replicate and the values averaged to give the number of nodules per plant and measured fresh weight of nodules with the help of electrical balance and kept in an oven at 50°C for about 48 hrs still constant weights.

Leghemoglobin (LHb) in nodules

The nodules were detached immediately after sampling and their LHb concentration was determined by the method of Sadasivam and Manickam (1992), which is based upon the conversion of hematin to pyridine hemochromogen.

Extraction

500 mg of fresh nodular tissue was homogenized in 5 ml of 0.05 M phosphate buffer (pH 7.0). The homogenate was filtered through two layers of cheesecloth. The nodule debris was discarded, and the turbid reddish brown filtrate was centrifuged at 10,000g for 30 minutes.

Procedure: A 3 ml aliquot of alkaline pyridine reagent was added to 3 ml of nodule extract and mixed well; the solution became greenish yellow due to the formation of hemochrome. The hemochrome was divided equally into two test tubes. To one test tube, a few crystals of potassium hexacyanoferrate were added to oxidize the hemochrome, and the absorbance was measured at 539 nm using a spectrophotometer (Spectronic 20D, Milton Roy, USA). To the other test tube, a few crystals of sodium dithionite were added to reduce the hemochrome, and the absorbance of this mixture was measured at 556 nm after an interval of 25 minutes. The leghemoglobin content (mM) was calculated using the following formula:

Lb concentration (mM)= A556-A539/23.4× 2D

where D is the initial dilution and A556 and A539 are the absorbance at 556 and 539 nm, respectively.

Nitrogenase (EC 1.18.6.1) activity in the nodules

Nitrogenase activity in nodules was measured using the acetylene reduction method as outlined by Herdina and Silsbury (1990). The nitrogen-fixing complex (nitrogenase) of legumes is able to reduce C_2H_2 to C_2H_4. The assay was performed immediately after harvesting the plants. The nodulated roots were cut from the base and shaken slowly in water to remove the attached soil particles and was incubated at room temperature in vials containing acetylene (C_2H_2 10 percent, v/v) in air and sealed with serum caps. The samples were flushed with acetylene gas by gently shaking the bottles and were incubated for 1 hr. The sample of 1ml of gas from the incubation mixture was analyzed for ethylene in a Shimadzu gas chromatograph equipped with a Porapak R column (Ligero *et al.,* 1986). Although the use of such a "closed" system for measuring acetylene reduction does create problems related to an acetylene induced decline in nitrogenase activity (Minchin *et al.,* 1983), it is still useful for comparative purposes, especially when the assay time is short (Vessey, 1994). From the standard values, the number of moles of ethylene produced in each case was calculated and the rate of ARA was calculated as number of C_2H_4 moles produced per mg dry weight of nodules per hr (nmol C_2H_4 mg^{-1} nodule DW. hr^{-1}).

Extraction and assay of nitrate reductase and nitrite reductase activity

Approximately 0.5 g of the frozen root material was ground into a fine powder in an ice bath. The powder was extracted in 4 ml of ice-cold extraction buffer containing 25 mM potassium phosphate buffer (pH 7.5), 5 mM cysteine and 5 mM EDTA-Na_2. The extract was centrifuged at 4,000 rpm for 15 min at 4°C.

Nitrate reductase (E.C.1.6.6.1) was determined following the *in vivo* method described by Jaworski (1971). Roots from different treatments were taken separately and were cut into small pieces. About 0.5 gm of root material was incubated in the medium containing 1 ml of 1 M potassium nitrate, 2 ml of 0.5% Triton X-100 for 1 hour, in dark under anaerobic conditions. After one-hour, 1 ml reaction mixture was transferred to another test tube and mixed with 1 ml of 1% sulfanilamide in 2N hydrochloric acid and 1 ml of 0.2% NEEDA (N-Cl-napthylethylene diamide dihydrochloride). 1 ml sulfanilamide and 1 ml NEEDA served as blank. The absorbance was read at 540 nm in SCHIMADZU UV-1800 Spectrophotometer. Standard curve was prepared with the help of different concentrations of potassium nitrite and enzyme activity was expressed as micromoles of NO_2 liberated h^{-1} g^{-1} fresh weight.

Nitrite reductase (NiR; EC 1.7.2.1) activity was measured as a reduction in the amount of NO_2^- in the reaction mixture according to Miflin (1967) with slight modifications. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.8), 0.4 mM $NaNO_2$, 2.3 mM methyl viologen, enzyme extract. The reaction was started by addition of 4.3 mM sodium dithionite in 100 mM $NaHCO_3$. The reaction was incubated for 30

min at 27°C and was stopped by vigorously mixing the content of the assay tube on a vortex mixer until the methyl viologen was completely oxidized and boiling for 1 min. The concentration of NO_2^- remaining in the reaction mixture was determined at 540 nm after reaction with SA and NEDD as described above using a standard curve of known $NaNO_2$ concentrations. One unit of NiR activity is defined as 1 mM NO_2^- reduced mg^{-1} protein h^{-1}.

Extraction and estimation of Glutamine synthetase (GS), Glutamine (amide) 2-oxoglutarate aminotransferase: (GOGAT) and NADH specific Glutamate dehydrogenase (NADH-GDH) enzymes

About 0.3 g frozen root were powdered in liquid N_2 and homogenized with 6 ml 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM Mg^{2+}, 2 mM DTT, and 0.4 M sucrose. The homogenate was centrifuged at 8000 rpm for 10 min at 4 ºC. The reactions were performed in 3 ml (final volume) of the media indicated below.

NADH specific glutamate dehydrogenase/L-Glutamate NAD-oxidoreductase (NADH-GDH; EC 1.4.1.2). NADH-GDH activities were determined by the mehod of Loulakakis and Roubelakis-Angelakis (1990). The 3 ml reaction mixture consist of 300 µmol Tris-HCl buffer (pH 8.0), 600 µmol ammonium chloride, 3 µmol calcium chloride, 0.6 µmol NADH, and 0.1 ml enzyme. The reaction was started by adding enzyme extract and carried out at 30 ºC. The absorbance at 340 nm was monitored for 300 s, and the activity of GDH was expressed as nmol NADH·mg^{-1} Pro·min^{-1}.

Glutamine synthetase (GS; EC 6.3.1.2)

The enzyme activity was determined as per the method of O'Neal and Joy, (1973) based on the formation of γ- glutamylhydroxamate. The incubation mixture contained in a total volume of 3 ml: 0.6 ml of imidazole-muriatic acid buffer (0.25 M, pH 7. 0), 0.4 ml of glutamic acid-Na (0.30 M, pH 7.0), 0.4 ml of ATP-Na (30 M, pH 7.0) and 0.2 ml of $MgSO_4$ (0.5 M) and 1.2 ml of extract. The mixture was incubated for 5 min at 25°C. Subsequently, 0.2 ml of hydroxylamine hydrochloride (a 1:1 mixture of 1 M hydroxylamine hydrochloride and 1 M HCl) was added, and the reaction was incubated for 15 min at 37°C. The reaction was terminated by adding 0.8 ml of acidic $FeCl_3$ (2% (W/V) in TCA and 3.5% (W/V) $FeCl_3$ in 2% HCl). The reaction mixture was centrifuged at 4,000 rpm for 15 min to remove precipitated proteins. The colour of ferric hydroxymate was measured at 540 nm. The amount of γ- glutamylhydroxamate formed was determined through a comparison with a standard curve that was generated after measuring authentic glutamylhydroxamate in the presence of all assay components. One unit of GS activity was determined as the amount of enzyme required to catalyze the formation of l µM γ-glutamylhydroxamate (GH)/min under the present conditions.

Glutamate synthase/ (Glutamine (amide) 2-oxoglutarate aminotransferase: (GOGAT; EC: 1.4.1.13) activity was measured according to the methods of Singh and Srivastava (1986). Glutamate synthase activity was assayed at 30°C. In a 3ml final volume of reaction mixture consist of 10 µmol a-ketoglutarate, 1 µmol potassium chloride, 37.5 µmol Tris-HCl buffer (pH 7.6), 0.6 µmol NADH, 8 µmol L-glutamine and 0.3 ml enzyme. The absorbance of initial rate of oxidation of NADH was monitored for 300s at 340 nm. The activity of GOGAT was estimated using the molar extinction coefficient of NADH (6.22 $mM^{-1}\cdot cm^{-1}$), and expressed as nmol NADH·mg^{-1} Pro·min^{-1}.

Nitrate

Root samples were dried in an oven at 70 °C until constant weight was obtained. The dried material was ground to a powder and samples of 200 mg were suspended in 10 ml of deionized water. The suspensions were incubated at 45°C for 2 h. After mixing, the samples were centrifuged at 5,000 g for 15 minutes,0.2 ml of supernatant was mixed thoroughly with 0.8 ml of 5% (w/v) salicylic acid in concentrated H_2SO_4 (SA-H_2SO_4). After incubation at room temperature for 20 min, 19 ml of 2 M NaOH was added to raise the pH to above 12. Samples were cooled to room temperature and NO_3^- concentration was measured by spectrophotometry at 410 nm with respect to its standard curve (Cataldo et al., 1975). The SA- H_2SO_4 reagent was made fresh at least once each week and stored in a brown bottle. Nitrate standards were stored at 4°C.

Ammonium

Concentrations of NH_4^+ in the roots were calculated based on the Berthelot reaction according to Brautigam et al., (2007). Roots were harvested and ground to a fine powder under liquid nitrogen. About 100 mg of the powder was homogenized in 1 ml of 100 mM HCl, and 500 µL of chloroform. The samples were rotated for 15 min at 4 °C, and the phases were separated by centrifugation at 10,000 g 4 °C for 10 min. The aqueous phase was transferred to a fresh tube containing 50 mg of acid-washed activated charcoal (activated charcoal), thoroughly mixed, and centrifuged (20,000g, 5 min, 4 °C). For ammonia quantification, the supernatant obtained after charcoal treatment was diluted 1:1 (v/v) in 100 mM HCl. Then 20 µl of this solution is mixed with 100 µl of a 1% (w/v) phenol–0.005% (w/v) sodium nitroprusside solution in water, and 100 µl of a 1% (v/v) sodium hypochlorite–0.5% (w/v) sodium hydroxide solution in water was added. The samples were incubated at 37 °C for 30 min, and absorbance was measured at 620 nm.

Nitrite

The NO_2^- concentration in samples was quantified as described by Ogawa et al. (1999). Snap-frozen roots were ground to a fine powder under liquid nitrogen. About 100 mg was extracted with a buffer containing 50 mM TRIS-HCl (pH 7.9), 5 mM cysteine, and 2 mM EDTA. The amount of NO_2^- produced was measured by combining 500 µl of the supernatant with 250 µl of 1% sulfanylamide prepared in 1.5 N HCl and 250 µl of 0.02% N-(1-naphtyl)ethylene-diamine dihydrochloride and reading at 540 nm in a spectrophotometer.

RESULTS AND DISCUSSION

Nodulation: Effect of EBL and/or SA on nodule number, nodule fresh mass and dry mass in chickpea plants grown under water-deficit stress at reproductive stage are presented in Table 1,2 and 3, Fig 1,2 and 3 .

Drought stress at reproductive stage decreased the nodule number considerably as compared to control. Although, nodule fresh mass and dry mass was found to be significantly decreased by 45.6% and 53.8% respectively over the control plants. Exogenous application of EBL and SA marginally increased the nodule number in drought stressed plants in comparison to stress control. However, EBL and SA together able increase the nodule number significantly by 62% (0.0287, $p \leq 0.05$) compared to drought stressed control.

Exogenous EBL and SA alone and their combination accounted for the considerable increase in nodule number than unstressed control. Drought stressed plants treated with EBL and SA exhibited the significant enhancement in nodule fresh mass (by 61.4% and 47.7% respectively) and dry mass (by 72.2% and 44.4% respectively) in comparison to stress control. Supplementation of EBL and SA to drought stress was able to reduce negative effect of drought stress on fresh and dry mass of nodule and increased it by 70% (0.0310, $p \leq 0.05$) and 94.4% (0.0430, $p \leq 0.05$) respectively compared with stress alone, suggesting a complex effect of co-application of EBL and SA on modulation of nodule biomass. Unstressed plants treated with exogenous EBL and SA exhibited the significant increase in nodule fresh mass and dry mass by over the normal control plants. Whereas co-application of EBL and SA was more significantly increased the nodule fresh mass and dry mass by 23% (0.0281, $p \leq 0.05$) and 33.3% (0.0391, $p \leq 0.05$) compared with control than their respective individuals.

Table: 1

	Nodule No./plant	
Control	22.1	0.93
EBR	26.8	1.54
SA	25.3	2.81
EBR+SA	28.4	1.24
Drought	**14.6**	**0.89**
D+EBR	18.3	1.71
D+SA	16.1	2.61
D+EBR+SA	20.4	1.56

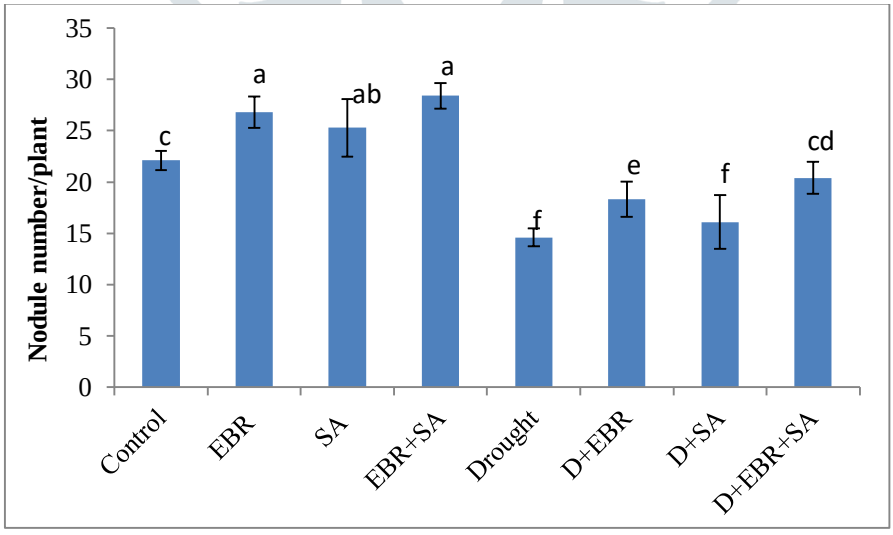

Fig :1 Nodule number/ plant

Table:2

	Nodule fwt (mg)/plant	
Control	362	15.85
EBR	422	28.21
SA	397	24.66
EBR+SA	445	10.75
Drought	197	17.10
D+EBR	318	15.55
D+SA	291	10.54
D+EBR+SA	335	18.02

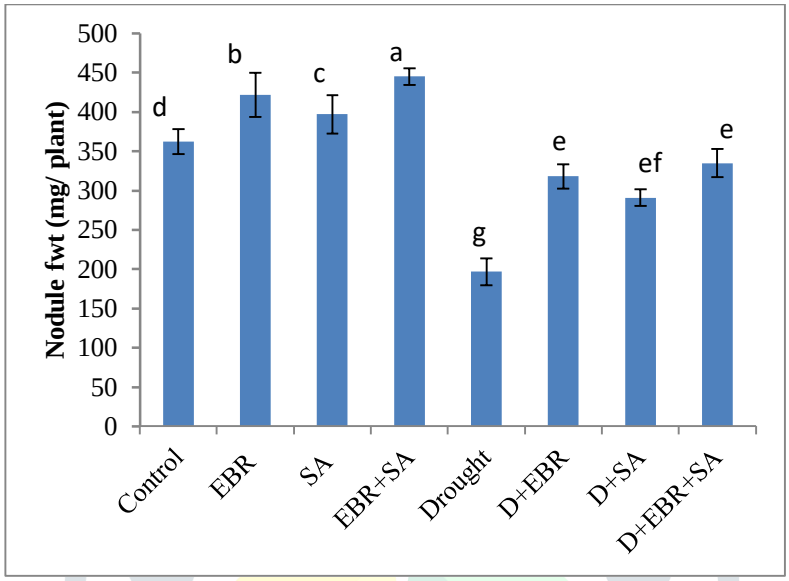

Fig: 2 Nodule fresh weight/Plant

Table:3 Nodule Dry weight/Plant

	Nodule dwt (mg)/plant	
Control	39	4.22
EBR	47	3.11
SA	43	2.41
EBR+SA	52	3.21
Drought	18	**1.08**
D+EBR	31	2.13
D+SA	26	4.11
D+EBR+SA	35	2.56

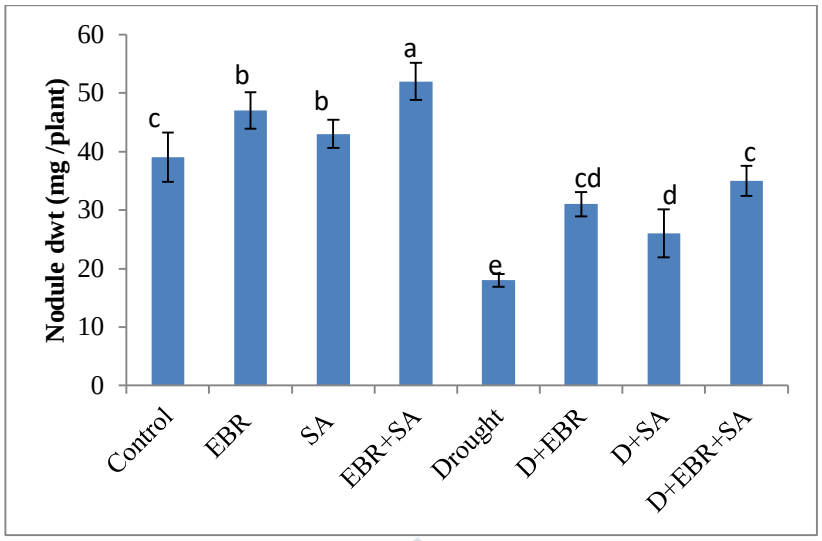

Fig:3 Nodule Dry weight/Plant

Leghemoglobin (LHb) in nodules

A sharp decrease in Lb content (70.2%; 0.0376 $p \leq 0.05$) was recorded in plants subjected to water-deficit stress at reproductive stage compared to control. However, addition of EBL negated drought effect on Lb content and further improved the Lb content significantly by 163.6% over the stress control. Similarly, SA treatment also reversed he drought stress effect on Lb content and improved by 118.6% in comparison to stress control. However, supplementation of both EBL and SA to drought plats showed a significant increase in Lb content (190%; 0.0216 $p \leq 0.05$) when compared with drought treatment alone. Control plants treated with exogenous EBL and SA alone exhibited the significant improved levels of Lb by 46% and 30% respectively over the unstressed control. However, EBL and Spd applied together enhanced Lb content (81%; 0.0372 $p \leq 0.05$) more efficiently than their individual applications compared to control plants. Table 4, Fig:4

Table:4

	Lb (mg/ protein)	
Control	0.37	0.007
EBR	0.54	0.007
SA	0.48	0.018
EBR+SA	0.67	0.008
Drought	0.11	0.009
D+EBR	0.29	0.017
D+SA	0.24	0.006
D+EBR+SA	0.32	0.027

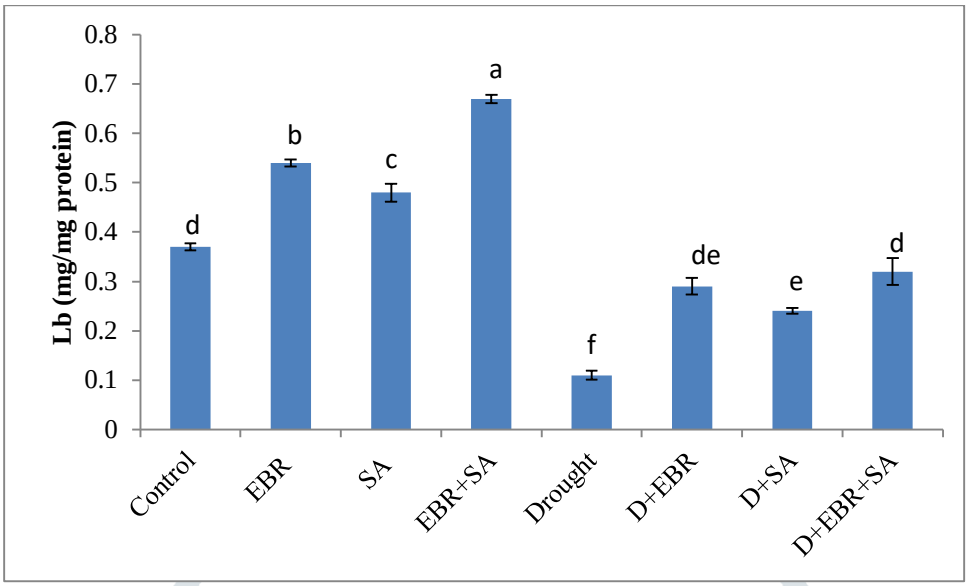

Fig:4 Leghemoglobin (LHb) in nodules

Nitrogen fixation: Effect of EBL and/or SA on nodule nitrogenase (N_2ase) activity and leghaemoglobin (Lb) content in chickpea plants under drought stress are presented in Table

In response to drought stress at reproductive stage nodule N_2ase activity was decreased significantly by 33.4% compared to control plants. Drought stressed plants treated with EBL improved the N_2ase activity by 41.1% over the stress control. Similarly, exogenous SA application also reversed the drought effect on N_2ase activity and improved it significantly (26.2%) in comparison to stress control. Co-application of EBL and SA was more significantly enhanced the N_2ase activity by 51.7% (0.0189, $p \leq 0.05$) than their individual applications in comparison to stress control. Exogenous EBL and SA alone application also accounted for little increase in NR activity by 14 % (p=0.0543) and 10.5 % (p=0.0713) respectively over the control. Co-application of EBL and SA was showed the considerable effect on N_2ase activity (16%; p=0.0412) than their individual applications over unstressed plants, Table 5, Fig 5.

Table:5

	Nitrogenase (µmol C_2H_4/ mg nodule DW/h)	
Control	0.625	0.016
EBR	0.713	0.036
SA	0.691	0.066
EBR+SA	0.725	0.045
Drought	0.416	0.026
D+EBR	0.587	0.044
D+SA	0.525	0.026
D+EBR+SA	0.631	0.044

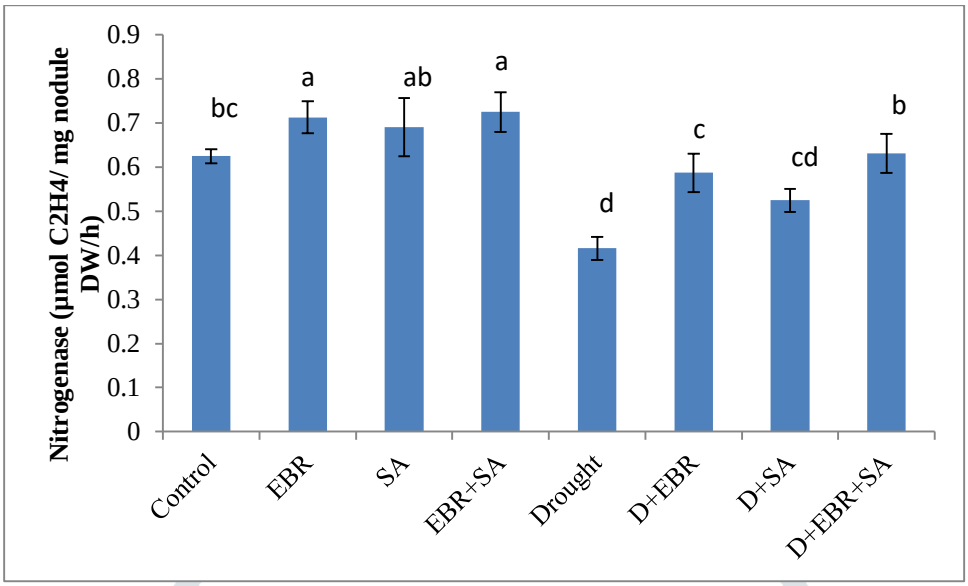

Fig:5 Nitrogenase mg/nodule

Activity of enzymes involved in NO_3^- reduction: Effect of EBL and/or SA on root NR and NiR activities of chickpea plants under stress and stress free conditions are presented in Table.

Water-deficit stress at reproductive stage significantly decreased the root NR activity by 32.4% compared to the control plants. However, addition of EBL and SA restored the NR activity in drought stressed plants. Exogenous EBL supplementation significantly increased the NR activity by 30.4% (0.0253 $p \leq 0.05$) in drought stressed plants over the stress control. Similarly, SA application also accounted for the enhancement of NR activity (16%) in drought stressed plants but it was not statistically significant. However, co-application of EBL and SA was enhanced the NR activity by 48.4% (0.0323, $p \leq 0.05$) 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 NR activity by 21.4 % ($p=0.0423$) and 9.3 % ($p=0.123$) respectively over the control. Co-application of EBL and SA was more effective (28.2%; 0.0265, $p \leq 0.05$) over unstressed control than their individual applications in enhancing NR activity in control plants.

Root NiR activity was declined (27.57%; 0.0267, $p \leq 0.05$) in chickpea plants when subjected to drought stress at reproductive stage. Exogenous application of EBL and SA negated the drought stress effect on root NiR activity and improved by 29% and 18.3% over the stress control. It was found that the root NiR activity was more significant (30.2%; 0.0297 $p \leq 0.05$) in drought stressed plants treated with combined EBL+SA than their individuals over the stress control. Unstressed plants treated with EBL and SA alone also exhibited the significant increase in root NiR activity by 26.48% and 18% respectively over the normal control. About 48.3% (0.0187, $p \leq 0.05$) of root NiR activity was recorded in plants supplemented with EBL+ SA, suggesting that co-application of EBL and SA has a more significant effect than their individual applications in comparison to control. Table 6,7 Fig 6,7

Table: 6

NR (µmol NO_2^- formed/g FW/ h)		
Control	5.59	0.18
EBR	6.79	0.27
SA	6.11	0.38
EBR+SA	7.17	0.86
Drought	3.78	0.32
D+EBR	4.93	0.56
D+SA	4.38	0.47
D+EBR+SA	5.61	0.64

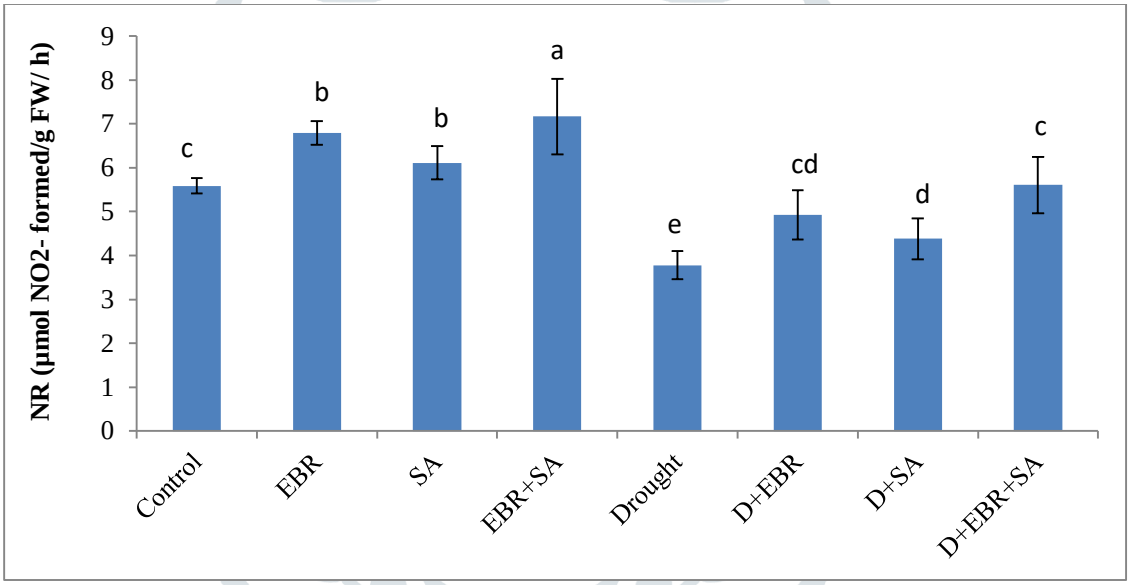

Fig: 6 NR (µmol NO_2^- formed/g FW/ h)

Table: 7

NiR (µmol NO_2^- reduced/ g FW/h)		
Control	0.457	0.051
EBR	0.578	0.032
SA	0.539	0.026
EBR+SA	0.678	0.047
Drought	0.331	0.018
D+EBR	0.427	0.051
D+SA	0.391	0.028
D+EBR+SA	0.431	0.014

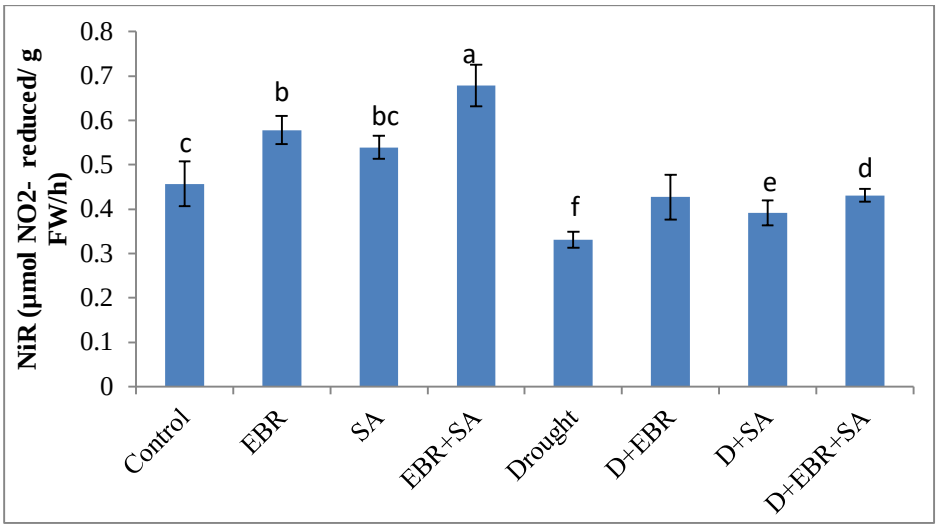

Fig: 7 NiR (µmol NO_2^- reduced/ g FW/h)

Activity of enzymes involved in NH_4^+ utilization: Effect of EBL and/or SA on root GS, GOGAT and NADH-GDH enzyme activities of chickpea plants under drought stress and stress free conditions are presented in Table 8,9,10 Fig 8,9,10

At reproductive stage water-deficit stress significantly decreased the root GS activity by 21% compared to control. However, addition of EBL reversed the decrease in GS activity and further enhanced the activity by 18.11% (0.0322, $p \leq 0.05$) over the stress control. Whereas, exogenous SA application accounted for the small increase GS activity in drought stressed plants but not significantly compared to stress control. However, co-application of EBL and SA was significantly enhanced the GS activity (25.1%; 0.0410, $p \leq 0.05$) 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 slightly increased the GS activity compared to the control. Plants treated with EBL+ SA showed a marked increase in GS activity (18%; 0.0471 $p \leq 0.05$) over unstressed control reflecting the combined effect on modulation of GS activity than their individual applications.

The activity of GOGAT was reduced by 36.4% (0.0378 $p \leq 0.05$) in drought stressed plants than the control. Exogenous application of EBL and SA negated the drought stress on GOGAT activity and improved it by 23% and 24% respectively in comparison to stress control. However, co-application of EBL and SA was enhanced the GOGAT activity by 43% (0.0270, $p \leq 0.05$), 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 NR activity by 21.4 % ($p=0.0423$) and 9.3 % ($p=0.123$) respectively over the control. Unstressed plants treated with exogenous EBL and EBL+SA exhibited the significant increase in GOGAT activity by 17% and 24.6% respectively over the normal control plants. SA application was able induce the GOGAT activity marginally over the control plants.

A significant reduction in the specific activity of NADH-GDH (49.3%; 0.0259, $p \leq 0.05$) was noted in response to drought stress at reproductive stage in comparison to control. Individual supplementation of EBL and SA to drought stressed plants increased the NADH-GDH activity significantly by 62.5 % and 31.2 %

respectively over the stress control. However, co-application of EBL and SA was able induced the NADH-GDH activity more significantly by 86.2% (0.0211, p≤0.05) than their individuals compared to the stress control. Exogenous EBL and SA alone and their combination application to unstressed plants exhibited small enhancement in NADH-GDH activity but not statistically significant over the control. Table 8,9,10 Fig 8,9,10

Table 8

GS (nmolγ-GH/ mg protein/min)		
Control	846	22.81
EBR	901	21.87
SA	897	10.19
EBR+SA	998	41.78
Drought	668	28.19
D+EBR	789	10.57
D+SA	722	29.37
D+EBR+SA	836	31.44

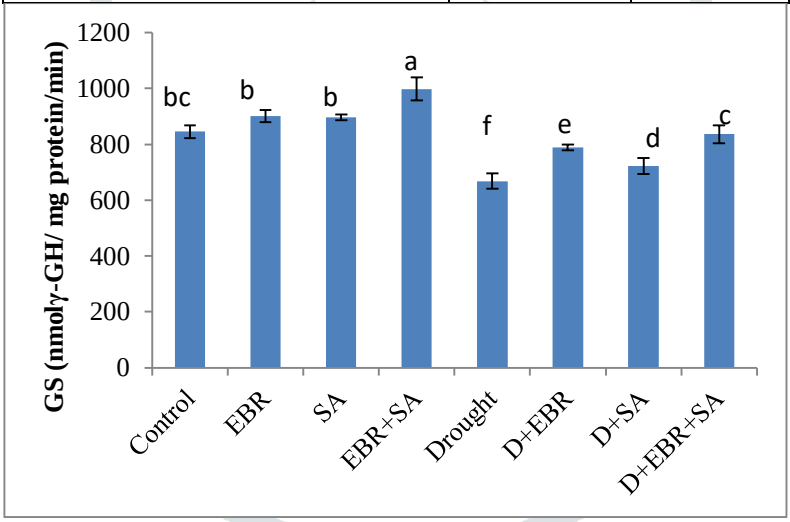

Fig:8 GS (nmolγ-GH/ mg protein/min)

Table:9

	GOGAT(nmol $NADH_{ox}$/mg protein/ min)	
Control	458	11.157
EBR	536	10.1
SA	503	20.47
EBR+SA	571	19.56
Drought	291	11.01
D+EBR	358	10.99
D+SA	361	8.16
D+EBR+SA	416	19.97

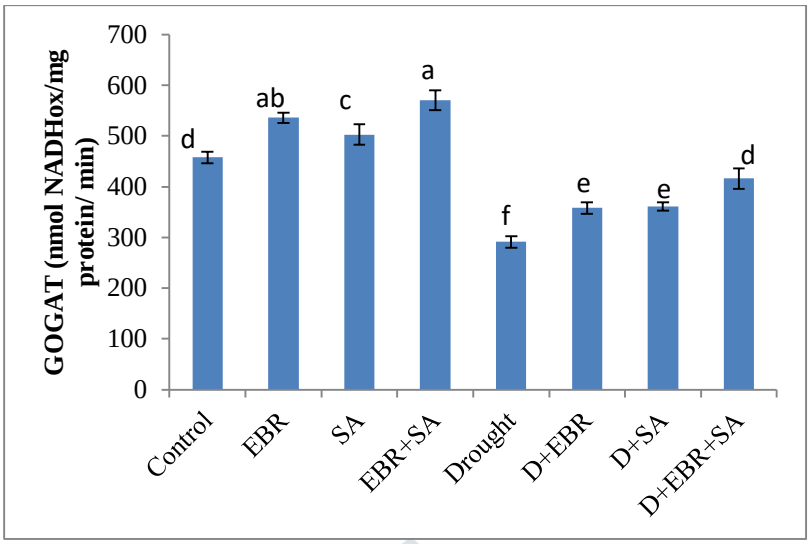

Fig 9 GOGAT(nmol $NADH_{ox}$/mg protein/ min)

Table 10

	NAD-GDH	NAD-GDH (nmol $NADH_{ox}$/mg protein/min)
Control	233	9.43
EBR	271	9.67
SA	267	7.17
EBR+SA	298	10.26
Drought	161	5.74
D+EBR	194	10.11
D+SA	204	9.51
D+EBR+SA	221	11.04

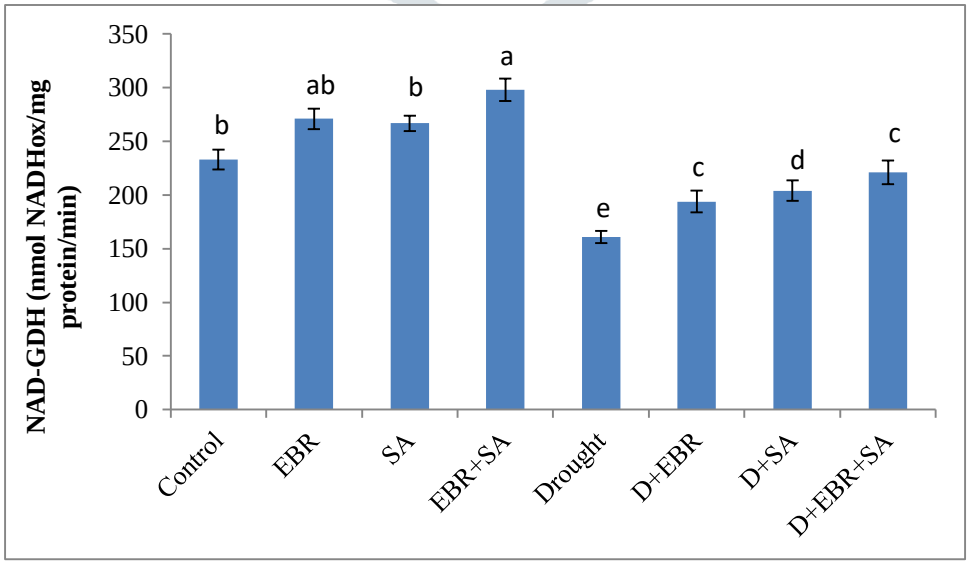

Fig 10: NAD-GDH (nmol NADHox/mg protein/min)

Accumulation of NO_3^-, NO_2^- and NH_4^+: Effect of EBL and/or SA on the accumulation of NO_3^-, NO_2^- and NH_4^+ in chickpea plants under drought stress at reproductive stage and stress free conditions are presented in Table. 11, 12, 13 Fig 11,12,13.

Drought stress at reproductive stage significantly decreased the NO_3^- and NO_2^- levels by 35.58% and 33.04% respectively in comparison to well-watered plants. Exogenous application of EBL and SA reversed the drought effect on the accumulation of NO_3^- and NO_2^- content and improved near to the control levels. Foliar spray of EBL to drought stressed plants enhanced the accumulation of NO_3^- and NO_2^- levels significantly by 34% and 41.42% over the stress control. Similarly, in comparison to well-watered plants SA application also improved the NO_3^- and NO_2^- accumulation significantly by 21.55% and 45.3% respectively. The accumulation of NO_3^- and NO_2^- levels were more significant (48.54%; 0.0410 $p \leq 0.05$ and 46.12%; 0.0335 $p \leq 0.05$) in drought stressed plants treated with EBL+SA than their individual application compared to stressed control. A small increase in NO_3^- levels for EBL alone treatment was observed whereas significant accumulation of NO_2^- levels was noted in well watered plants compared to control. Exogenous SA alone application was found to be marginally enhanced the NO_3^- and NO_2^- accumulation over the control plants. On the other hand, when plants treated with EBL+SA showed a significant accumulation of NO_3^- (28.4; 0.0256 $p \leq 0.05$) and NO_2^- (36%; 0.0189 $p \leq 0.05$) levels than their individual application compared to normal control.

In contrast, about 31.75% of higher NH_4^+ accumulation was noted in drought stressed plants as compared to well-watered plants. Exogenous application of EBL significantly increased the NH_4^+ accumulation in drought stressed plants by 17.47% over the stressed control. No significant enhancement of NH_4^+ accumulation was recorded in droughted plants treated with exogenous SA application. A significant accumulation of NH_4^+ (20.63%; 0.0315 $p \leq 0.05$) was observed in drought stressed plants treated with EBL and SA together over the stress control. Well-watered plants treated with EBL alone application increased the NH_4^+ accumulation by 16.67% over the control. Whereas, SA alone application showed no significant improvement on NH_4^+ accumulation. However, plants treated with EBL+SA exhibited the significant NH_4^+ accumulation in plants in comparison with untreated control, reflecting that co-application of EBL and SA has a more significant effect than their individual applications on the NH_4^+ accumulation Table 11, 12 and 13 Fig 11, 12 and 13.

Table:11

	NO3 (µmol /g DW)	
Control	7.42	0.34
EBR	8.21	0.74
SA	8.17	0.57
EBR+SA	9.53	0.87
Drought	4.78	0.67
D+EBR	6.4	0.45
D+SA	5.81	0.89
D+EBR+SA	7.1	0.38

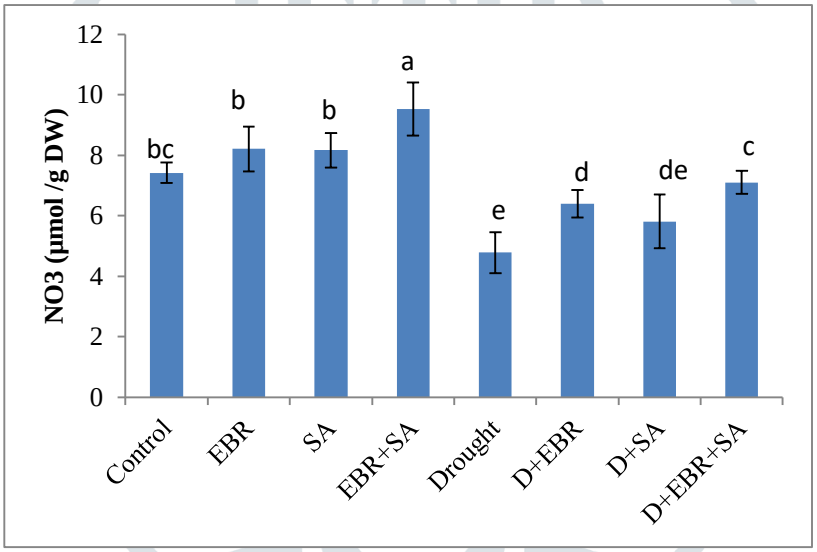

Fig: 11 NO3 (µmol /g DW)

Table: 12

	NO2 (µmol/g DW)	
Control	9.23	0.57
EBR	11.26	0.64
SA	10.89	0.21
EBR+SA	12.55	0.37
Drought	6.18	0.61
D+EBR	8.74	0.52
D+SA	8.98	0.28
D+EBR+SA	9.03	0.28

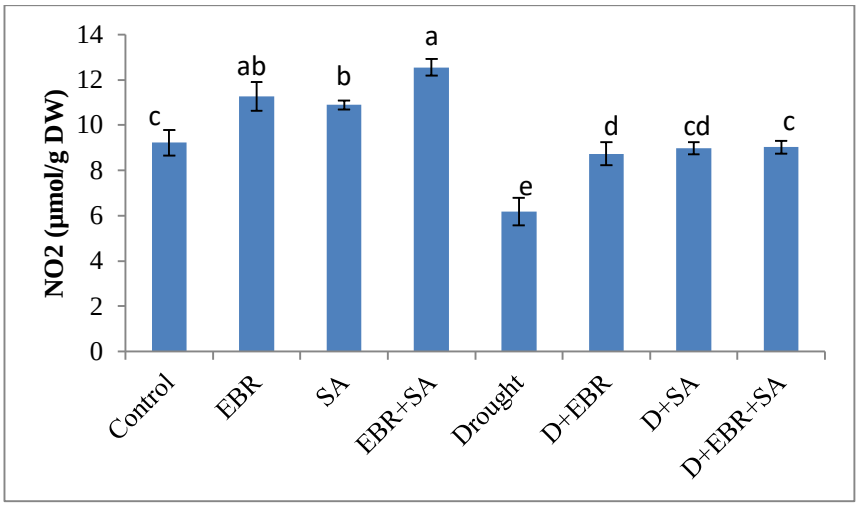

Fig: 12 NO^2 (µmol/g DW)

Table:13

	NH4 (µmol /g DW)	
Control	12.6	0.77
EBR	14.7	0.64
SA	13.1	1.71
EBR+SA	15.2	0.87
Drought	16.6	0.64
D+EBR	19.5	0.58
D+SA	17.9	0.88
D+EBR+SA	21.8	1.28

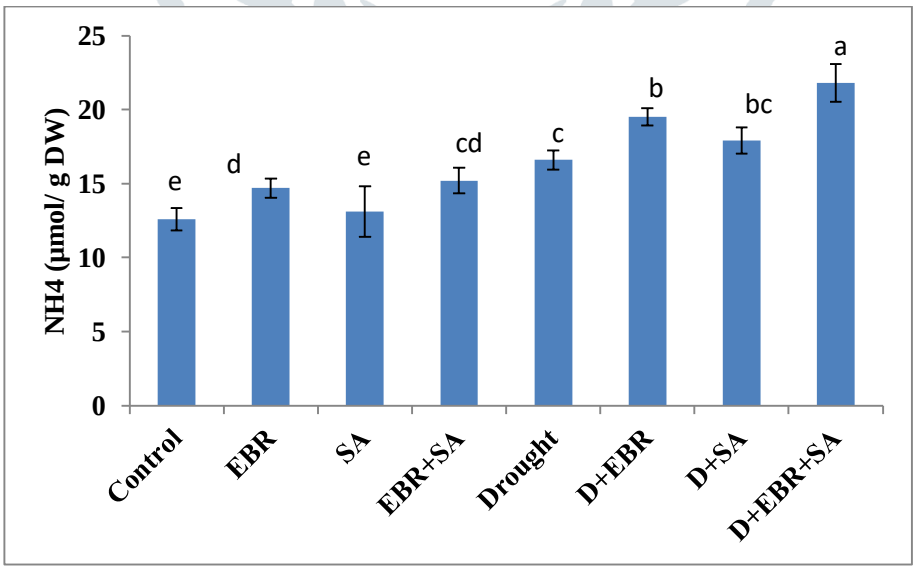

Fig: 13 NH4 (µmol /g DW)

Conclusion

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