Plant Archives Volume 20 No. 2, 2020 pp. 8535-8545 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

AMELIORATING EFFECT OF PROLINE IN VITRO ON PHYSIOLOGICAL AND SECONDARY METABOLITE ANALYSIS OF *SILYBUM MARIANUM* L. GROWN UNDER ABIOTIC STRESS CONDITION (WATER DEFICIT STRESS)

Somvir Singh*, Shalini Jaswal, Rahul Bhatia, Vishav Kiran, Sakshi Gupta, Priyanka Ratwan, Vandna Devi, Arti Thakur and Sunil Puri

School of Biological and Environmental Sciences, Faculty of Sciences, Shoolini University of Biotechnology and Management Sciences, Solan (Himachal Pradesh), India.

Abstract

Physiological and secondary metabolite profile of *Silybum marianum* L. grown in hydroponic cultures in controlled condition of light (PAR: 40µmol m^{-2} s^{-1}) and temperature (25±2ºc) evaluated to recognize the ameliorative role of proline against the negative effects of water deficit stress. Physiological analysis (carbohydrate, protein, proline, relative water content, chlorophyll) and secondary metabolite profile (phenol, flavonoid, alkaloid, saponin and glycosides) were determined after 45 days. Exogenous application of proline 50µg/l, 100µg/l and 150µg/l were standardized and applied to different PEG-6000 concentration 0.2, 0.4, 0.6, 0.8 and 1% in hydroponic solution. *Proline enhanced the physiological and secondary metabolites in stressed plant; it is play a major role in cellular osmotic adjustment.* Proline in stressed plants may have a significant role in osmotic adjustment. It could concluded that the addition of proline can ameliorate the oxidative stress in *Silybum marianum* L. stressed plants. This study also provided evidence for the ability of *Silybum marianum* L. plants grown in water deficit condition due to its capacity for osmotic adjustment.

Key words: Abiotic Stress, Antioxidant, Medicinal Plants, Proline, Water Deficit Stress

Introduction

Silybum marianum L. is an annual medicinal plant belonging to the Asteraceae family. It also known as milk thistle and native to Southern Europe. In India, Western Himalayas and Kashmir are its natural habitat. Its medicinal effects documented among the alternative medicines referred to as liver and bile- related diseases remedy (Fraschini *et al.,* 2002; Kurkin, 2003). Milk thistle oil has suggested for suitable edible oil and a vitamin E rich source it contains a phenolic compound known as silibinin, which is a major constituent of silymarin an extract of milk thistle seeds. (El- Mallah *et al.,* 2003). Abiotic stresses such as high temperature, freezing/cold, drought/water, salinity, heavy metals, UV radiation and nutrient stresses have adverse effects on the physiology, biochemistry, molecular mechanisms, and survival of plants (Atkinson and Urwin 2012; Jeandroz and Lamotte 2017). In the scenario of global climatic change, different biotic and abiotic stresses are severe threats to the agricultural production worldwide. In nature, plants continuously stressed by exposure to multiple adverse conditions. Reactive oxygen species due to unfavorable environmental conditions such as drought, salinity, heavy metals, herbicides, nutrient deficiency, or radiation. Abiotic stress also lead to oxidative stress in the plant cell resulting in a higher leakage of electrons towards O_2 during photosynthetic and respiratory processes, which cause enhancement of reactive oxygen species (ROS) generation. Several ROS continuously produced in plants as byproducts of many metabolic reactions such as photosynthesis, photorespiration and respiration. Depending on the nature of the ROS, some are highly toxic and rapidly detoxified by various cellular enzymatic and non-enzymatic mechanisms. Abiotic stresses especially drought has the greatest effect on medicinal plants and do not generally adapt quickly to these stresses. Water stress may arise either due to excess of water

***Author for correspondence :** E-mail : somvirsingh@shooliniuniversity.com

or water-deficit in which water-deficit is a meteorological term also known as drought stress (Jaleel *et al*., 2009). Generally, drought stress occurs in plants when the available water in the soil reduced and atmospheric conditions cause permanent wilting by transpiration or evaporation processes. Around the world agricultural productivity may affected by major environmental factor i.e., drought which may result in yield reduction. Under drought conditions, the ability of plant to grow and reproduce satisfactorily known as drought resistance and plant's ability to slowly modify its structure and function so that it can better tolerate drought known as drought acclimation. To ensure the prosperity of their offspring and their own survival and plants have evolved a range of strategies to cope with various abiotic stresses (Jaleel *et al*., 2009). Those were controlled by various antioxidant defense systems CAT, APX, POX, SOD, MDHAR, DHAR, GR, ascorbate, glutathione, carotenoids, phenolic compounds, proline, glycine betaine, sugar, and polyamines (Gill and Tuteja, 2010; Karuppanapandian *et al.,* 2011). Antioxidant defense mechanisms allow plants to adapt and survive stressful events. Organic osmolytes play a vital role in minimizing the harmful effects of abiotic stresses, including amino acids and their derivatives, polyols and sugars, methylamines, and methylsulfonium compounds (Yancey 2005). Initially, the term osmolyte referred to molecules that are overproduced and accumulate during osmotic stress to maintain homeostasis in a cell or in the surrounding fluid. Later, the term osmolyte included any solute or metabolite produced and accumulated for protecting the cell environment against harmful effects caused by abiotic stress. The osmolytes include various biochemical such as sugars, polyamines, secondary metabolites, amino acids, methylamines, and polyols, which protect or neutralize the damaging effects of abiotic stresses, and protect cells and make them tolerant of the particular abiotic stress (Roychoudhury and Banerjee 2016). Amino acid, proline is most important and well known for its role in changing abiotic stress environments. Proline is the most extensively studied osmolyte because of its great importance in stress tolerance (Kamran *et al*., 2009). The exogenous application of proline can increase its endogenous levels in plant tissues subjected to water stress conditions, which help maintain osmotic adjustment in plant tissues. It may be a good source of minimizing the adverse effects of water stress on plants, and triggering their growth depends upon the type of plant species and its concentration (Ali *et al.,* 2007). For osmotic adjustment, proline contributes to stabilizing subcellular structures (*e.g.,* membranes and proteins), scavenging free radicals, and buffering cellular redox potential under stress conditions. A rapid breakdown of proline upon relief of stress may provide sufficient reducing agents that support mitochondrial oxidative phosphorylation and generation of ATP for recovery from stress and repairing of stress-induced damages (Hare and Cress, 1997). The accumulation of proline in drought-tolerant and drought-sensitive cultivars has revealed the significance of this osmolyte (Iqbal *et al.,* 2016). The role of proline in induced PEG experiment gave evidence that the higher levels of proline are due to the emergent need of stressed plant. This osmolyte is able to control the osmotic regulation of the cellular environment because of its high water solubility and protects cell membranes against adverse effects of drought stress. It is also functional as a protein compatible hydrotrope and as a hydroxyl radical scavenger (Fahad *et al.,* 2015).

Materials and Methods

Plant growth

Seeds of *Silybum marianum* L. selected for uniformity (based on color and size). Before germination, damaged and insect infected seeds discarded and the empty ones eliminated using floating method in distilled water. Surface sterilization of seeds of *S. marianum* done with 0.1% $HgCl_2$ for 2-5 minutes, after which the seeds rinsed three times with distilled water. The surface sterilized seeds were soaked in distilled water (control), 0.2, 0.4, 0.6, 0.8 and 1%, PEG-6000 for 24 hours. Thereafter, the seeds transferred to petriplates lined with three layers of filter paper moistened by distilled water (control) and different concentration of PEG-6000. The seeds allowed for germination in an incubator at 25 ± 2°C under continuous illumination provided by fluorescent white light. The 10 days old seedling shifted to hydroponic culture containing Hoagland nutrient solution. Plastic pots of 300 ml capacity used for plant culture. Three plants per pot grown by holding the plants in holes into the thermocol lids with the help of cotton wool. Fresh one at regular intervals appropriately replaced the nutrient medium during the growth of the plants. The plastic pots along with plants shifted to the BOD incubator at 25 ±2°C for further growth. After 10 days of shifting to BOD, the plants treated with 0.2, 0.4, 0.6, 0.8 and 1%, PEG-6000 through appropriate addition to the nutrient medium and different concentration of proline 50, 100 and 150 µg/l were standardized and applied though foliar spray. After 45 days of treatment, various physiological (carbohydrate, protein, proline, relative water content, chlorophyll) and secondary metabolite profile (phenol, flavonoid, alkaloid, saponin, glycosides) analysis were done.

Estimation of Carbohydrate

Total carbohydrate were determined in plant tissue

method described by (Hedge and Hofreiter, 1962). Weighed 100 mg of the sample. Hydrolyzed via keeping it in hot water bath aimed at 180 minutes through 5 mL of 2.5N HCl then cooled. Deactivated it through dense sodium carbonate until the bubbliness finishes. Centrifuged at 10,000 rpm for 5 minutes. Collected the supernatant and took 0.5 then 1ml aliquots for examination. Made up the volume toward 1ml in all the tubes comprising the sample tubes by addition of refined water. Then, added 4 ml of anthrone reagent. Heated aimed at eight minutes in a hot water bath. Cooled rapidly and read the green to dark green color at 630nm. Drawn a normal graph by scheming absorption of the standard on the X-axis versus absorbance proceeding the Y-axis from the graph calculated the quantity of carbohydrate existing in the sample tube.

Estimation of Protein Content

Protein estimated by method as described by (Lowry *et al*., 1951).Weighed 0.5gm of the sample then grind well through a pestle, mortar in 5-10 ml of the phosphate buffer. Centrifuged and cast-off the supernatant aimed at protein approximation. Pipette obtainable 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard hooked on a series of test tube. Pipette obtainable 0.2 ml of the sample extract in test tubes. A tube through 1mL of water aided as the blank. Added 5 ml of mixture C (alkaline copper solution) to each tube including the blank. Mixed well and permissible to stance for 10min. Then added 0.5 ml of reagent D (Folin-Ciocalteau Reagent) mixed well and kept at room temperature in the dark aimed at 30min. Blue color developed. Took the reading at 660nm. Drawn a standard graph using BSA and calculated the amount of protein in the sample.

Estimation of Proline Content

Proline measured by the method given by (Bates *et al.,* 1973). Extracted 0.5g of plant material by homogenizing in 10 ml of 3% aqueous sulphosalicylic acid. Filtered the homogenate through Whattman No. 2 filter paper. Took 2 ml of remainder in a test tube and added 2 ml of glacial acetic acid and 2 ml acid ninhydrin. Heated it in the boiling water bath for 1h. Terminated the reaction by retaining the tube in ice bath. Added 4 ml toluene to the reaction mixture and stirred well for 20-30 second separated the toluene layer and warmed to room temperature. Measured the red color intensity at 520nm. Ran a series of standards with pure proline in a similar way and prepared a standard curve.

Relative Water Content (RWC)

The fresh weight of top leaves from each treatment recorded. The leaves immersed in distilled water in beakers and left for 24 h. Thereafter, fully turgid leaves weighed again. The leaves dried in oven for 72 h at 70 ºC, until constant weight of leaves obtained. Relative water content (RWC) of leaves calculated according to (Wheatherley, 1950).

$$RWC = \frac{fresh\ mass - dry\ mass}{saturated\ mass - dry\ mass} \times 100$$

Chlorophyll

Chlorophyll extraction done by using dimethyl sulphoxide (DMSO) chlorophyll extraction technique (Hiscox and Israelstam, 1979). Instead of the extractions, glass centrifuge vessels containing 7 ml DMSO heated to 65°C in a water bath. The spectrophotometer calibrated to zero utilizing an outright of pure DMSO. The absorbance of individually blank as well as sample measured at 645 and 663 nm.

Phenol

Phenol content estimated by (Malick and Singh, 1980). 1g of the sample (leaf and root) and ground it with a pestle and mortar in 10-time volume of 80% ethanol centrifuged at 10,000 rpm for 20 min. Re-extracted the residue with five times the volume of 80% ethanol, centrifuged and pooled the supernatants. Supernatant then evaporated to dryness residues dissolved in distilled water. Different aliquots (0.2 to 2 ml) into test tubes pipetted out and volume made up in each tube to 3 ml with water. Folin-Ciocalteau reagent added after 3 min, added 2 ml of 20% Na_2CO_3 solution to each tube and mixed thoroughly. Placed the tubes in a boiling water bath for one minute, cooled and measured the absorbance at 650 nm against a reagent blank. Standard curve using different concentrations of gallic acid was prepared. From the standard curve, the concentration of phenols in the test sample was determined and expressed as mg/g material.

Flavonoids

The samples homogenized at the rate of 0.1 g per ml of 80% methanol. The methanolic extract (250 µl) mixed with 1.25 ml of distilled water and 75 µl of a 5% $NaNO_2$ solution. After 5 min, 150 µl of a 10% $AlCl_3.H_2O$ solution added and filtered for 6 min. About 500 µl of 1M NaOH and 275µl of distilled water added to the mixture well and the intensity of pink colour measured at 510 nm. The level of total flavonoids concentration was calculated using quercetin (QU) as a standard. The results expressed as mg of quercetin/g fresh weight of plant sample (Jia *et al.,* 1999).

Determination of Alkaloid

Adopted the method given by (Omoruyi *et al.,* 2012).

5 g of plant extract mixed with 200 mL of 10% acetic acid in ethanol. The mixture covered then permissible toward stand for 4 h. This mixture filtered than the remainder stood concentrated on a hot water bath to a quarter of its original volume. Rigorous ammonium hydroxide added in droplets to the extract until precipitation (cloudy fume) accomplished. The solution remained permissible to settle, washed through diluted ammonium hydroxide then filtered. The residue collected was dried and weighed then the alkaloid content calculated by means of the equation:

% Alkaloid =Weight of precipitate/Weight of original sample ×100

Determination of Saponin

Saponin content estimated as method described by (Obadoni and Ochuko, 2001). Briefly, 5 g of the crushed plant sample added to 50 mL of 20% ethanol, retained on a shaker aimed at 30 min and then heated in a water bath on 55^{0}C for 4 h. The subsequent mixture filtered and then remainder re-extracted through additional 200 mL of 20% aqueous ethanol. The remainders were collective and condensed to 40 mL in a boiling water bath at 90^{0}C. The concentrate shifted into a splitting funnel, 20 mL of diethyl ether added and then shaken enthusiastically. The ether film, which was the upper film, discarded and then the aqueous (bottom) layer retained in a beaker. The retained layer re-introduced into a splitting funnel and 60 mL of n-butanol added then shaken enthusiastically. The butanol extract, which is the upper layer, reserved although the bottom layer thrown away. The butanol layer was wash away twice with 10 mL of 5% aqueous sodium chloride. The residual solution collected and heated to evaporation in a boiling water bath, formerly dehydrated to constant weight at 40°C in an oven. The saponin content remained calculated by means of the equation:

% Saponin content = Weight of residue/Weight of original sample × 100

Glycosides

Cardiac glycosides estimated by the method given by (El-Olemy *et al*., 1994). It develop an orange red colour complex with Baljet's reagent. The intensity of colour produced is proportional to the concentration of glycosides.10ml of the extract and 10ml of Baljet's reagent taken and allowed to stand for one hour dilute the solution with 20ml distilled water and mix. Read the absorbance of the colour obtained against blank at 495nm. The difference between test and control taken for calculation. Standard graph prepared by using standard digitoxin. Concentration (%) = Absorbance × 100 g % 17.

Results

Carbohydrate

The effects of osmolyte that is proline and water deficit stress treatment on carbohydrate content of *Silybum marianum* L. Whereas different concentration of proline 50, 100and 150µg/l applied with PEG-6000, stress0.2, 0.4, 0.6, 0.8 and 1%, than the carbohydrate content was enhanced. The proline treatment increased carbohydrate content under stress as compared to control 1.102±0.596 by 1.836±0.652, 2.345±0.845, 2.686±0.745, 2.746±0.985, 2.982±0.798mg/gin 50µg/l along with different stress 0.2, 0.4, 0.6, 0.8 and 1%, treatment respectively. Similarly, proline 100 and 150µg/l with stress treatment increased carbohydrate content by 2.714±0.654, 2.956±0.731, 3.234±0.528, 3.638±0.684, 3.964±0.964, 3.463±0.854, 3.621±0.746, 3.958±0.542, 4.462±0.984, 4.889±0.561mg/g compared to control respectively that explained in table 1.

Protein

Exogenous application of proline provided osmoprotection and enhanced the tolerance of plants exposed to drought stress. The proline treatment increased protein content under stress as compared to control 1.162±0.676 by 1.445±0.564, 1.852±0.843, 2.321±0.728, 2.654±0.529, 2.915±0.654mg/g in 50µg/l along with different stress 0.2, 0.4, 0.6, 0.8 and 1%, treatment respectively. In the same way, proline 100 and 150µg/l with stress treatment increased protein by 2.854±0.785, 3.284±0.521, 3.459±0.947, 3.654±0.854, 3.863±0.983, 3.554±0.749, 3.857±0.529, 4.295±0.647, 4.645±0.974, 4.874±0.572mg/g compared to control respectively that elucidated in table 1.

Proline

Proline, applied exogenously at different concentration, ameliorated the adverse effects of drought stress in *Silybum marianum* L. and enhanced the tolerance to stress. Exogenously provided proline treatment increased proline content under stress as compared to control 0.395±0.528 by 1.485±0.635, 1.554±0.582, 1.698±0.694, 1.756±0.657, 1.854±0.598mg/g in 50µg/l along with different stress 0.2, 0.4, 0.6, 0.8 and 1%, treatment respectively. Similarly, proline 100 and 150µg/l with stress treatment increased proline by 1.696±0.653, 1.785±0.845, 1.795±0.974, 1.879±0.617, 1.912±0.561, 1.798±0.479, 1.842±0.947, 1.878±0.653, 1.891±0.784, 1.962±0.698 mg/g compared to control respectively that elucidated in table 1.

Relative water content

The proline treatment increased relative water content

Table 1: Effect of proline to drought stress on physiological analysis of *Silybum marianum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by one way Anova followed by Tukey post-test P<0.05*, P<0.01**, P<0.001*** significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	Carbohydrate mg/g	Protein mg/g	Proline mg/g	RWC cm^2	Chlorophyll mg/g
Control	1.102±0.596	1.162±0.676	0.395±0.528	64.370±0.740	30.662±0.584
Proline 50µg/l and PEG 0.2%	1.836±0.652	1.445±0.564	1.485±0.635	68.567±0.964a***	32.896±0.698a*
Proline 50µg/l and PEG 0.4%	2.345±0.845	1.852±0.843	1.554±0.582	69.667±0.895b***	33.569±0.478b***
Proline 50µg/l and PEG 0.6%	2.686±0.745	2.321±0.728	1.698±0.694	69.889±0.597c***	33.896±0.574c***
Proline 50µg/l and PEG 0.8%	2.746±0.985	2.654±0.529	1.756±0.657	70.894±0.896d***	34.575±0.896d***
Proline 50µg/l and PEG 1%	2.982±0.798a*	2.915±0.654	1.854±0.598	71.756±0.984e***	34.986±0.749e***
Proline 100µg/l and PEG 0.2%	2.714±0.654	2.854±0.785	1.696±0.653	70.854±0.542e***	33.754±0.875e***
Proline 100µg/l and PEG 0.4%	2.956±0.731	3.284±0.521a*	1.785±0.845	71.698±0.621f***	33.986±0.984f***
Proline 100µg/l and PEG 0.6%	3.234±0.528a*	3.459±0.947b**	1.795±0.974	71.995±0.785g***	34.659±0.587g***
Proline 100µg/l and PEG 0.8%	3.638±0.684b**	3.654±0.854c**	1.879±0.617	72.465±0.785h***	34.824±0.697h***
Proline 100µg/l and PEG 1%	3.964±0.964c***	3.863±0.983d**	1.912±0.561a*	72.889±0.964i***	35.459±0.749i***
Proline 150µg/l and PEG 0.2%	3.463±0.854c**	3.554±0.749d**	1.798±0.479	72.586±0.847i***	34.984±0.774i***
Proline 150µg/l and PEG 0.4%	3.621±0.746d**	3.857±0.529e**	1.842±0.947	72.896±0.749j***	34.554±0.872i***
Proline 150µg/l and PEG 0.6%	3.958±0.542e***	4.295±0.647f***	1.878±0.653	73.719±0.549k***	34.970±0.974j***
Proline 150µg/l and PEG 0.8%	4.462±0.984f***	4.645±0.974g***	1.891±0.784	73.804±0.679l***	35.785±0.674k***
Proline 150µg/l and PEG 1%	4.889±0.561g***	4.874±0.572h***	1.962±0.698b*	74.634±0.875m***	35.894±0.596l***

under stress as compared to control 64.370±0.740 by 68.567±0.964, 69.667±0.895, 69.889±0.597, 70.894±0.896, 71.756±0.984cm^2 in 50µg/l along with different stress 0.2, 0.4, 0.6, 0.8 and 1%, treatment respectively. Correspondingly, proline 100 and 150µg/l with stress treatment increased relative water content by 70.854±0.542, 71.698±0.621, 71.995±0.785, 72.465±0.785, 72.889±0.964, 72.586±0.847, 72.896±0.749, 73.719±0.549, 73.804±0.679, 74.634±0.875cm^2 compared to control respectively that explained in table 1.

Chlorophyll

Exogenous application of proline to *Silybum marianum* L. that subjected to drought stress resulted in an increased in chlorophyll content which were shown in table 1. The exogenous proline treatment enhanced chlorophyll content under stress as compared to control 30.662±0.584 as a result of32.896±0.698, 33.569±0.478, 33.896±0.574, 34.575±0.896, 34.986±0.749mg/g in 50µg/l along with different stress 0.2, 0.4, 0.6, 0.8 and 1%, treatment correspondingly. Likewise, proline 100 and 150µg/l with stress treatment increased chlorophyll by 33.754±0.875, 33.986±0.984, 34.659±0.587, 34.824±0.697, 35.459±0.749, 34.984±0.774, 34.554±0.872, 34.970±0.974, 35.785±0.674, 35.894±0.596mg/g.

Phenol

Exogenous application of proline enhanced phenolic content in a concentration dependent manner and maintained plant tolerance that exposed to drought stress. Exogenously applied proline treatment increased phenolic content in *Silybum marianum* L. under stress as compared to control 0.250±0.193 as a result of 0.283±0.153, 0.323±0.197, 0.346±0.185, 0.368±0.152, 0.393±0.185mg/g in 50µg/l along with different stress 0.2, 0.4, 0.6, 0.8 and 1%, treatment respectively. Similarly, proline 100 and 150µg/l with stress treatment increased phenol by 0.375±0.184, 0.396±0.186, 0.424±0.185, 0.462±0.178, 0.494±0.153, 0.454±0.184, 0.496±0.153, 0.529±0.184, 0.568±0.173, 0.591±0.135mg/g respectively that described in table 2.

Flavonoid

Exogenous application of proline to plants which were subjected to drought stress resulted in an increase in flavonoid content that is secondary plant product accumulate during plants facing stress. The exogenously applied proline treatment increased flavonoid content under stress as compared to control 0.218±0.133 as a result of 0.265±0.162, 0.293±0.189, 0.319±0.186, 0.359±0.198, 0.392±0.154mg/g in 50µg/l along with different stress 0.2, 0.4, 0.6, 0.8 and 1%, treatment respectively. Correspondingly, proline 100 and 150µg/l with stress treatment increased flavonoid by 0.363±0.196, 0.396±0.143, 0.423±0.154, 0.472±0.181, 0.513±0.193, 0.453±0.121, 0.496±0.198, 0.535±0.131, 0.564±0.184, 0.589±0.135mg/g respectively that described in table 2.

Alkaloid

Proline, an amino acid, plays an important role in

plants. It protects the plants from drought stress and help plants to recover from stress more rapidly. When applied exogenously to plants exposed to drought stress, results enhanced an alkaloid content of *Silybum marianum* L. as compared to control 0.216±0.163 as a result of 0.396±0.121, 0.449±0.296, 0.585±0.146, 0.654±0.154, 0.784±0.237 % in 50µg/l along with different stress 0.2, 0.4, 0.6, 0.8 and 1%, treatment respectively. Correspondingly, proline 100 and 150µg/l with stress treatment increased alkaloid by 0.465±0.229, 0.554±0.293, 0.696±0.249, 0.746±0.185, 0.854±0.265, 0.563±0.254, 0.623±0.235, 0.784±0.294, 0.864±0.243, 0.951±0.284 % respectively which were elucidated in table 2.

Saponin

Exogenous application of proline mitigated the drought induced inhibitory effects on the saponin content that is secondary plant product and enhanced it under stress as compared to control 0.144±0.152 as a result of 0.215±0.184, 0.250±0.149, 0.286±0.116, 0.295±0.172, 0.324±0.179 % in 50µg/l along with different stress 0.2, 0.4, 0.6, 0.8 and 1%, treatment respectively. Similarly, proline 100 and 150µg/l with stress treatment increased saponin by 0.356±0.149, 0.384±0.198, 0.393±0.139, 0.425±0.146, 0.446±0.196, 0.397±0.142, 0.426±0.149, 0.459±0.163, 0.485±0.149, 0.514±0.139 % respectively which were explained in table 2.

Glycosides

Proline acts as a compatible solute to protect plant cells under osmotic stress and as a molecular chaperone, proline has been demonstrated to protect plants to drought stress. The exogenously applied proline treatment increased glycosides content under stress as compared to control 0.115±0.190 as a result of0.159±0.165, 0.181±0.184, 0.194±0.198, 0.226±0.143, 0.254±0.156 % in 50µg/l along with different stress 0.2, 0.4, 0.6, 0.8 and 1%, treatment respectively. Similarly, proline 100 and 150µg/l with stress treatment increased glycosides by 0.252±0.174, 0.283±0.196, 0.314±0.149, 0.334±0.143, 0.351±0.173, 0.385±0.119, 0.396±0.139, 0.415±0.194, 0.437±0.136, 0.458±0.146 % respectively that elucidated in table 2.

Discussion

Exogenous application of compatible osmolytes such as proline, had gained considerable attention in mitigating the effect of stress (Ashraf and Foolad, 2007). Under stress condition, exogenous proline application improved tolerance of stressed plants (Deivanai *et al*., 2011). Proline has proposed to act as a compatible solute that adjusts the osmotic potential in the cytoplasm, it considered to play an important role in defense mechanisms of stressed cells (Arshi *et al.,* 2005). Exogenously provided proline is facilitating growth in highly stressed environments (Yancey, 1994). The present experiment carried out on *Silybum marianum* L. plant under water deficit stress and exogenous application of proline through hydroponic culture in controlled condition of light and temperature. The results indicating of this experiment that there is enhancement of carbohydrate

Table 2: Effect of proline to drought stress on secondary metabolite profile of *Silybum marianum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by one way Anova followed by Tukey post-test P<0.05*, P<0.01**, P<0.001*** significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	Phenol mg/g	Flavonoid mg/g	Alkaloid %	Saponin %	Glycosides %
Control	0.250±0.193	0.218±0.133	0.216±0.163	0.144±0.152	0.115±0.190
Proline 50µg/l and PEG 0.2%	0.283±0.153	0.265±0.162	0.396±0.121	0.215±0.184	0.159±0.165
Proline 50µg/l and PEG 0.4%	0.323±0.197	0.293±0.189	0.449±0.296	0.250±0.149	0.181±0.184
Proline 50µg/l and PEG 0.6%	0.346±0.185	0.319±0.186	0.585±0.146	0.286±0.116	0.194±0.198
Proline 50µg/l and PEG 0.8%	0.368±0.152	0.359±0.198	0.654±0.154	0.295±0.172	0.226±0.143
Proline 50µg/l and PEG 1%	0.393±0.185	0.392±0.154	0.784±0.237	0.324±0.179	0.254±0.156
Proline 100µg/l and PEG 0.2%	0.375±0.184	0.363±0.196	0.465±0.229	0.356±0.149	0.252±0.174
Proline 100µg/l and PEG 0.4%	0.396±0.186	0.396±0.143	0.554±0.293	0.384±0.198	0.283±0.196
Proline 100µg/l and PEG 0.6%	0.424±0.185	0.423±0.154	0.696±0.249	0.393±0.139	0.314±0.149
Proline 100µg/l and PEG 0.8%	0.462±0.178	0.472±0.181	0.746±0.185	0.425±0.146	0.334±0.143
Proline 100µg/l and PEG 1%	0.494±0.153	0.513±0.193	0.854±0.265a*	0.446±0.196	0.351±0.173
Proline 150µg/l and PEG 0.2%	0.454±0.184	0.453±0.121	0.563±0.254	0.397±0.142	0.385±0.119
Proline 150µg/l and PEG 0.4%	0.496±0.153	0.496±0.198	0.623±0.235	0.426±0.149	0.396±0.139
Proline 150µg/l and PEG 0.6%	0.529±0.184	0.535±0.131	0.784±0.294	0.459±0.163	0.415±0.194
Proline 150µg/l and PEG 0.8%	0.568±0.173	0.564±0.184a*	0.864±0.243b*	0.485±0.149a*	0.437±0.136
Proline 150µg/l and PEG 1%	0.591±0.135a*	0.589±0.135b*	0.951±0.284c**	0.514±0.139b*	0.458±0.146a*

content in *Silybum marianum* L. under drought stress and exogenously applied proline, which shown in table 1. Carbohydrates are a major category of compatible solutes, which accumulated during stress. The results obtained here were in agreement with those obtained by (Carvalho *et al*., 2005) whom reported that water stress caused an increase in sugar and total carbohydrates concentrations in seed's dry weight as compared with non-stressed control plants of two *Lupinus species*. In addition, (Khalid, 2006) pointed out that total carbohydrate of *Ocimum* spp. increased under water stress. Similar results obtained by (El-Sayed *et al.,* 2008). *Tribulus* species and (Khalil *et al.,* 2012) *Jatropha curcas* L. Treated plants with low and moderate proline concentrations exhibited significant decrease in total carbohydrates percent (Gamal El-Din and Abd El-Wahed, 2005) reported a decrease in total carbohydrates % of chamomile *(Matricaria chamomilla* L.) plant under low proline concentrations followed by an increase under the highest concentrations (Ali *et al*., 2007). Exogenous application of proline as foliar spray significantly increased the protein, contents in both the cultivars of maize under water stress and non-stress conditions showing the ameliorating effects of exogenously applied proline on seed proximate composition. Furthermore, this enhancement in the seed proximate parameters due to foliar application of proline more pronounced under water deficit conditions as compared with non-stress conditions. This ameliorating effect of exogenously applied proline on altering these seed chemical parameters might have been due to its role to maintain turgor in plants both under non-stress and water deficit conditions, thereby maintaining high photosynthetic efficiency and as a result more allocation of assimilates to developing seeds (Makela *et al*., 1998). Similar results were obtained where protein content is enhanced by exogenous application of proline under drought stress which shown in table 1. Proline accumulation in *Lepidium sativum* leaves increased progressively by increasing the drought period. Such increases in proline values with increasing stress was attributed to one of the defense mechanisms in which stressed plants used to reduce cell osmotic potential, which resulted in increasing cell water uptake with concomitant increases in both cell turgidity and its activity. These results were documented by (Hossein *et al.,* 2009; Khalil *et al.,* 2012). Proline treatment increased tolerance of maize and broad bean plants through osmoregulation using the organic solutes (Yamada *et al.,* 2005; Cuin and Shabala, 2005). As similar result observed in present study where proline content progressively increased in stressed plant as compared to their respective control table 1. Relative water content (RWC) of leaves has reported as an uninterrupted indicator of plant water contents under water deficit circumstances. Water stress prime to the reduction of water status throughout crop growth, soil water potential, and plant osmotic potential for water and nutrient uptake, which ultimately reduces leaf turgor pressure, which results in distressed plant metabolic activities. Beneath water stress, condition reduction in water status, and osmotic potential in plants exists the eventual consequence of lower relative water content. (Lugojan and Ciulca, 2011). Similar result found in present study where relative water content enhanced by exogenous application of proline, which explained in table 1. Chlorophyll is one of the major chloroplast components for photosynthesis (Rahdari *et al.,* 2012). The decrease in chlorophyll content under drought stress has considered a typical symptom of pigment photo oxidation and chlorophyll degradation (Anjum *et al,* 2011). Decreased of chlorophyll content during drought stress depending on the duration and severity of drought level (Zhang and Kirkham, 1996). A decrease of total chlorophyll content with drought stress implies a lowered capacity for light harvesting. Since the production of reactive oxygen species mainly driven by excess energy absorption in the photosynthetic apparatus, this might avoided by degrading the absorbing pigments (Mafakheri *et al.,* 2010). In relation to drought effect on chlorophyll a and b in leaf, we can express that drought is due to chloroplastic proteins hydrolysis, decreasing of leaf pigments and chlorophyll destruction as a primary stage in degradation of proteins (Synerri *et al.,* 1993). Similar result found here the chlorophyll content enhanced by exogenous application of proline in a concentration dependent manner which shown in table 1. The antioxidative defense system of plants helps in reducing the stress-induced ROS accumulation, which comprises the phenolics and flavonoid content (Ali *et al.,* 2018). Furthermore, cellular metabolites such as leaf phenolics and flavonoid contents help in regulating several plants metabolic processes including playing a role in reducing ROS-induced oxidative impairment (Habib *et al.,* 2016). The present experiment carried out on *Silybum marianum* L. plant under water deficit stress and exogenous application of proline. The results indicating of this experiment that there is enhancement of (catalase, peroxidase, superoxide dismutase, ascorbic acid, phenolics, and flavonoid) in *Silybum marianum* L. under drought stress and exogenously applied proline, overcome their enhancements whenever stress is ceased which shown in table 2. The total alkaloid accumulation in shoot of *Catharanthus roseus* found increased significantly under drought stress. The content of alkaloids in *C. roseus* has been found influenced by individual factor, such as stage of plant growth due to drought stress (Misra and Gupta, 2006; Osman, *et al.,* 2007). The leaves and stem are the sources of the natural dimeric alkaloids vinblastine and vincristine that are essential parts of most anti-cancer

Control | Proline 50 µg/l and PEG 0.2% | Proline 50 µg/l and PEG 0.4%

Proline 50 µg/l and PEG 0.6% | Proline 50 µg/l and PEG 0.8% | Proline 50 µg/l and PEG 1%

Proline 100 µg/l and PEG 0.2% | Proline 100 µg/l and PEG 0.4% | Proline 100 µg/l and PEG 0.6%

Proline 100 µg/l and PEG 0.8% | Proline 100 µg/l and PEG 1% | Proline 150 µg/l and PEG 0.2%

Proline 150 µg/l and 0.4% Proline 150 µg/l and PEG 0.6% Proline 150 µg/l and PEG 0.8%

Proline 150 µg/l and PEG 1%

Fig. 1: Showing effect of proline and PEG-6000 on *Silybum marianum* L. plants at 45 days before collecting sample.

chemotherapies (Heijden *et al.,* 2004). Similarly result of present study revealed that the alkaloid content enhanced as compared to respective control in water deficit condition to avoid the deleterious effects of stress but it overcome by exogenous application of proline in an concentration dependent manner which shown in table 2. Previous reports have demonstrated that saponin concentration increased under severe water deficient (Odjegba and Alokolaro, 2013), but in others reports, their concentration decreased (Ramakrishna and Ravishankar, 2011), suggesting that not all species respond in the same way to water stress. Saponins also known to be synthetized due to a general defense mechanism in plants against both abiotic and biotic stress, and it has been suggested that their concentration and localization may depend on the protection needed by the plant, thus explaining their accumulation on the leaves(Upadhyay *et al.,* 2014). The present study result revealed that saponin content increased in water deficit condition but it overcome by exogenous application of proline in an concentration dependent manner which shown in table 2. In *Stevia, rebaudiana* Bertoni analysis of total glycosides revealed that abiotic stress increased glycosides. On the other hand, *stevia* plants treated with polyamines had more increased glycosides. It concluded that the polyamines supplement could induced a considerable tolerance in *Stevia, rebaudiana* Bertoni in an abiotic stress tolerance (Peynevandi *et al.,* 2018). The present study also revealed that glycosides percentage increased in water deficit condition but it more enhanced by exogenous application of proline in a concentration dependent manner which shown in table 2. In order to deal with the deleterious impacts of water stress; plants undergo osmotic regulation by increasing the synthesis of potential osmolyte (proline) in the cytosol and in other organelles. Proline is the key osmolyte that contribute significantly in cellular osmotic adjustment. Leaf proline, an important secondary metabolite, performs dual functions in plants as an antioxidant as well as an osmoprotectant (Habib *et al.,* 2016). As an antioxidative compound, it has the potential to protect the membranes of the organelles, and as an osmolyte, it plays a key role in improving cellular water relations under water-stressed conditions (Ali *et al.,* 2013). It well known that under osmotic stress, a high concentration of proline acts as a water substitute to stabilize the cellular structures through their hydrophobic interactions and hydrogen bonding, which protects the membranes from dehydration (Kavi Kishor *et al.,* 2005). Furthermore, the proline metabolism produces other stress-related compounds that are directly involved in the antioxidative defense mechanism (Hossain *et al.,* 2014).

Conclusion

Osmolytes play pivotal role in plants for improving abiotic stress tolerance. The overproduction and accumulation of osmoprotectants is universal process for combating abiotic stress. Various abiotic stresses trigger the biosynthesis and accumulation of osmoprotectants such as proline, glycinebetaine, and soluble sugars. The present study focused on *Silybum marianum* L. plant under water deficit stress and exogenous application of proline through hydroponic culture in controlled condition of light and temperature. The results of this experiment indicating that there is enhancement of physiological analysis (carbohydrate, protein, proline, relative water

content, chlorophyll) and secondary metabolite profile (phenol, flavonoid, alkaloid, saponin, glycosides) in *Silybum marianum* L. under drought stress whereas proline were applied through exogenously. Therefore, exogenous application of proline might have acted as a direct scavenger of ROS or boosted the antioxidation mechanism by playing a key role as a signaling molecule. As a result, it was elective in maintaining better stabilization of sub-cellular structures and membranes, stabilization of proteins, as well as the maintenance of cellular functions.

Acknowledgements

The authors acknowledge to Shoolini University of Biotechnology and Management Sciences, Solan, H.P. for providing platform for this work and thanks to the Department of Basic Sciences for supporting and provide lab facility to carry out this work.

References

Ali, G., P.S. Srivastava and M. Iqbal (2007). Proline accumulation, protein pattern and photosynthesis in regenerants grown under NaCl stress. *Biologia Plantarum*, **42:** 89-95.

Ali, Q., F. Anwar, M. Ashraf, N. Saar and R. Perveen (2013). Ameliorating ejects of exogenously applied proline on seed composition, seed oil quality and oil antioxidant activity of maize (*Zea mays* L.) under Drought Stress. *International Journal of Molecular Sciences*, **14:** 818-835.

Ali, Q., M.T. Javed, A. Noman, M.Z. Haider, M. Waseem, N. Iqbal, M. Wasim, M.S. Shah, F. Shahzad and R. Perveen (2018). Assessment of drought tolerance in mung bean cultivars/lines as depicted by the activities of germination enzymes, seedling's antioxidative potential and nutrient acquisition. *Archives of Agronomy and Soil Science*, **64:** 84-102.

Anjum, S., X. Xie, L. Wang, M. Saleem, C. Man and W. Lei (2011). Morphological, physiological and Biochemical responses of plants to drought stress. *African Journal of Agricultural Research*, **6:** 2026-2032.

Arshi, A., M.Z. Abdin and M. Igbal (2005). Ameliorative effects of $CaCl_2$ on growth, ionic relations and proline content of senna under salinity stress. *Journal of Plant Nutrition*, **28:** 101-125.

Ashraf, M. and M.R. Foolad (2007). Roles of glycinebetaine and proline in improving plant abiotic stress tolerance. *Environmental and Experimental Botany*, **59:** 206-216.

Atkinson, N.J. and P.E. Urwin (2012).The interaction of plant biotic and abiotic stresses**:** from genes to the field. *Journal of Experimental Botany*, **63:** 3523–3543.

Bates, L.S., R.P. Waldren and I.D. Teare (1973). Rapid determination of free proline for ware stress studies. *Plant and soil*, **39:** 205-207.

Carvalho, I.S., M. Chaves and C.P. Ricardo (2005). Influence of water stress on the chemical composition of seeds of two lupins *Lupinus albus* and *Lupinus mutabilis*. *Journal of Agronomy & Crop Science*, **191:** 95-98.

Cuin, T.A. and S. Shabala (2005). Exogenously supplied compatible rapidly ameliorate NaCl-induced potassium efflux from barley roots. *Pant and Cell Physiology*, **46:** 1924-1933.

Deivanai S., R. Xavier, V. Vinod, K. Timalata and O.F. Lim (2011). Role of exogenous proline in ameliorating salt stress at early stage in two rice cultivars. *Journal of Stress Physiology & Biochemistry,* **7:** 157-174.

EL-Mallah, M.H., S.M. EL-Shami and M.M. Hassanein (2003). Detailed studies on some lipids of *Silybum marianum* seed oil. *Grasasy Aceites*, **54:** 397-402.

El-Olemy, M.M., F.J. Al-Muhtadi and A.F.A. Afifi (1994). Experimental photochemistry**:** A laboratory manual. *King Saud University Press, Saudi Arabia*, 21-27.

Fahad S., L. Nie, Y. Chen, C. Wu, D. Xiong, S. Saud, L. Hongyan, K. Cui and J. Huang (2015). Crop plant hormones and environmental stress. *Sustainable Agriculture Reviews*, **15:** 371-400.

Fraschini, F., G. Damartini, D. Esposti (2002). Pharmacology of silymarin. *Drug Investigation*, **22:** 51-65.

Gamal EL-Din, K.M. and M.S.A. Abd EL-Wahed (2005). Effect of some amino acids on growth and essential oil content of chamomile plant. *International Journal of. Agriculture and Biology*, **7:** 376-380.

Gill, S.S. and N. Tuteja (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants, *Plant Physiology Biochemistry*, **48:** 909-930.

Habib, N., M.S. Akram, M.T. Javed, M. Azeem, Q. Ali, H.L. Shaheen and M. Ashraf (2016). Nitric oxide regulated improvement in growth and yield of rice plants grown under salinity**:** Antioxidant defense system. *Applied Ecology* and Environmental *Research,* **14:** 91-105.

Hare, P.D. and W.A. Cress (1997). Metabolic implications of stress induced proline accumulation during stress. *Plant Growth Regulations*, **21:**79-102.

Hedge, J.E. and B.T. Hofreiter (1962). In**:** Carbohydrate Chemistry, 17 (Eds. Whistler R.L. and Be Miller, J.N.), Academic Press, New York.

Heijden, R.V.D., I. Jacobs Denise, W. Snoeijer, D. Hallard and R. Verpoorte (2004). The Catharanthus Alkaloids**:** Pharmacognosy and Biotechnology. *Current Medicinal Chemistry*, **11:** 607-628.

Hiscox, J.D. and G.F. Israelstam (1979). A method for the extraction of chlorophyll from leaf tissue without maceration. *Canadian journal of botany*, **57:** 1332-1334.

Hossain, M.A., M.A. Hoque, D.J. Burritt and M. Fujita (2014). Proline protects plants against abiotic oxidative stress**:** biochemical and molecular mechanisms. In Oxidative Damage to Plants; Academic Press**:** Cambridge, MA, USA, 477-522.

Hossein, A.F., A.V. Sayed, D. Jahanfar, H.S. Amir and A.K. Mohammad (2009). Medicinal and aromatic plants farming under drought conditions. *Journal of Horticulture and Forestry*, **1:** 086-092.

Iqbal, M.J., Y. Maqsood, Z.U. Abdin, A. Manzoor, M. Hassan and A. Jamil (2016). SSR markers associated with Proline in drought tolerant wheat germplasm. *Applied Biochemistry and Biotechnology*, **178:** 1042-1052.

Jaleel, C.A., P. Manivannan, A. Wahid, M. Farooq, H.J. Al-Juburi and Somasundaram, R (2009). Drought Stress in plants**:** A review on morphological characteristics and pigments composition. International *Journal of*

Agriculture and Biology, **11:** 100-105.

Jeandroz, S. and O. Lamotte (2017). Plant responses to biotic and abiotic stresses**:** lessons from cell signaling. *Frontiers of Plant Science*, **8:** 1772.

Jia Z., M. Tang and J. Wu (1999). The determination of flavonoid contents in mulberry and their scavenging**:** effects on superoxide radicals. *Food Chemistry*, **64:** (4)555-559.

Kamran M., M. Shahbaz, M. Ashraf and N.A. Akram (2009). Alleviation of drought-induced adverse effects in spring wheat (*Triticum aestivum* L.) using proline as a pre-sowing seed treatment. *Journal of Botany*, **41:** 621-632.

Karuppanapandian, T., J.H. Moon, C, Kim, K. Manoharan and W. Kim (2011). Reactive oxygen species in plants**:** their generation, signal transduction and scavenging mechanisms. *Australian Journal Crop Science*, **15:** 709-725.

Kavi Kishor, P.B., S. Sangam, R.N. Amrutha, P.S. Laxmi, K.R. Naidu, K.R.S.S. Rao, S. Rao, K.J. Reddy, P. Theriappan and N. Sreenivasulu (2005). Regulation of proline biosynthesis, degradation, uptake and transport in higher plants**:** Its implications in plant growth and abiotic stress tolerance. *Current Science*, **88:** 424-438.

Khalid, A.K. (2006). Influence of water stress on growth, essential oil and chemical composition of herbs *Ocimum spp*. *International Agrophysics*, **20:** 289-296.

Khalil, S.E., M.M. Hussein and J.T. da Silva (2012). Roles of anti transpirants in improving growth and water relations of *Jatropha curcas* L. grown under water stress conditions. *Plant Stress*, **6:** 49-54.

Kurkin, V.A., (2003). Saint–Mary Thistle**:** A source of medicinal. *Pharmacy chemistry*, **37:** 189-202.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951). (The Original Method).*Journal of Biological Chemistry*, **193:** 265.

Lugojan, C. and S. Ciulca (2011). Evaluation of relative water content in winter wheat. *Journal of Horticulture, Forestry and Biotechnology*, **15:** 173-177.

Mafakheri, A., A. Siosemardeh, B. Bahramnejad, P.C. Struik and Y. Sohrabi (2010). Effect of drought stress on yield, proline and chlorophyll content in three Chickpea cultivars. *Journal of Australian Crop Science*, **4:** 580-585.

Makela, P., R. Munns, T.D. Colmer, A.G. Condon and P. Peltonen-Sainio (1998). Effects of foliar applications of glycinebetaine on stomatal conductance, abscisic acid and solute concentrations in leaves of salt- or drought-stressed tomato. *Australian Journal of Plant Physiology*, **25:** 655-663.

Malick, C.P. and M.B. Singh (1980). Phenolics. Plant Enzymology and Histoenzymology Kalyani Publishers, New Delhi 286.

Misra, N. and A.K. Gupta (2006). Effect of salinity and different nitrogen sources on the activity of antioxidant enzymes and indole alkaloid content in *Catharanthus roseus* seedlings. Journal of Plant Physiology, **163:** 11-18.

Obadoni, B.O. and P.O. Ochuko (2002). Phytochemical studies and comparative efficacy of the crude extracts of some hemostatic plants in Edo and Delta States of Nigeria. Global *Journal of Pure and Applied Sciences*, **8:** 203-208.

Odjegba, V.J. and A. Alokolaro (2013). Simulated drought and salinity modulates the production of phytochemicals in *Acalypha wilkesiana*. *Journal of Medicinal Plant Studies*, **2:** 105-112.

Omoruyi, B.E., G. Bradley and A.J. Afolayan (2012). Antioxidant and phytochemical properties of *Carpobrotus edulis* (L.) bolus leaf used for the management of common infections in HIV/AIDS patients in Eastern Cape Province. *BMC Complementary and Alternative Medicine*, **12:** 215.

Osman, M.E.H., S.S. Elfeky, K. Abo El-Soud and A.M. Hasan (2007). Response of *Catharanthus roseus* Shoots to Salinity and Drought in Relation to Vincristine Alkaloid Content. *Asian Journal of Plant Sciences*, **6:** 1223-1228.

Peynevandi, M.M, S.M. Razavi and S. Zahri (2018). The ameliorating effects of polyamine supplement on physiological and biochemical parameters of *Stevia rebaudiana* Bertoni under cold stress. *Plant Production Science*, **21:** 123-131.

Rahdari, P., S.M. Hoseini and S. Tavakoli (2012). The studying effect of drought stress on germination, proline, sugar, lipid, protein and chlorophyll content in Purslane (*Portulaca oleraceae* L.) leaves. *Journal of Medicinal Plants Research*, **6:** 1539-1547.

Ramakrishna, A. and G.A. Ravishankar (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling and Behavior*, **6:** 1720-1731.

Roychoudhury, A. and A. Banerjee, (2016). Endogenous glycine betaine accumulation mediates abiotic stress tolerance in plants. *Tropical Plant Research*, **3:** 105-111.

Synerri, C.L.M., C. Pizino and F. Navariizzo (1993). Chemical changes and O_2 production in thylakoid membrane under water stress. *Plant Physiology*, **87:** 211-216.

Upadhyay, S., U.J. Phukan, S. Mishra and R.K. Shukla (2014). De novo leaf and root transcriptome analysis identified novel genes involved in steroidal sapogenin biosynthesis in *Asparagus racemosus*. *BMC Genomics*, **15:** 746.

Weatherley, P. (1950). Studies in the water relation of the cotton plant**:** I. The field measurement of water deficits in leaves. *New Phytologist*, **49:** 81-97.

Yancey, P.H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology*, **208:** 2819-2830.

Yancey, P.H. (1994). Compatible and counteracting solutes. In K. Strange (ed)**:** Cellular and Molecular Physiology of Cell Volume Regulation. *CRC Press, Boca Raton, FL*, 81-109.

Zhang, J. and M.B. Kirkham (1996). Antioxidant response to drought in Sunflower and Sorghum seedlings. *New Phytologist*, **132:** 361-373.