Morpho-physiological and Biochemical Responses of Flax (*Linum usitatissimum* L.) to Exogenously Applied Glycinebetaine under Water Deficit Conditions

Zyma Basharat[1] and Muhammad Shahbaz[1*]

Department of Botany, University of Agriculture, Faisalabad.

ABSTRACT

Water shortage is the key aspect of dropped yield and production all over the world. Two pot trials was conducted at University of Agriculture Faisalabad to discover the role of foliar applied glycinebetaine (GB) on two flax varieties as foliar spray under water stress circumstances. Two flax varieties i.e. Chandini and Roshini were analyzed against drought (controlled and 50% FC). Four level of glycinebetaine were sprayed such as no spray, water spray, 10 mM and 20 mM. Drought was maintained after 60 days of seed implanting. After 15 days of ex-foliar spray with GB, morphological data was acquired and harvesting was done at crop maturity. Water scarcity adversely declined the flax crop yield by dropping the flax fresh weight, dry weight, shoot length, photosynthetic pigments (chl. *a*, *b*, *a/b*, total chlorophyll and carotenoids). Under stress circumstances MDA and H_2O_2 concentration was increased in both varieties. Enzymatic (SOD, POD, CAT) and non- antioxidants (leaf ascorbic acid, total soluble protein, total free amino acids, flavonoids, glycinebetaine, total phenolic) action was improved under stress situation. Ions concentration such as sodium was improved but potassium and calcium concentration was decreased. Foliar applied GB increased the yield by increasing no. of pods per plant and total seed weight of both flax varieties. MDA and H_2O_2 content declined and enzymatic and non-enzymatic antioxidants activity enhanced along with flax morphological characteristics (shoot fresh and dry weight, shoot length). Potassium content was boost up and sodium ratio was diminished via foliar spray. Overall, 10 mM of GB and variety chandini performed better in controlled and stress situation.

I. INTRODUCTION

Flax/linseed (*Linum usitatissimum* L.) is a versatile crop that used for food and pharmacological requirements of humans (*Goyal et al*., 2014)Despite being cultivated since 9000 BC, it was a "Neglected crop" in the arena of molecular study till 2012, when international collaborations embarked on a comprehensive, multidisciplinary research venture (Rizvi *et al*., 2021). Flaxseed is a uniquely abundant plant-based source of alpha-linolenic acid (ALA), a vital omega-3 fatty acid source (Tonon *et al*., 2011), and it also contains a significant amount of lignans, a group of phytoestrogens with potential health aids (Singh *et al*., 2011).

Water stress can exert a detrimental impact on flax cultivation, leading to diminished yields, reduced fiber content, and compromised fiber quality, thereby imperiling the crop's economic viability and industrial utility (Soto-Cerda *et al*., 2020). Drought stress situation play a leading role in all non-living stresses. The growth rate of Pakistani inhabitant's raised on and average 2.4% annually, hence different approaches are considers to overcome the effect of abiotic stresses and improved the yield of several crops to attain the food requirement (Mahajan and Tuteja, 2006). Water scarcity significantly reduces crop yields, with losses ranging from 50% to 60% (Luqman *et al*., 2023).

Exogenous application of innumerable compounds can increase stress tolerance in plants. Glycinebetaine and proline are two well-known stress-alleviating compounds (Ashraf and Foolad, 2007). Glycinebetaine is a quaternary ammonium compound that is non-toxic, environmental friendly and water soluble, found in many plant species at varying concentrations (Kumar *et al*., 2009). Glycinebetaine (GB) is a crucial stress-ameliorating compound, particularly for plants that cannot produce it endogenously. Exogenous GB application is essential for stress tolerance in these plants. GB plays a vital role in plant defense against drought, saline, temperature, and light stress (Farooq *et al*., 2008), maintaining enzyme and protein structure intact, preserving membrane integrity, and protecting them against any abiotic injury (Sakamoto and Murata, 2002). Glycinebetaine (GB) boosts resistance to drought stress by improving osmotic regulations in plant cells, decreasing damage to the cell membranes which triggered by osmotic water loss, stabilizing subcellular activities and structures by scavenging reactive oxygen species (ROS) (Ashraf and Foolad, 2007). Furthermore, roles of GB comprises of preserving the thermodynamic firmness of macromolecules and retreating protein misfolding and accretion without compromising the protein's native functions (Hussain *et al*., 2009) It also

reduces the level of plasma membrane instability, improving ROS scavenging capability and maintaining cell membrane permanency (Cicek and Cakirlar, 2008). Additionally, it serves as a catabolic source of methyl groups, facilitating various biochemical processes. GB acts as an osmoprotectant, protecting cells against osmotic shock and enhancing water retention capacity, thereby maintaining cellular turgor and integrity (Ashraf and Foolad 2007).

II. MATERIALS AND METHODS

2.1 Experimental layout

2.1.1 Seeds curtsey and varieties

The experimental seeds of 2 flax varieties i.e, Roshini and Chandani were found from the Ayub Agricultural Research Institute (AARI) Faisalabad

2.1.2 Experimental site

Experimental test was conducted in University of Agriculture Faisalabad's old botanical garden in the years of 2020-2021.

2.1.3 Treatment level of glycinebetaine

For pre-sowing seed treatment available varieties of flax were soaked in the glycinebetaine (GB) solution for almost 8 hours. 4 levels of GB were used i-e (non-soaked, water soaked, 10 and 20 mM) as pre-sowing seed treatment and 4 GB level (Non-spray, water spray, 10, 20 mM) was used as foliar application. Twenty seeds of each of the varieties were seeded in each plastic pot.

2.1.4 Stress level of drought

Two water stress levels were retained i.e. well watering and 50% field capacity after 60 days of seeds planting.

2.1.5 Data collection

Following 15 days of foliar application with glycinebetaine, morphological data were collected, and the crops were harvested at maturity, marking the culmination of the experiment.

2.1.6 Field capacity (FC) determination

Two soil samples each of which contains of 200 g were kept in oven for seven days at 105 ºC. After that dried weight of soil sample was noted. Saturation fractions of both samples were determined by making paste of 100 g of desiccated soil from both samples by evaluating the water used during paste formation. The FC was determined by using given formula and maintained

Field capacity (FC) = Saturation % / 2

2.2 Morphological attributes

All morphological attributes like fresh weight of plant shoot, shoot length and dry weight were calculated. To perform these, two plants from each replicate were taken from the pots very carefully and rinsed with plain water to get rid of soil residues after that meter rod was used to measure the plant length. Plant fresh weigh was measured by weighing scale. Samples were placed for drying in oven at 65 degree centigrade.

2.3 Chlorophyll estimation

Arnon (1949) method was executed for the assessment of the pigments of chlorophyll in leaves of flax plants. Hundred mg of green fresh leaves were extracted in 5 ml of 80% acetone and incubated samples overnight. The absorbance density of the material was documented by means of spectrophotometer (IRMECO U2020) at 645, 663 and 480 nm optical density (OD).

2.3 Quantification and Calculation of enzymatic antioxidants

Fresh flax leaves (0.25g) were crushed by adding 5 ml of phosphate buffer (50 m*M*) of pH 7.8 for the assessment of enzymatic antioxidants. After crushing, plant leaves extract obtained centrifuged the material at 12000 rpm for 15 minutes at 4ºC. Supernatants were poured into eppendrofs for enzymes quantification and left over material was thrown away.

2.3.1. Superoxide dismutase (SOD)

The activity of superoxide dismutase (SOD) was evaluated using the protocol described by Giannopolitis and Ries (1977). The reaction mixture consisted of 400 µl of distilled water, 100 µl of L-methionine, 250 µl of potassium phosphate buffer (pH 7.8), and 100 µl of Triton X-100. Subsequently, 50 µl of nitroblue tetrazolium (NBT), 50 µl of enzyme extract, and 50 µl of riboflavin were added to the cuvette. The samples were then incubated under a lamp (3000 lux) for 15 minutes at 25°C. A reaction mixture without enzyme extract served as the control. The absorbance of each sample was measured at 560 nm using a spectrophotometer (U2020 IRMECO). The SOD activity was determined, based on the enzyme concentration that inhibited 50% of the photochemical reduction of NBT.

2.3.2. Catalase (CAT)

Chance and Maehly (1955) methodology was practiced to determine the catalase activity. The reaction mixture was prepared by using, 1.9 ml of potassium phosphate buffer, 0.1 ml of sample extract and 1 ml distilled H_2O_2. Sample was analyzed at 240 nm by using spectrophotometer in decreasing manner, taking interval of 20 seconds for 2 minutes. Per minute variation in optical density is the basis catalase enzyme activity.

2.3.3. Peroxidase (POD)

By using the procedure of Chance and Maehly (1955), peroxidase activity was determined. For this purpose, 1 ml reaction mixture for the activity of peroxidase included 100 µl H_2O_2, 0.1 ml of guaiacol, 50 ml of sample extract and 750 µl of phosphate buffer was added. Absorbance was noted by using spectrophotometer at 470 nm with regular interval of 20 seconds for 2 minutes.

2.4 Total soluble proteins

Bradford (1976) technique was used for the quantification of total soluble proteins. Fresh flax leaves (0.25g) were teased into the 5 ml solution of potassium phosphate buffer (pH 7) in pre-chilled pestle and mortar. The material was centrifuged at 4ºC for 10 minutes at 12000 rpm. In test tubes 100 micro litter of the extract along with 5 ml of Bradford reagents were added. Test tubes were vortexed and readings were taken at 595 nm on spectrophotometer.

2.5 Total Phenolics estimation

Total leaf phenolics were estimated by using the protocol that given by Julkenen-Titto (1985) in which 0.1 g of flax plant leaves were teased in 1ml of 80 % acetone. After that the material was centrifuged at 12000 rpm for 15 minutes. Leaves sample were separated and stored the material at 20 degree centigrade before used. For further analysis 100 µl from the stored material was mixed with1 ml of Folin-Ciocalteau's phenol reagent and shake vigorously. After that immediately added 5 ml solution of 20 % Na_2CO_3 in reaction mixture and distilled water was added and made volume up to 10 ml. Absorbance was observed of vortexed mixture after 20 minutes of wait at 750 nm on UV-Visible spectrophotometer (IRMECO U2020).

2.6 Estimation of leaf ascorbic acid

Mukherjee and Choudhuri (1983) protocol was used to evaluate the flax leaves ascorbic acid. Flax leaves (0.25g) extracted in 10 ml solution of 6% TCA. Four milliliter extracted material were further added with 2 ml of acidic soln. of 2% dinitrophenyl hydrazine. Furthermore single drop from the solution of 10% thiourea was added into the reaction tubes. Mixture was put into the water bath for 20 minutes at boiling temperature. After that, the mixture was incubated at room temperature then 5 ml of H_2SO_4 was mixed into the reaction tubes at 4ºC. OD was noted at 530 nm.

2.7 Malondialdehyde (MDA) concentration

Carmak and Horst (1991) method with little amendments was used to quantify the MDA concentrations in flax plant tissue. Fresh leaf material (0.25 g) was extracted in 3 ml of trichloroacetic acid (TCA) solution. To homogenate the plant tissues material, centrifuged the sample at 12000 rpm for 15 minutes. Three milliliter of thiobarbituric acid (0.5% TBA) was added in 20% TCA. The samples were heated at 95 ºC for 50 minutes. After that cooled the samples in chilling water, 10 minutes centrifuging process was completed at 10000 rpm. Absorbance was detected at 532 nm and 600 nm.

2.8 Hydrogen peroxide (H_2O_2)

To observe the content of hydrogen peroxide in plant tissues, the method of Velikova *et al*. (2000) was followed. For this purpose, 0.5 g of fresh leaves sample was crushed by adding 5 ml of 0.1 % tri-chloroacetic acid (TCA). The samples were centrifuged at 12000 rpm for 15 minutes. In test tubes 0.5 ml of the prepared above material, 0.5 ml of potassium phosphate buffer of pH 7.0 and 1 ml of KI were also added. After vortexed the material its optical density was noted at 390 nm. Distilled was used as blank.

2.9 Total free proline

Total free proline was determined by using Bates *et al*., (1973) protocol. For this purpose 0.25 g fresh leaf material was homogenized in 5 ml sulfosalicyclic acid (3%). The material was filtered through Whatman No. 2 filter paper. One mL extract was taken in test tube and 1 ml acid ninhydrin and 1 ml glacial acetic acid were added. Vortexed the sample after that at 100°C, sample was incubated in water bath for 60 min, and then cooled in ice. After cooling, 4 ml toluene was added and vortexed for 15-20 s. The absorbance was obtained at 520 nm on spectrophotometer (IRMECO U2020).

2.10 Glycinebetaine

For determination of Glycinebetaine, Grieve and Gratten (1983) procedure was observed. For this purpose, took 0.25 g of freeze plant material and homogenized in 5 ml of distilled water. The extract was centrifuged at 12000 rpm for 10 min, mixed the 1 ml supernatant with 1 ml of 2 normal H_2SO_4 in test tube. To 0.5 ml of solution, added 2 ml of potassium tri-iodide. Put the test tubes in ice bath for 90 minutes. After chilling, added 1.4 ml of distilled water followed by addition of 6 ml of 1-2-dichloroethane in chilled condition. As a result, two layers were formed, upper layer was discarded and lower red layer was taken for glycinebetaine determination at 365 nm wavelength by using spectrophotometer (IRMECO U2020).

2.11 Total free amino acids

Hamilton and Van Slyke (1943) protocol was used to calculate the total free amino acid content in plant leaves. One milliliter of reaction mixture was taken (as extracted for protein estimation) in tubes and added 1ml of 10 % pyridine along with 1 ml of 2 % acidic ninhydrin solution. Heat up the mixture for 30 minutes in water bath at 100 ºC. Cool down the reaction tubes and make the volume up to 50 ml. By using spectrophotometer OD was noted at 570 nm.

2.12 Flavonoids estimation

The protocol of Kim *et al* (1999) was followed to evaluate the concentrations of flavonoids. Hundred milligrams of fresh leaves was ground in 2 ml of 80% acetone with the help of pistil and mortar. Centrifuge the extracted material for 15 minutes at 12000 rpm. Add 0.5 ml of supernatant in test tube along with 2 ml of distilled water. After 5 minutes 0.5 ml of $AlCl_3$ (10%) and 0.6 ml of $NaNO_2$ (5%) were also added. Wait for further 1 minute and then 2 ml of NaNOH (1M) and 2.4 ml of distilled water were also added. Absorbance was noted at 510 nm by using spectrophotometer (IRMECO U2020).

2.13 Inorganic ions

Dried material of shoot (0.1g) was ground and put into the reaction flask along with 2 ml of H_2SO_4 (sulphuric acid). Reaction flasks were kept at room temperature and incubated for overnight. Put the flask on hot plate and heated up at 150ºC. One ml of H_2O_2 was poured into the digestion flasks. Fumed were generated from the reaction mixture. Carried out the procedure until the samples became clear and colorless solution was obtained. After filtration made the volume up to 50 ml by adding distilled water. Ions (Na^+, Ca^{2+} and K^+) content were determined by using flame photometer.

2.14 Yield related attributes

At plant maturity, different yield related parameters e.g. number of seeds pods/plant and per plant seed weight of flax was determined.

2.15 Statistical analysis

Three factor factorial completely randomized design (CRD) was used to arrange the both experiments. CO- STAT computer software by Snedecor and Cochran (1980) method was used to analyze the data with 3-way ANOVA.

3. Results:

Imposition of water scarcity exhibited a noticeable ($P \leq 0.001$) reduction in shoot fresh biomass of respective flax varieties. Significant ($P \leq 0.001$) varietal variance was witnessed as Roshini revealed enhanced SFW in both situations (controlled and drought) as likened to Chandini. Ex-foliar practice of glycinebetaine conspicuously ($P \leq 0.001$) augmented the SFW in both flax varieties. Of different glycinebetaine levels, 20 mM showed high SFW in Roshini under both circumstances but 10 mM level of GB performed better in Chandini in both conditions. Over all Roshini exhibited more shoot fresh weight as related to Chandini under normal situations. (Table 3.1; Fig 3.1)

Significant ($P \leq 0.001$) decline was witnessed in shoot dry weigh (SDW) under water stress circumstances in both flax varieties. Exogenously practice of glycinebetaine noticeably ($P \leq 0.001$) boosted the SDW in both flax varieties under stress and controlled circumstances. Over all 10 mM of GB did slightly ($P \leq 0.05$) better role in Roshini but 20 mM GB revealed high SDW in Chandini under stress situations (Table 3.1; Fig 3.1)

Imposition of 50% moisture threshold pointedly ($P \leq 0.001$) abridged the shoot length (SL) in both varieties. A substantial ($P \leq 0.001$) varietal variation was practiced as Chandini showed better performance according to SL enhancement. GB practice as exogenous spray boosted the SL considerably ($P \leq 0.001$). Both flax varieties showed uniform behavior with respect to drought imposition, but 20 mM GB improved the shoot length in both varieties under stress circumstances. Collective interaction V×D×GB also shown significant ($P \leq 0.05$) value (Table 3.1; Fig 3.1)

Implementation of 50% field capacity substantially ($P \leq 0.001$) abridged the chlorophyll *a* content in both flax varieties. Considerably less significant ($P \leq 0.05$) varietal difference was practiced as Chandini exhibited better chlorophyll *a* content as compared to Roshini. Exogenously applied GB significantly ($P \leq 0.001$) enhanced the content of chlorophyll *a* in both varieties of flax. Under water stress circumstances Chandini exhibited more chlorophyll *a* as compared to Roshini (Table 3.1; Fig 3.2)

Considerable reduction ($P \leq 0.001$) in chlorophyll *b* content was observed in both flax varieties under controlled and drought conditions. Foliar applied GB significantly ($P \leq 0.001$) increased the chlorophyll *b* content in both flax varieties under controlled and drought conditions. In drought conditions, a remarkable increase was observed in chlorophyll *b* content in Chandini as compared to Roshini. Under drought conditions 20 mM performed better to increased chlorophyll *b* content in both flax varieties (Table 3.1; Fig 3.2)

Drought stress imposition reduced the total chlorophyll prominently ($P \leq 0.001$) in Roshini and Chandini. Slightly significant ($P \leq 0.05$) variation was observed in Chandini regarding this parameter. Exogenous application GB considerably ($P \leq 0.001$) enhanced the total chlorophyll in both conditions. Chandini showed significantly ($P \leq 0.01$) more total chlorophyll content then Roshini in stress conditions. Maximum total chlorophyll was observed in both varieties with 20 mM glycinebetaine application under controlled and stress situations (Table 3.1; Fig 3.2)

Table 3.1: Mean squares from analysis of variance of data for growth attributes and chlorophyll pigments of flax (Linum usitatissimum L.) when 60 days old plants treated as foliar treatment with glycinebetaine under drought stress conditions.

SOV	**df**	**SFW**	**SDW**	**SL**
Varieties (V)	1	1.722***	0.006 ns	235.085***
Drought (D)	1	146.59***	6.497***	6083.22***
Glycinebetaine (GB)	3	1.660***	0.125***	117.478***
V × D	1	2.449***	0.013 ns	74.476***
V × GB	3	0.140 ns	0.019*	1.500 ns
D × GB	3	0.261 ns	0.020*	4.330 ns
V × D × GB	3	0.100 ns	0.0043 ns	5.958*
Error	48	0.114	0.005	1.610
SOV	**df**	**Chl *a***	**Chl *b***	**Total Chlorophyll**
Varieties (V)	1	0.238*	0.002 ns	0.190*
Drought (D)	1	1.362***	4.517***	10.835***
Glycinebetaine (GB)	3	0.587***	0.305***	1.736***
V × D	1	0.016 ns	0.00005 ns	0.023 ns
V × GB	3	0.075 ns	0.062 ns	0.218**
D × GB	3	0.104 ns	0.120**	0.212**
V × D × GB	3	0.050 ns	0.157***	0.098 ns
Error	48	0.046	0.023	0.035

*,** and *** significant at 0.05, 0.01 and 0.001 levels, respectively; ns = non-significant

SFW = Shoot fresh weight, SDW = Shoot dry weight, SL = Shoot length, Chl = Chlorophyll

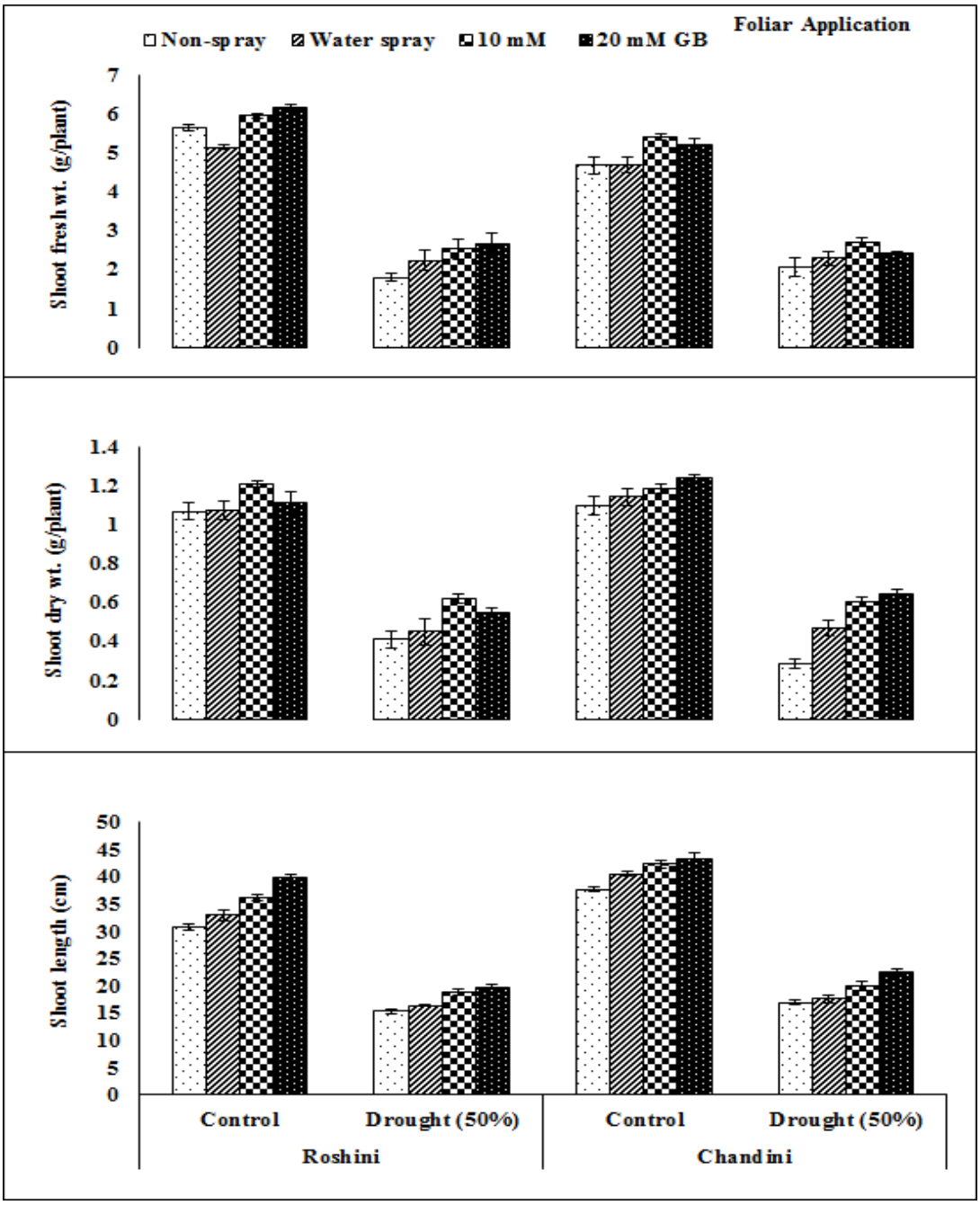

Figure 3.1: Growth attributes of flax plant (*Linum usitatissimum* L.) varieties (Roshini and Chandani) when 60 day old plants treated as foliar treatment with glycinebetaine under control and drought stress conditions.

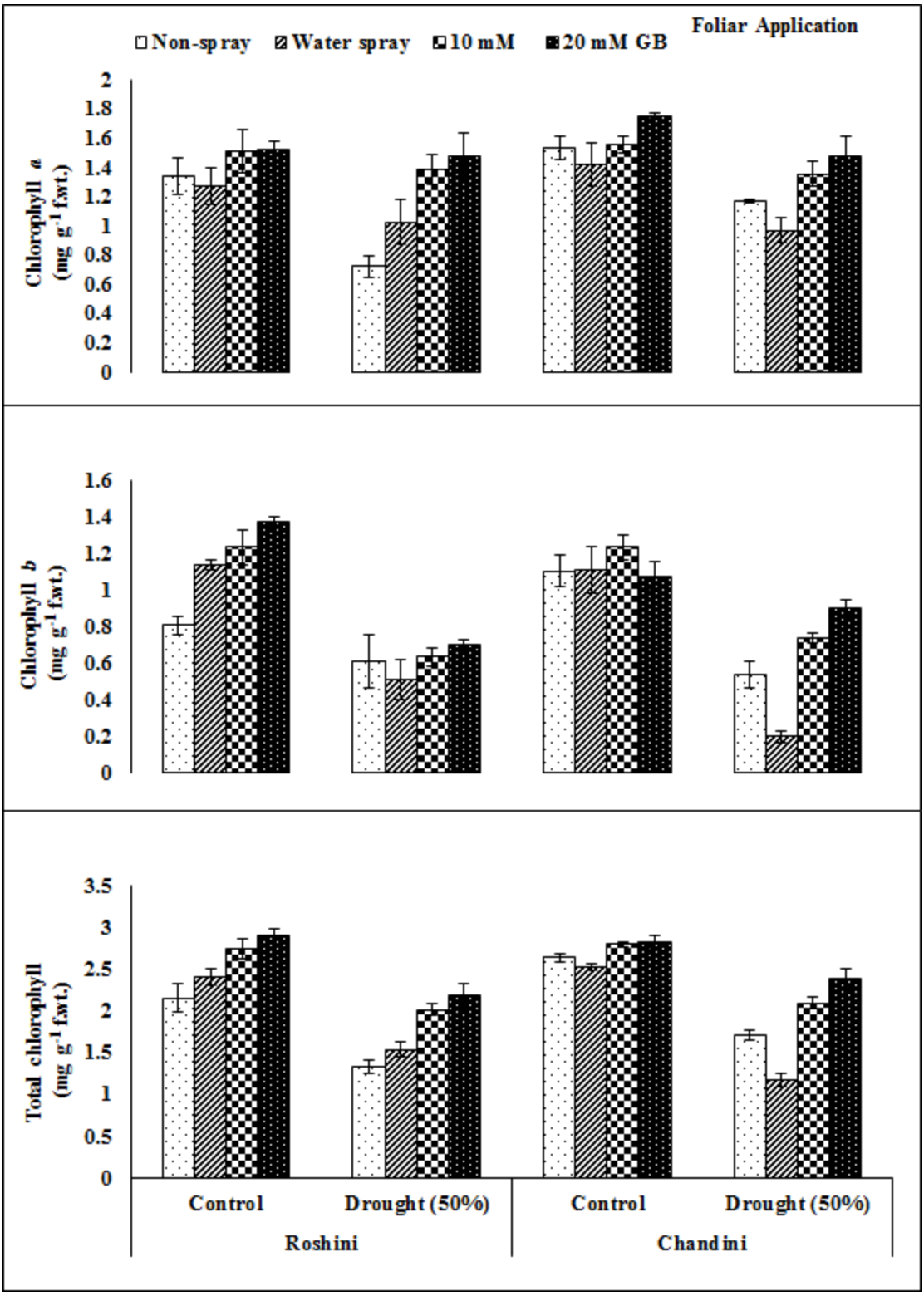

Figure 3.2: Chlorophyll *a*, chlorophyll *b* and total chlorophyll of flax plant (*Linum usitatissimum* L.) varieties (Roshini and Chandani) when 60 day old plants treated as foliar treatment with glycinebetaine under control and drought stress conditions.

Imposition of moisture stress considerably ($P \leq 0.001$) improved the chl. *a*/*b* (chlorophyll) proportion in both varieties. Foliar use of glycinebetaine significantly ($P \leq 0.01$) abridged the chl. *a*/*b* ratio in both flax varieties under water shortage situation. The interaction between V×D was prominently significant but overall relations between V×D ×GB was observed non-significant (Table 3.2; Fig 3.3)

Moisture stress exhibited a significant ($P \leq 0.001$) decline in carotenoids. Varietal variation was also significant ($P \leq 0.001$) as Chandini exhibited better carotenoids content related to Roshini. GB spray also boosted the carotenoids value in both varieties. Chandini exposed better carotenoids value as compared to Roshini under drought stress. In both varieties, 20 mM GB caused more enhancements in carotenoids content under stress situation (Table 3.2; Fig 3.3)

Imposition of drought (50% FC) deliberatively ($P \leq 0.001$) amplified the superoxide dismutase (SOD) action in both varieties. Varietal change was also significant ($P \leq 0.001$) as SOD action was maximum in Chandini as compared to Roshini. GB application as foliar spray considerably ($P \leq 0.001$) increased the activity of SOD in both varieties but 10 mM GB performed better under stress conditions. Over all, in drought condition Chandini showed better response as compared to Roshini (Table 3.2; Fig 3.3)

Moisture stress application significantly ($P \leq 0.001$) increased the catalase (CAT) activity in flax. Significant ($P \leq 0.001$) varietal difference was noticed as maximum CAT activity was observed in Roshini as compared to Chandini. GB application as an exogenous spray increased the catalase activity significantly ($P \leq 0.001$) in both varieties. Of various GB levels 10 mM showed better performance in both varieties under water shortage condition. V×D×GB interaction also showed highly significant ($P \leq 0.001$) (Table 3.2; Fig 3.4)

Imposition of 50% field capacity increased the value of peroxidase (POD) significantly ($P \leq 0.001$) in both flax varieties. A significant varietal ($P \leq 0.01$) difference was observed as Chandini showed more peroxidase activity as compared to Roshini. Foliar application of glycinebetaine enhanced the activity of POD significantly ($P \leq 0.001$) in both varieties as 10 mM GB level performed better in Chandini and 20 mM performed better in Roshini under stress conditions (Table 3.2; Fig 3.4)

Total phenolics content was improved significantly ($P \leq 0.001$) in both flax varieties. Varietal difference was also noticeably significant ($P \leq 0.05$) as total phenolics content escalated in Chandini as compared to Roshini under stress circumstance. Exogenously applied GB amazingly ($P \leq 0.001$) improved the total phenolics content in both varieties but 10 mM GB performed better in Roshini and 20 mM in Chandini in both situations (Table 3.2; Fig 3.4)

Table 3.2: Mean squares from analysis of variance of data for chlorophyll pigments, SOD, CAT, POD and Total phenolics of flax (*Linum usitatissimum* L.) when 60 days old plants treated as foliar treatment with glycinebetaine under drought stress conditions.

SOV	**df**	**Chl *a*/*b***	**Carotenoids**	**SOD**
Varieties (V)	1	992.169***	0.015***	3144.411***
Drought (D)	1	3522.75***	0.016***	2559.012***
Glycinebetaine (GB)	3	40.878 ns	0.004***	100.907***
V × D	1	355.203***	0.0003 ns	635.869***
V × GB	3	56.514*	0.0006**	2.397 ns
D × GB	3	68.342*	0.0004*	24.594**
V × D × GB	3	35.598 ns	0.001***	8.240 ns
Error	48	17.102	0.0001	5.647
SOV	**df**	**CAT**	**POD**	**Total phenolics**
Varieties (V)	1	0.00003***	0.0001**	15.056**
Drought (D)	1	0.0003***	0.011***	5.265 ns
Glycinebetaine (GB)	3	0.00002***	0.00005***	1.615 ns
V × D	1	0.00001 ns	0.0004***	8.993*
V × GB	3	0.0000004 ns	0.00005*	12.090***
D × GB	3	0.000006***	0.0001**	1.297 ns
V × D × GB	3	0.000003***	0.00006*	5.464*
Error	48	0.0000003	0.00001	1.460

***,** and *** significant at 0.05, 0.01 and 0.001 levels, respectively; ns = non-significant**

Chl = Chlorophyll; SOD = Superoxide dismutase; CAT = Catalase; POD = Peroxidase

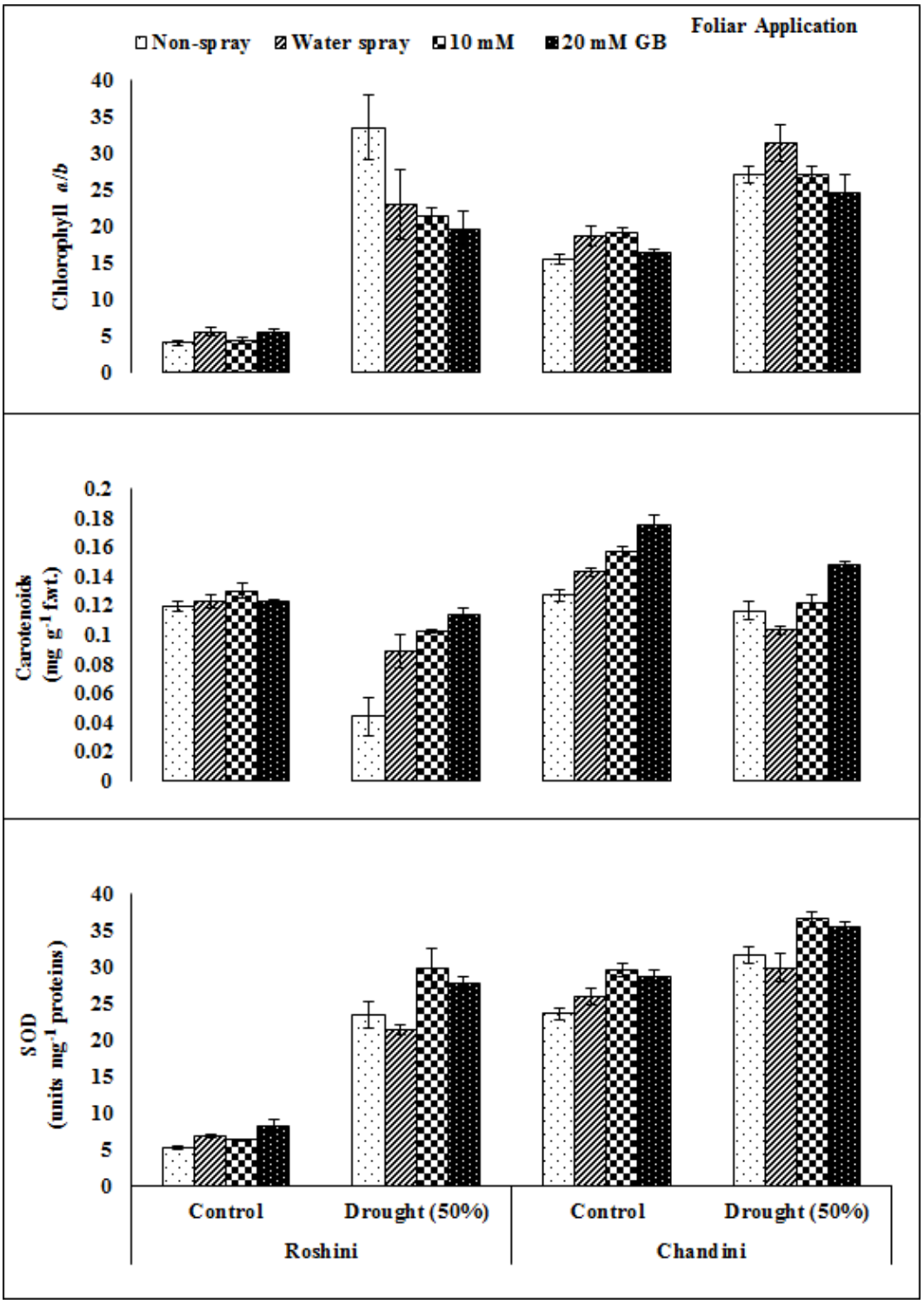

Figure 3.3: Chlorophyll *a*/*b*, carotenoids and superoxide dismutase of flax plant (*Linum usitatissimum* L.) varieties (Roshini and Chandani) when 60 day old plants treated as foliar treatment with glycinebetaine under control and drought stress conditions.

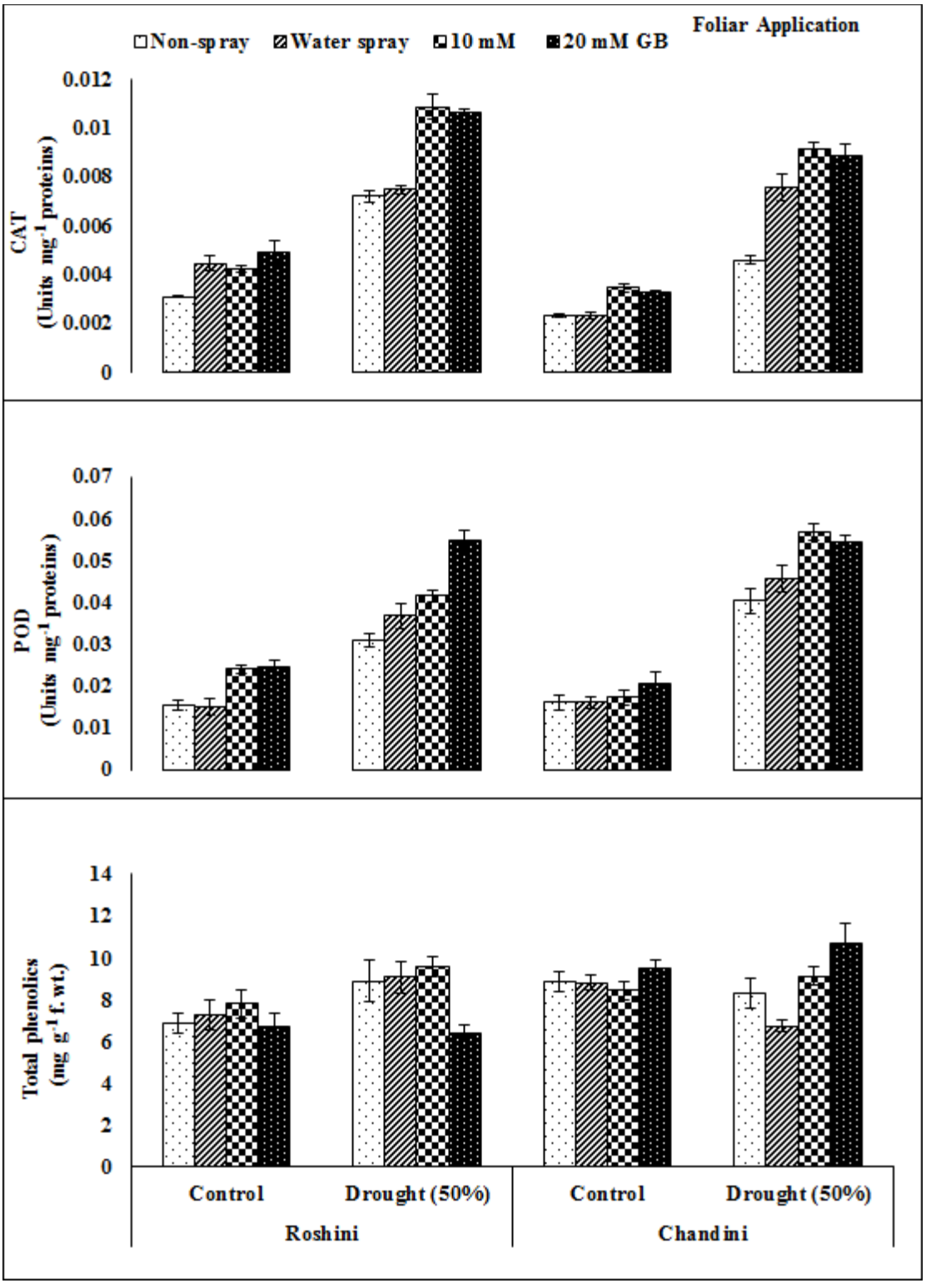

Figure 3.4: Catalase, peroxidase and total phenolics of flax plant (*Linum usitatissimum* L.) varieties (Roshini and Chandani) when 60 day old plants treated as foliar treatment with glycinebetaine under control and drought stress conditions

Leaf ascorbic acid profoundly ($P \leq 0.001$) enhanced in both flax varieties but Roshini exhibited better ascorbic acid ratio as compared to Chandini under both controlled and drought stress situation. Glycinebetaine showed non-significant impact on leaf ascorbic acid content. V×D×GB interaction was also non-significant (Table 3.3; Fig 3.5).

Under water stress conditions, total free proline content exhibited a significant increase ($P \leq 0.001$) in both flax varieties. A highly significant varietal difference ($P \leq 0.001$) was noted as Chandini exhibiting superior total free proline content as compared to Roshini in both controlled and stress circumstances. Foliar application of glycinebetaine (GB) significantly ($P \leq 0.001$) improved total free proline content. 20 mM GB application yielding the most pronounced effect in both varieties under both conditions (Table 3.3; Fig 3.5)

The exogenous application of glycinebetaine (GB) significantly augmented ($P \leq 0.001$) the internal GB content in both flax varieties. Chandini exhibited greater tolerance to stress and non-stress conditions showing a superior adaptive response as compared to Roshini. Furthermore, GB application significantly enhanced ($P \leq 0.001$) the endogenous GB level, off various level, 20 mM GB eliciting the most pronounced effect in both varieties under both stress and non-stress situations (Table 3.3; Fig 3.5). Under drought stress conditions total soluble proteins enhanced significantly ($P \leq 0.01$) in both flax varieties. Exogenously applied glycinebetaine ($P \leq 0.001$) enhanced the total soluble protein in both flax varieties but 20 mM performed better in both varieties under all situations (Table 3; Fig 6)

Imposition of water stress profoundly ($P \leq 0.001$) increased the flavonoids content in both flax varieties. Foliar application of glycinebetaine significantly ($P \leq 0.001$) increased the flavonoids content in both varieties under controlled and stress situations and 10 mM concentration of GB performed best under both conditions in both varieties. Over all, varietal difference was non-significant (Table 3.3; Fig 3.6). Co-relation between drought stress and variety was highly ($P \leq 0.001$) significant as Chandini contain more total free amino acid content as compared to Roshini in stress situation. Interaction between glycinebetaine and variety was also observed significant ($P \leq 0.001$) as 10 mM GB concentration performed better in Chandini and 20 mM in Roshini under drought conditions. Over all interaction between V×D×GB was showed highly significant ($P \leq 0.001$) (Table 3.3; Fig 3.6).

Table 3.3: Mean squares from analysis of variance of data for leaf ascorbic acid, total free proline, glycinebetaine, total soluble proteins, Flavonoids and Total free amino acids of flax (*Linum usitatissimum* L.) when 60 days old plants treated as foliar treatment with glycinebetaine under drought stress conditions.

SOV	**df**	**Leaf ascorbic acid**	**Total free proline**	**Glycinebetaine**
Varieties (V)	1	0.004***	4.322***	0.419***
Drought (D)	1	0.00007 ns	6.065***	0.081 ns
Glycinebetaine (GB)	3	0.0003 ns	4.053***	1.070***
V × D	1	0.00001 ns	0.066 ns	0.103 ns
V × GB	3	0.00001 ns	2.412***	0.042 ns
D × GB	3	0.00005 ns	0.733 ns	0.141**
V × D × GB	3	0.00002 ns	0.630 ns	0.051 ns
Error	48	0.0001	0.342	0.026
SOV	**df**	**Total soluble proteins**	**Flavonoids**	**Total free amino acids**
Varieties (V)	1	0.014 ns	0.001 ns	0.096 ns
Drought (D)	1	0.683**	1.140***	0.115 ns
Glycinebetaine (GB)	3	0.685***	1.035***	0.218*
V × D	1	0.208 ns	0.254 ns	3.134***
V × GB	3	0.064 ns	0.089 ns	0.552***
D × GB	3	0.03 ns	0.319*	0.448***
V × D × GB	3	0.040 ns	0.111 ns	0.481***
Error	48	0.089	0.082	0.054

*,** and *** significant at 0.05, 0.01 and 0.001 levels, respectively; ns = non-significant

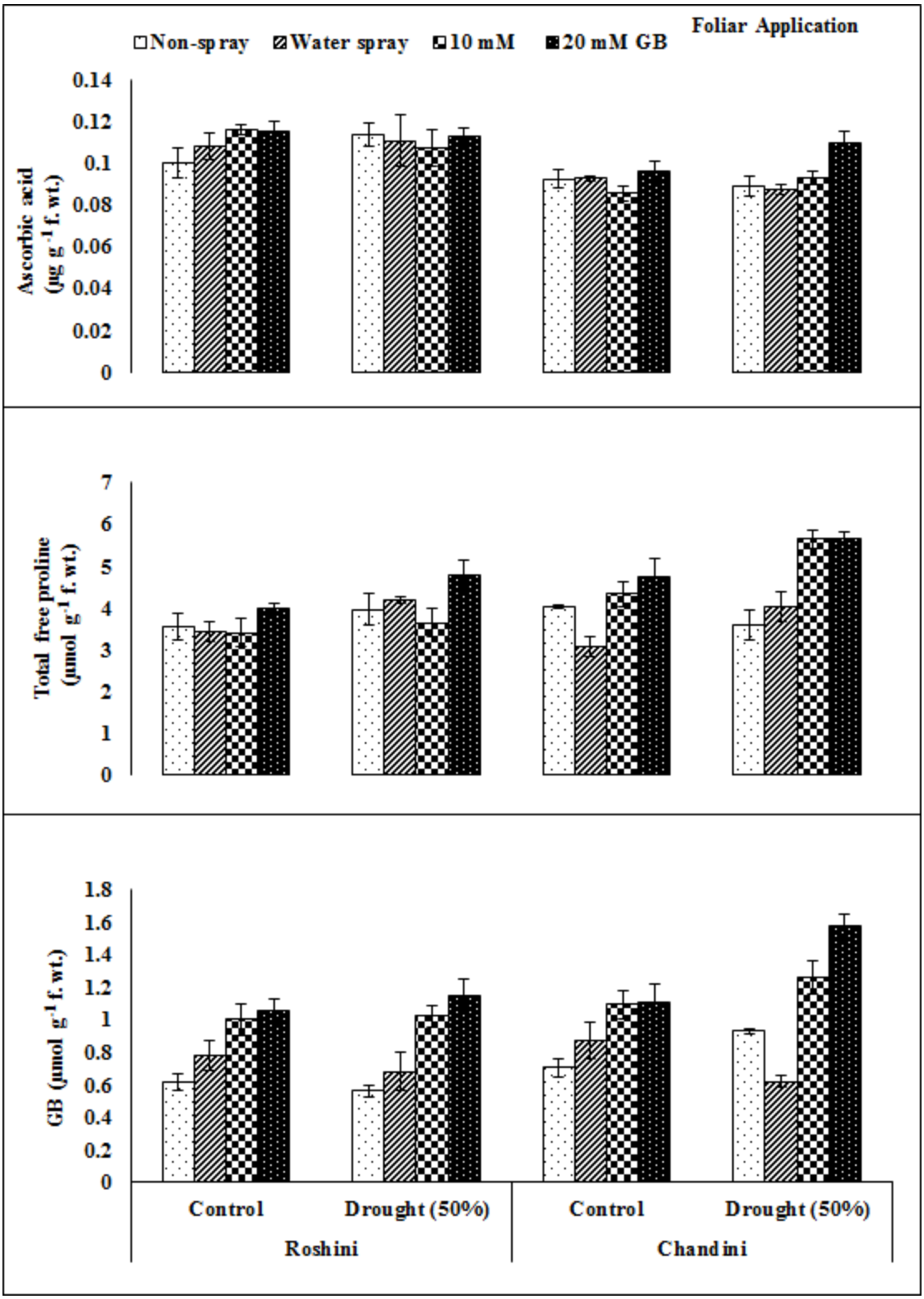

Figure 3.5: Ascorbic acid, total free proline and glycinebetaine of flax plant (*Linum usitatissimum* L.) varieties (Roshini and Chandani) when 60 day old plants treated as foliar treatment with glycinebetaine under control and drought stress conditions.

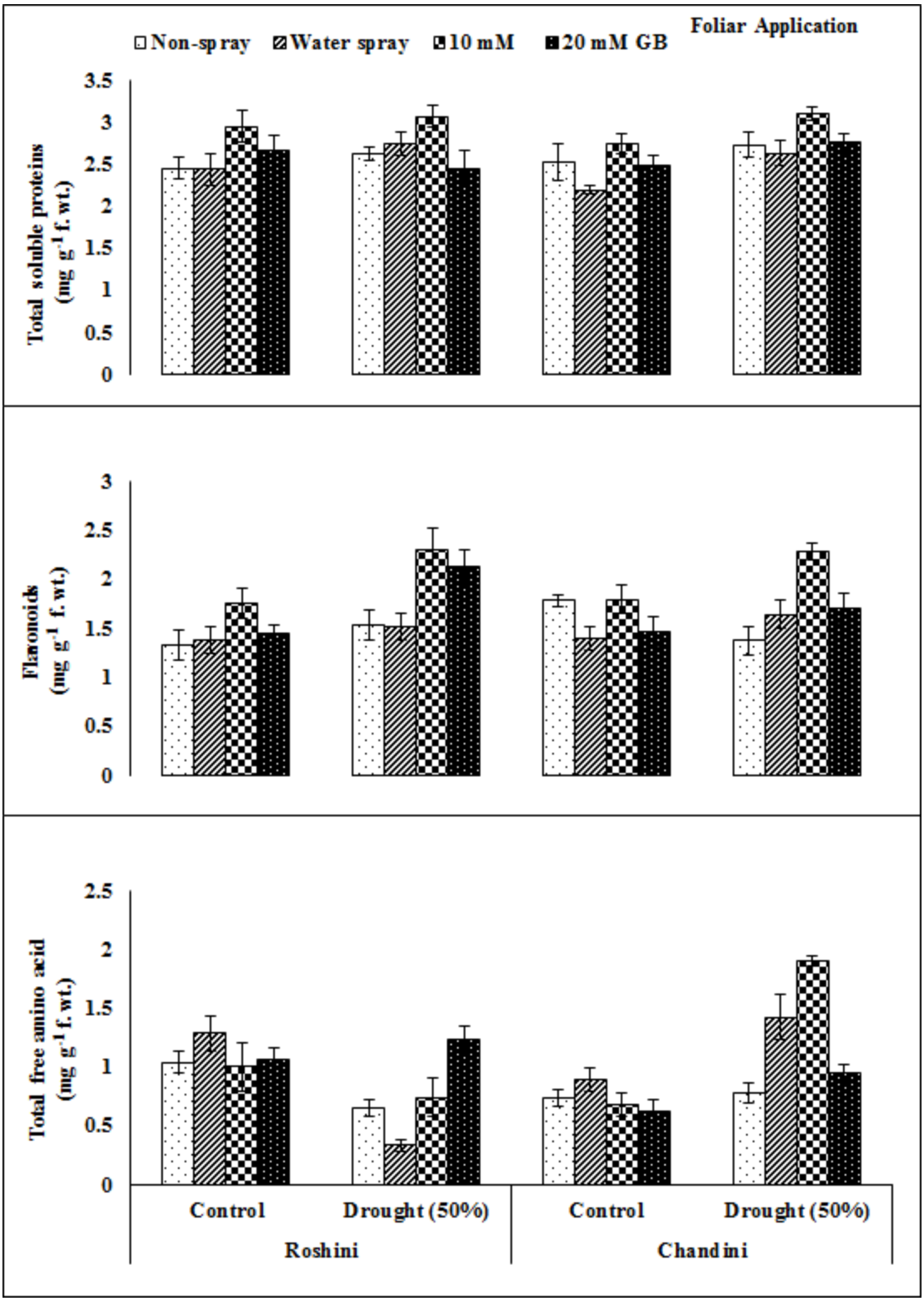

Figure 3.6: Total soluble proteins, flavonoids and Total free amino acids of flax plant (*Linum usitatissimum* L.) varieties (Roshini and Chandani) when 60 day old plants treated as foliar treatment with glycinebetaine under control and drought stress conditions.

MDA (Malondialdehyde) content was significantly ($P \leq 0.01$) increased in Roshini. Under stress condition MDA profoundly ($P \leq 0.001$) increased in both flax varieties. Exogenously applied GB decreased the MDA concentration in Roshini. Overall, 10 mM GB concentration performed best in both flax varieties under drought situations (Table 3.4; Fig 3.7)

Hydrogen peroxide (H_2O_2) level enhanced considerably ($P \leq 0.001$) in both flax varieties when drought was applied. Foliar application of glycinebetaine significantly ($P \leq 0.001$) decreased the H_2O_2 in both flax varieties. Roshini showed significantly ($P \leq 0.01$) lower H_2O_2 content as compared to Chandini in water stress conditions. Application of GB decreased the hydrogen peroxide level in both varieties under stress conditions. Overall, 20 mM GB level performed better in all conditions (Table 3.4; Fig 3.7)

Application of 50% field capacity increased the leaf sodium (Na^+) significantly ($P \leq 0.01$) in both varieties. Varietal difference was non-significant. Foliar application of GB profoundly ($P \leq 0.01$) lowered the level of leaf sodium (Na^+) in both flax varieties but 20 mM GB level performed better under drought conditions. Interaction of V×D×GB was non-significant (Table 3.4; Fig 3.7)

Application of 50% field capacity decreased the leaf potassium (K^+) significantly ($P \leq 0.001$) in both flax varieties. Varietal difference was non-significant. Both levels of foliar applied glycinebetaine considerably ($P \leq 0.001$) increased the shoot potassium under drought and controlled conditions but 10 mM GB level performed better in Roshini and 20 mM in Chandini in all conditions (Table 3.4; Fig 3.8)

Drought stress reduced the shoot calcium level profoundly ($P \leq 0.001$) in both flax varieties (Roshini and Chandini). Varietal difference was non-significant. No interaction was observed between V×D×GB as co- relation was also observed non-significant (Table 3.4; Fig 8)

Number of pods/plant decreased significantly ($P \leq 0.001$) in both flax varieties under moisture stress situation. Varietal difference was also highly significant ($P \leq 0.001$) as Chandini showed more number of pods/plant. Exogenously applied GB significantly ($P \leq 0.001$) increased the number of pods in both varieties but 10 mM showed better in Roshini but 20 mM performed best in Chandini in controlled and stress conditions (Table 3.4; Fig 3.8)

Table 3.4: Mean squares from analysis of variance of data for malondialdehyde, hydrogen per oxide, shoot Na^+, K^+,Ca^{2+} and number of pods/ plants of flax (*Linum usitatissimum* L.) when 60 days old plants treated as foliar treatment with glycinebetaine under drought stress conditions.

SOV	**df**	**Malondialdehyde**	**Hydrogen peroxide**	**Shoot Na^+**
Varieties (V)	1	1.669**	0.00001 ns	3.062 ns
Drought (D)	1	9.117***	0.0001***	11.390**
Glycinebetaine (GB)	3	1.398***	0.00005***	5.276**
V × D	1	0.815*	0.00004**	1.562 ns
V × GB	3	0.569*	0.000002 ns	0.927 ns
D × GB	3	1.158***	0.00001 ns	1.359 ns
V × D × GB	3	0.917***	0.000007 ns	0.010 ns
Error	48	0.141	0.000005	0.997
SOV	**df**	**Shoot K^+**	**Shoot Ca^{2+}**	**Number of pods/ plants**
Varieties (V)	1	0.765 ns	0.140 ns	95.062***
Drought (D)	1	124.557***	26.265***	4192.562***
Glycinebetaine (GB)	3	161.317***	0.317 ns	53.562***
V × D	1	1.429 ns	0.140 ns	105.062***
V × GB	3	14.359 ns	0.463 ns	1.729***
D × GB	3	6.072 ns	0.317 ns	1.729***
V × D × GB	3	17.197 ns	0.213 ns	5.895*
Error	48	6.294	0.380	1.5

*,** and *** significant at 0.05, 0.01 and 0.001 levels, respectively; ns = non-significant

Na = Sodium; K^+ = Potassium; Ca^{2+} = Calcium

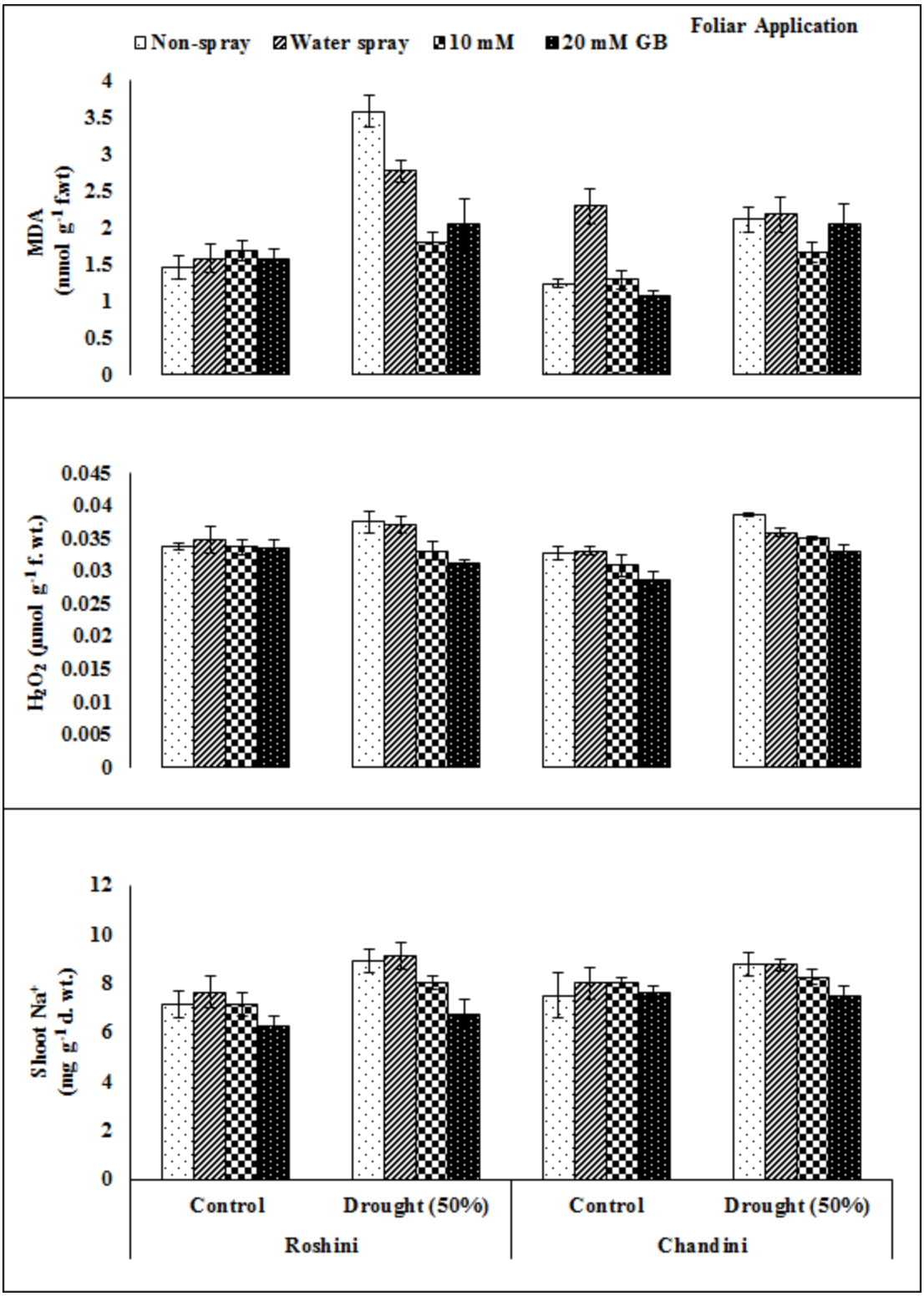

Figure 3.7: Malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and shoot Sodium (Na^+) of flax plant (*Linum usitatissimum* L.) varieties (Roshini and Chandani) when 60 day old plants treated as foliar treatment with glycinebetaine under control and drought stress conditions.

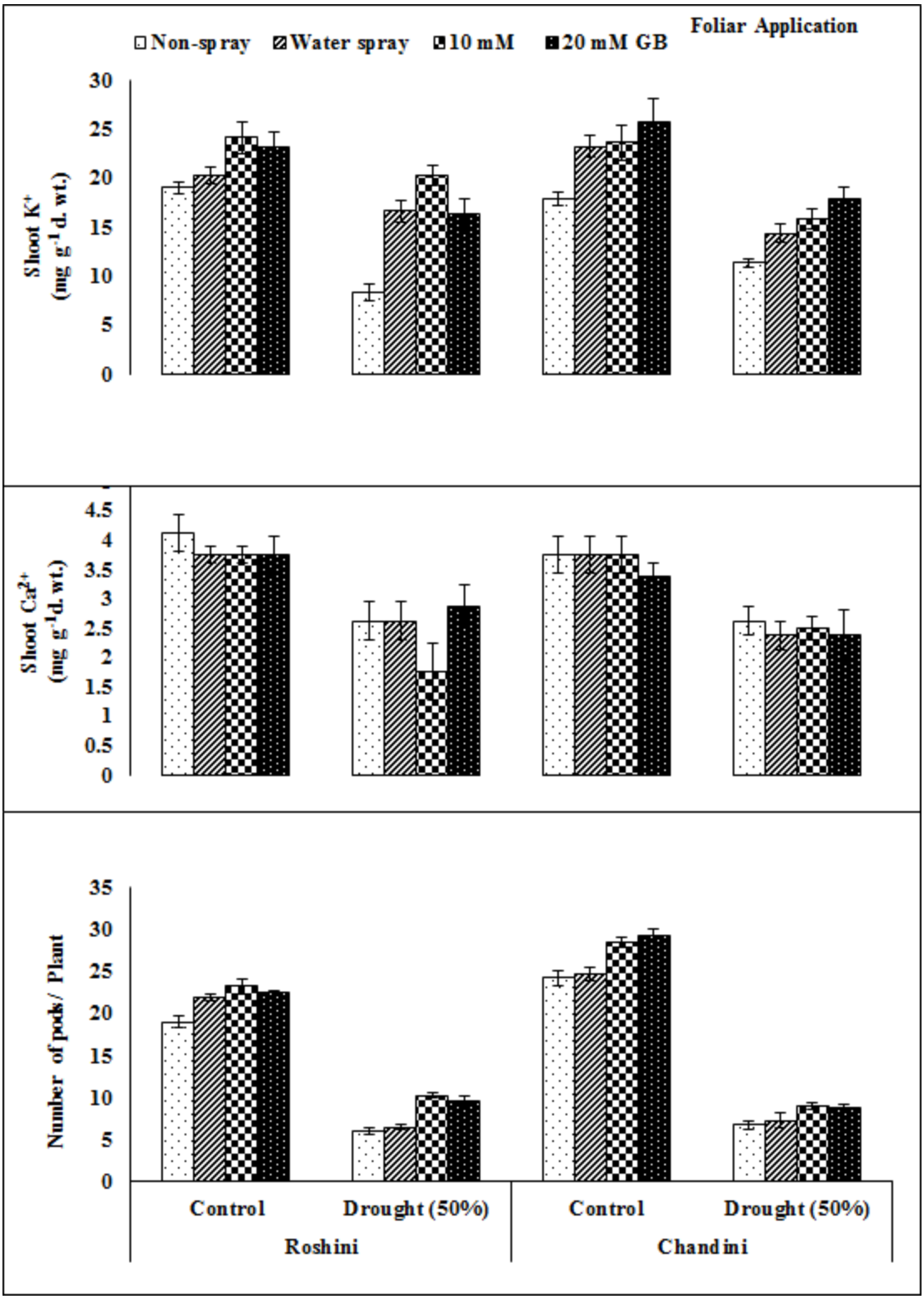

Figure 3.8: Shoot potassium (K^+), Shoot Calcium (Ca^{2+}) and number of pods per plant of flax (*Linum usitatissimum* L.) varieties (Roshini and Chandani) when 60 day old plants treated as foliar treatment with glycinebetaine under control and drought stress conditions.

Drought stress profoundly ($P \leq 0.001$) reduced the total seed weight in both flax varieties. GB treatment as foliar application remarkably ($P \leq 0.001$) increased the total seed weight in both varieties. Varietal difference was also significant ($P \leq 0.01$) as Roshini performed better in controlled conditions. Interaction between variety and drought which described Roshini performed slightly ($P \leq 0.05$) better in stress situation. Off various GB levels 20 mM performed better in Roshini and 10 mM in Chandini in controlled conditions but 10 mM in Roshini and 20 mM in Chandini in stress situation (Table 3.5; Fig 3.9)

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Table 3.5: Mean squares from analysis of variance of data for total seed weight/ plants of flax (*Linum usitatissimum* L.) when 60 days old plants treated as foliar treatment with glycinebetaine under drought stress conditions.

SOV	df	Total seed weight/plant
Varieties (V)	1	0.1620**
Drought (D)	1	56.655***
Glycinebetaine (GB)	3	0.326***
V × D	1	0.088*
V × GB	3	0.040 ns
D × GB	3	0.025 ns
V × D × GB	3	0.051*
Error	48	0.014

*,** and *** significant at 0.05, 0.01 and 0.001 levels, respectively; ns = non-significant

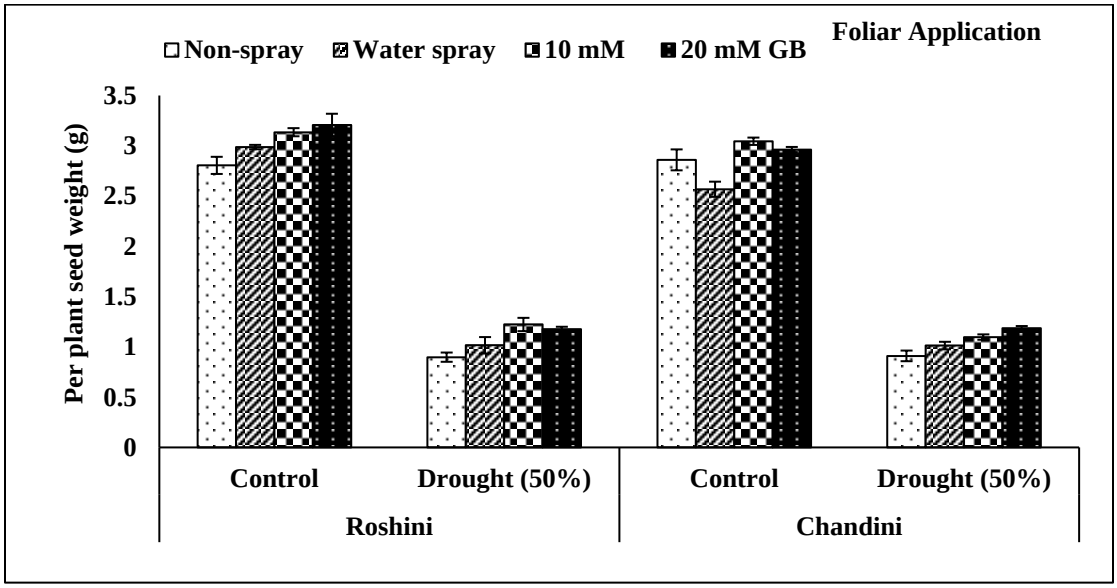

Figure 3.9: Per plant seed weight of flax plant (*Linum usitatissimum* L.) varieties (Roshini and Chandani) when 60 day old plants treated as foliar treatment with glycinebetaine under control and drought stress conditions.

IV. DISCUSSION

4.1 Growth parameters and photosynthetic pigments

The diverse mechanism of plants disturbed due to less moisture availability such as photosynthetic machinery, metabolism and uptake of different indispensable nutrients, ions absorption and others important activities which eventually deteriorated the growth related parameters and wilting of plants (Li *et al*., 2011). In current investigations, it is absolutely practiced that in moisture shortage circumstance the abnormal diminished of the shoot fresh and dry weight was witnessed as well as it also repressed the shoot length and chlorophyll pigments in flax plant analogous findings were seen safflower (Rahmani *et al*., 2019) and maize (Shafiq *et al*., 2021) and flax (Abid and Shahbaz 2022). This was may be the after effects of interruption or impaired biosynthesis of chlorophyll molecules and other accessory molecules (Shafiq *et al*., 2021). Foliage application of glycinebetaine (GB) as foliar spray enhanced the chlorophyll pigments and its content ratio in both flax varieties such as Roshini and chandini under drought and controlled environments. Earlier findings also revealed clearly the enhanced content of chlorophyll pigments in rapeseed (Bhuiyan *et al*., 2019) and maize as GB also stabilize the chlorophyll pigments, its structure and activity and also protect from damage under water scarce circumstances. Moreover, it also soothing out the structures of Rubisco enzyme, other natural membranes and tissues in plants under stress situations. Moreover, glycinebetaine boost up the activity of photosynthesis (Shafiq *et al*., 2021, Khan *et al*., 2015).

4.2 Reactive oxygen species and Enzymatic Antioxidants

Under water deficit situation reactive oxygen species (ROS) gathered in excess quantity in *Camellia Sinensis* L. (Kumar and Yadav 2009) such as MDA and H_2O_2 which are very relatable with our recent observations as ROS produced in bulk quantity in both flax varieties under drought stress. Excessive cellular interruption occurred due to ROS formation which causes loss in plant physical permeability (Chen *et al*., 2000). Moisture stress situation mean while improved the SOD, POD and CAT activity in current experimental conditions in both flax varieties relatable findings were previously observed in wheat (Huseynova *et al*., 2012) leaves as enzymatic antioxidants are produced in contracting with stress to nullify are reduced the impact of ROS (reactive oxygen species) (H_2O_2, MDA) and increase the tolerance in plants (Polle

2001). ROS are produced under normal circumstances too but its content is low. Under stressful situation the content of ROS increased in plants so enzymatic antioxidants functioning definitely increased in plants (Laloi and Apel, 2004). Glycinebetaine as foliar application also augmented the enzymatic antioxidants ability in stress situation in maize (Anjum *et al*., 2012) cotton (Prajapat *et al*., 2018) and decrease the ROS content in both flax varieties. These findings are similar with our current results. Enhanced ROS production cause excessive cellular breakdown (Chen *et al*., 2000). Exogenous applied GB increased the endogenous level of GB that delimited the antioxidants ability to ROS excessive degradation (Demiral and Turkan 2004).

4.3 Non- Enzymatic Antioxidants activity

Non- enzymatic antioxidants such as leaf ascorbic acid, total free proline, total phenolics, total free amino acids glycinebetaine flavonoids and total soluble protein proficiency increased in drought induced flax varieties in current experiment. Similar observations was detected in previous findings such as in wheat (Chandrasekar *et al*., 2000), millet (Assefa and Fatene 2013) and canna plant (Zhang *et al*., 2013) which increase the tolerance against stress in plants. Ascorbic acid keenly protect plants from any cellular damage via different processes and it eradicate the activity ROS by abolish H_2O_2 (Chugh *et al*., 2011). Different plants accumulate different inorganic and organic solutes under to cope with stress situation to uphold turgor pressure in cells. Total soluble protein increased in stress circumstances to improve water uptake in desiccating soil (Zhang *et al*., 2013). Total free proline, GB, total phenolics rose up under stress circumstances because these osmoprotactants managed the water stability in cells, protects enzymes and macromolecules from damage, boosted up the functioning of all enzymes meanwhile lower down the amount of ROS and develop the tolerance of plant from any oxidative injury (Molla *et al*., 2014). Externally applied glycinebetaine increased the level of total free proline, ascorbic acid, total free amino acids and internal level of glycinebetaine in current experimental findings. The reason behind this context is the positive role of GB which boosted up the stress tolerance level in both flax varieties and also increased the overall yield by up regulation the mechanism of growth and yield production in any stress situation. Similar findings were documented in quinoa (Yaqoob *et al*., 2019) and in pea (Osman, 2015). Glycinebetaine, total phenolics as well as total free proline are assumed to have great tendency to stabilize the DNA and gene structure and functioning in plants which also regulates the gene

expressions by scavenging reactive oxygen species. Total free amino acids content increased by ex-foliar practice of GB in current findings enhance the total free amino acids n flax leaves which not only help in osmoregulation but also play a fantastic role in ions absorption such as potassium and calcium (Rai, 2002). Under stress situation flavonoids content was increased in both varieties of flax which correlates with previous experimental findings such as in potato. Flavonoids act as an osmolytes. It has dual properties such as metal chelators and scavenger of free radicals and singlet oxygen which produce during metabolism in plants. It also prevent from lipid denaturation (Chu *et al*., 2000). Exogenous spray of GB boosts up the flavonoids amount in peach fruit which justified our current observations. Increased flavonoids under stress situation clearly disclosed that flavonoid generally increased under stress circumstances and protect plant from any oxidative harm and amplified the tolerance in plant against any stress (Wang *et al*., 2019).

4.4 Ions analysis

Drought stress decreased the potassium and decreased the potassium level in both flax varieties. Under stress situation plants accumulates different osmolytes such as GB, total free amines to regulates ions and sustain cellular water. On the other hand drought disturbs cation- anion equilibrium and plant destruct its turgor pressure (Flower and Yeo, 1986). Decreased potassium level observed in rice shoots which hindered the growth features under stress because potassium plays a tremendous role in enzymes stimulation followed by photosynthetic role. Low level potassium also lesser down the osmotic ability of plant and leads to stomatal closure which also observed in maize varieties (Andrade *et al*., 2018). Foliar applied GB augmented the potassium but lesser down the sodium content in both flax varieties in current findings. Similar result was justified in wheat cultivars it may be due to increased potassium content decreased the sodium content or other ions by persistent increase of glycinebetaine in tissues under any stress situation. Augmented content of potassium and GB supports in osmotic adjustment and leads to good plant growth and yield in stress (Raza *et al*., 2007).

4.5 Yield attributes

Water shortage situation declined the overall yield of plant in current experiment as number of pods/ plant and total yield of plant reduced in both flax varieties similar findings were observed

in sugarcane (Vasantha *et al*., 2005) and in legumes by declining the total number of flower (Fang *et al*., 2010). Low moisture content was the basic reason of closing stomata, which is the main indications to lesser CO_2 intake, distressing the proportion of photosynthesis and eventually diminishes growth and total yield output of any crop (Chaves *et al*., 2009). Foliar application of glycinebetaine boost up the yield of wheat crop which is similar with our latest findings as in previous reports it is clearly indicated that plant have natural ability to utilize and translocate the exogenously applied GB in different parts of plant and also increased the internal ratio of GB under stress circumstances (Díaz-Zorita *et al*., 2001). Glycinebetaine enhanced the water absorbing capacity and boost up the choline production in plants eventually prevent chlorophyll from degradation and increased the crop yield (Miri *et al*., 2021).

Conclusions

Drought stress (50%) significantly decreased morphological and physiological parameters in both flax varieties (Roshini and Chandini), including shoot (growth, fresh, dry weight) chlorophyll pigments ((chlorophyll *a*, *b*, total chorophyll and carotenoids), mineral ions (potassium and calcium) and yield attributes. However, enzymatic and non-enzymatic antioxidant activities increased in response to drought stress. Foliar application of glycinebetaine (GB) at various concentrations (0, 10, 20 mM) enhanced morphological, physiological, and biochemical attributes in both varieties under stress and controlled conditions. Exogenous GB application increased internal GB levels, boosted antioxidant enzymes activity (superoxide dismutase, catalase, and peroxidase), and reduced oxidative stress markers (MDA and H_2O_2). GB treated plants showed improved yield attributes in both stress and non-stress conditions. Optimization analysis revealed that 10 mM GB in the Chandini variety was the most effective, exhibiting enhanced resilience and adaptability under both controlled and stress conditions.

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