EFFECT OF DROUGHT STRESS ON ACTIVE SUBSTANCES RATE, SILYMARINE AND PROLINE OF MILK THISTLE (SILYBUM MARIANUM)

Amin Farnia, Ali Nematian, Researchers
Department of Agronomy, Islamic Azad University, Borujerd, Iran
Corresponding author email :E-mail: alinematian1982@gmail.com
Mohammad Ali Shariati, Researcher
Islamic Azad University, Tehran, Iran
E-mail: shariatymohammadali@gmail.com
postal address: No. 107-Shahid Asadi Razi-East 160- Tehranpars St.-Tehran-Iran,
Postal code:1654688513

Abstract: *Milk thistle(Silybum marianum) is an annual or biennial plant of the Asteraceae family, which is of great importance in the pharmaceutical industry to treat liver disorders like hepatitis and liver damage from alcohol and has a stomach upset. In order to examine the influence of drought stress on the rate of active substance Silymarin and Proline in Silybum marianum, an experiment was performed in Karaj experimental farm, Alborz province, 2011. The experiment was done as a randomized complete block design with three replications. Three levels of drought stress treatments T1: 100% of field capacity (without stress) T2: 85% of field capacity (mild stress) T3: 70% of field capacity (moderate stress) T4: 55% of field capacity (Severe stress), respectively. After extracting, the rate of active substances of Silymarin and Proline has been evaluated using high performance liquid chromatography(HPLC). Regarding the obtained results, drought stress treatments had a significant effect on the rate of active substances and the highest amounts of Silymarin and Proline were measured as 1311.2, 578.7 μg/g in the treatment T4: 55% of field capacity (Severe stress).*

Keyword: *Milk thistle(silybum marianum), Asteraceae, drought stress, active substance, silymarine, proline*

Introduction

Silybum marinum is a biennial or annual plant native to Europe and is also found in some parts of the United States. It grows in rocky soils at a height of three to 10 feet with an erect stem that bears large, alternate, spiny-edged leaves. The common name, milk thistle, is derived from the “milky white” veins in the leaves, which when broken open, producing a milky sap. The portion of the plant's fruit is glossy brown or gray spots. Modern plant extracts are produced from the small hard fruit (often referred to as seeds) that have the tuft of feathers removed (Tamayo and Diamond, 2007 & Gazak et al., 2007). It is deduced that silymarin contains a huge concentration of flavolignans that constitutes of silybin, also known as silybinin which are the effective elements, silydianin, isosilybin and silychristin (Khan et al., 2006). Nowadays, silymarin is widely used as a hepatoprotectant and as a supporting therapy of liver diseases such as cirrhosis, hepatitis and fatty acid infiltration from alcohol and toxic chemicals (Ball and Kowdley, 2005; Kren and Walterova, 2005). Silymarin also demonstrated beneficial effects in the case of radiation damage to the membranes of liver cells (Ramadan et al., 2002). It appears silymarin is able to protect liver cells directly by stabilizing membrane permeability by inhibiting lipid peroxidation and prevent depletion of liver glutathione (Skottova et al., 2003). Recent studies suggest that silymarin and its fraction of polyphenols may have beneficial effects on some risk factors of atherosclerosis. The results demonstrated that Silymarin has a hypolipidemic effect (Sobolova et al., 2006) and preventive effect on low density lipoprotein (LDL) cholesterol peroxidation in vitro (Locher et al., 1998). Silymarin also has a protective effect against stress induced by gastric ulcers and leads recovery of pancreatic function following alloxan damage in rats (Soto et al.,2004).

Direct drought stress may affect the biochemical processes related to the photosynthesis and reduce the input of carbon dioxide into the stomata which are blocked due to the dehydration mode. Therefore, the photosynthetic materials transfer has been affected by drought stress and causes the leaves to be saturated through these materials that may limit the photosynthesis. It is obvious that following the limitation of photosynthetic materials in the water leakage mode, the plant growth and ultimately the active substance value are diminished (Blokhina et al., 2003; Jubany-Mari et al., 2010).

The plants reflect the different defensive mechanisms for confronting the oxidative stress including enzymatic and non-enzymatic. The non-enzyme system includes ascorbate, tocopherol, carotenoids, and flavonoids (Ozkur et al., 2009). Phenol compounds include a large group of secondary metabolites such as cyclic compounds including phenol, flavons, flavonoids, tannins, lignins and even the cyclic amino acids such as tryptophan, tyrosine, and proline. These compounds have various ecologic and physiologic roles such as being the defensive factor and antioxidant (Andre et al., 2009).

The active gradient of milk thistle referred to as Silymarin is a kind of flauniod. According to the reports, the increment of these compounds' photosynthesis is by means of various peripheral simulators such as microbial attacks, ultraviolet rays and peripheral physical and chemical stresses. For instance, the analysis applied on Eucalyptus indicated that under drought stress mode, the phenol compounds in the plant are increased (Schwambach *et al*., 2008). Studying the flavoniod value on *Brassica napus* in the drought stress mode indicated that this material in increased in the plant as the secondary metabolite (Sangtarash et al., 2009). Efeoglu et al. (2009) reported the water leakage has significant impact on the growth, fatty acids and yield of Salvia essential oil, so that the middle stress increased the essential oil yield. Drought stress has significant effect on the growth and yield of citronella *(cymbopogon winterianus juwit*) essential oil, per acre of land (Fatima et al., 2000).

Proline is an amino acid, that increment of its concentration is the most frequent and general response that is observed upon stress creation (Cha-um and Kirdmanee,. 2009). The study applied on *Capsicum annum* implies the increase of proline in the plant (Koc *et al*., 2010). As well as, studying the sunflower under drought mode indicated that during the stress, pursuant to the increase of gamma glutamine kinas activity, the proline rate is increased as well (Manivannan et al., 2007).

The impact of drought stress is depending on the duration, durability and its shortage rate (Pandey et al., 2001). Identification of critical time and scheduling based on an exact and essential plan for the plant is considered as a key for water storing and improvement of irrigation operation and plant tolerability against water leakage (Ngouajio *et al*., 2007).

With respect to the Milk thistle the medicinal plant's responses to the different peripheral mode, low information is available. Thus, the extant study has been provided with the objective of studying the impact of drought stress on the active substance and Proline values in this valuable plant.

Materials and Methods

I) Planting

This research has been applied with the objective of measuring the rate of the active substance of *Silybum marianum* in the different ranges of drought stress as randomized complete block design with three replications, in Tehran Province, Karaj County on an experimental farm 450 m^2, each patches including 5 planting lines 4m in length, the interval between two planting lines 50cm (the area of each patch is 10 square meters and patch length is 4m and patch width is 2.5m). The climatic specifications and testing area soil have been provided in tables 1 and 2. The irrigation treatments used for applying the stress are as follows:

T1: Field capacity 100% (without stress), T2: field capacity 85% (mild stress), T3: field capacity 70% (moderate stress), T4: field capacity 55% (Severe stress).

At first, the field was plowed then was flattened and patched. The graft in the size of the plant has been cultivated on April 24th in the field with the relative density. For applying the drought stress ranges, time reflectometry method, TDR (Time-Domain Reflectometry) and (TIMER, IMKO) were used. The unique feature of this machine is its mobile sensor that provides the moisture measuring in 20cm layers at the soil depths. The length of zone transmitting the sensor of wave machine is 20cm. The produced waves by the machine are propagated in an area located in soil profile. The obtained number is recorded as soil moisture. The weeds have been controlled manually in several times. During the test, no pests and diseases were found.

II) Collecting

After stress ending, the harvest has been carried out manually on June 1st for extraction of Silymarin and proline active substances.

III) Extracting

The measured traits included the rate of active substances of Silymarin and proline.

HPLC assay

This experiment was carried out using a Knauer K2600A liquid chromatograph (Germany), equipped with a Nucleosil C18 (150 × 4.6 mm I.D, 5 µm) column. The analysis of samples was performed using silymarin Purospher stationary phase RP18 (15,034 mm 5 mm). A mixture of 85% phosphoricacid-methanol-water (0.5:46:64, v / v) was used as mobile phase. Elution was performed in an isocratic mode with a flow rate 1 ml / min and at 288 nm thedetection. One analysis requires 25 min (Kvasnicka et al., 2003).

Proline assay

The proline has been measured according to the method of Bates et al (1973), the absorption along the wavelength has been read on 532 nanometers and by means of the Proline standard curve, the amount of this material has been calculated and declared based on microgram to gram.

The data of this study were analyzed aiding MSTAT-C statistical software, and the mean values compared in accordance with Duncan method. Means were compared by using LSD test at 1% level of significance.

Results and Discussion

Silymarin

The analysis of variance indicates that the drought stress had a significant effect within 0.01 on the value of Silymarin active substance (table 3). Upon comparing the mean values, it was concluded that no significant difference existed between the active substance rate in all treatments except T1 and T2. By reducing the irrigation (increasing the stress area), the active substance rate was reduced (T4: field capacity 55% (Severe stress), so that the maximum rate of Silymarin's active substance was equaled to 1311.2 µg/g in the Severe stress treatment, and the minimum rate in stress-excluding treatment equaled to 843.6 µg/g (Fig 1).

In this study, the results of chemical analyses showed that drought stress had significant effect on the value of Silymarin active substance. In *Silybum mariantum* as a medicinal plant, the drought stress had the significant effect on the silymarin's active substance and caused the increase of silymarin in plant. Because when the plant is exposed to the stress, a lot of active oxygen species such as anion superoxide and hydrogen peroxide hydroxyl radical are produced (Jubany-Mari et al., 2010).

Identification of critical time and scheduling based on an exact and essential plan for the plant is considered as a key for water storing and improvement of irrigation operation and plant tolerability against water leakage (Ngouajio *et al*., 2007).

In drought stress mode that is the major physiological and biochemical aspect of plants, the secondary metabolites are affected and have significant effect on some yields of metabolites and compounds, consequently, production of active substance is increased due to the prevention from intercellular oxidation (Petropoulos et al., 2008).

The obtained results are corresponding to the results obtained from *Ocimum americanum* (Khalid, 2006), *Hypericum brasiliense* (De Abreu and Mazzafera, 2005). As well as, when *Brassica napus* was exposed to the drought stress and its active substance that is a type of flavonoid was studied, it was observed that the active substance value of the plant is increased under impact of drought stress (Sangtarash et al., 2009). In addition, similar results have been obtained on *Stellaria Longipes* (Singh et al., 2006).

proline

The analysis of variance indicated that the drought stress had significant effect within 0.01 on the proline value (table 3). Upon comparing the mean values, it was concluded that a significant difference existed between all treatments with respect to the proline value. By reducing the irrigation (increasing the stress area), the proline rate was increased (T4: field capacity 55% (Severe stress), so that the maximum rate of proline was equaled to 578.7 µg/g in the Severe stress treatment, and the minimum rate in stress-excluding treatment equaled to 308.8 µg/g(Fig 2).

Drough stress had significant effect on Proline value. In the extant paper, the proline concentration was increased under stress which is in compliance with the results of study applied on *Saccharum officinarum* L. (Cha-um and Kirdmanee, 2009). In addition to the increase of proline concentration in the different drought ranges to the control, the substance of aerial organs' proline was more than the roots, because the proline is synthesized at the vertex and root elongation area and transferred to the aerial parts through the transpiration, thus its accumulation value in the leaves is more than the other parts (Verslues and Sharp, 1999). Increment of proline during stress demonstrates the role of this amino acid for adjusting the osmotic pressure (Ashraf and Foolad, 2007). Osmotic adjustment in the plants is the major mechanism of avoiding the drought stresses in the dry and salty environment (Perez-Peez et al., 2009).

The researchers believe that the proline storage in the plant cells is related to the resistant to the drought mechanism (Yin et al., 2009). The high ranges of proline enable the plant to hold its water potential at a low rate (Valliyodan and Nguyen. 2006). The high rates of proline cause the reduction of free radicals responding the osmotic stress. These findings will clarify the proline biosynthesis adjustment in the plants and its role in reducing the induced oxidative stress more than ever, furthermore, confirms its accepted role as the osmolite (Hong et al., 2003).

Drought stress had positive effect on the *Capsicum annum* and increased the phenolic compounds and its spiciness (Estrada et al., 1999). Drought stress had significant effect on the growth and yield of essential oil in *cymbopogon winterianus juwit* (Fatima et al., 2000).

Therefore, secondary metabolites of the plants may be affected under impact of peripheral factors and water leakage that is the major physiological and biochemical aspect of the plants (Charles et al., 1994).

Conclusion

Results showed that drought stress had a significant effect on active substance and Proline of *Silybum marianum.*The best drought stress in this research was T4(field capacity 55% (Severe stress)) because of maximum Silymarin(1311.2µg/g) and Proline(578.7µg/g) values.

Acknowledgements

Thanks for Dr.ghooshchi and Dr.ariapour for supporting me in duration of this research.

Reference

1. Andre, C.M., R. Schafleitner, S. Legay, I. Lefevre and C.A.A. Aliaga et al.,. (2009). Gene expression changes related to the production of phenolic compounds in potato tubers grown under drought stress. Phytochemistry, 70(9): 1107-1116.
2. Ashraf, M. and M.R. Foolad, 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ. Exp. Bot., 59: 206-216.
3. Bates, L.S., R.P. Waldren and I.D. Teare,. 1973. Rapid determination of free proline for water-stress studies. Plant Soil, 39: 205-207.
4. Ball, K.R. and K.V. Kowdley, 2005. A review of *Silybum marianum* (milk thistle) as a treatment for alcoholic liver disease. J. Clin. Gastroenterol., 39: 520-528.
5. Blokhina, O., E. Virolainen and K.V. Fagerstedt,. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Annals of Botany, 91(2): 179-194.
6. Charles, D.J., R.J. Joly and J. E. Simon,. 1994. Effect of osmotic stress on the essential oil content and composition of peppermint. Phytochemistry, 29: 2837-2840.
7. Cha-um, S. and C. Kirdmanee,. 2009. Proline accumulation, photosynthetic abilities and growth characters of sugarcane (Saccharum officinarum L.) plantlets in response to iso-osmotic salt and water-deficit stress.. Agric. Sci. China, 8: 51 -58.
8. De Abreu, I.N. and P. Mazzafera,. 2005. Effect of water and temperature stress on the content of active constituents of Hypericum brasilienne Choisy.. Plant Physiol. Biochem., 43: 241–248.
9. Efeoglu, B., Y. Ekmekci and N. Cicek,. 2009. Physiological responses of three maize cultivars to drought stress and recovery. S. Afr. J. Bot., 75: 34-42.
10. Estrada, B., F. Pomar, J. Diaz, F. Merino and M.A. Bernal,. 1999. Pungency level in fruits of the Padron pepper with different water supply. Scientia Horticulturae, 81: 385-396.
11. Fatima, S., A.H.A. Farooqi and S. Sharma, 2000. Effect of drought stress and plant density on growth and essential oil metabolism in citronella java (Cymbopogon winterianus) cultivars. J. Med. Aromatic Plant Sci., 22: 563-567.
12. Gazak, R., D. Walterova and V. Kren,. 2007. Silybin and silymarin, new and emergining applications in medicine. Current Med. Chem. Schiphol., 14: 315-338.
13. Hong, S.W., S.J. Kwon, S.I. Sohn, N.S. Kim and J.C. Kim,. 2003.. Characterization of embryogenesisrelated *Pbmyb* genes during in vitro dif-

ferentiation of *Pimpinella brachycarpa*. Korean J. Genetics, 25: 293-300.

14. Kvasnicka , F., B. Biba, R. Sevcik, M. Voldrich and J. Kratka,. 2003. Analysis of the active components of silymarin. J. Chromatogr. A., 990: 239-245.

15. Jubany-Mari, T., S. Munne-Bosch and L. Alegre,. 2010. Redox regulation of water stress responses in field-grown plants. Role of hydrogen peroxide and ascorbate. Plant Physiology and Biochemistry, 48(5): 351-358.

16. Khan, S.A., B. Ahmad and T. Alam,. 2006. Synthesis and antihepatotoxic activity of some new chalcones containing 1, 4-dioxane ring system. Pak. J. Pharm. Sci., 19: 290-294.

17. Kren, V. and D. Walterova,. 2005. Silybin and silymarin: New effects and applications. Biomed. Pap. Med. Fac. Univ Palacky. Olomouc. Czech. Repub., 149: 29-41.

18. Koc E., C. Islek and A.S. Ustun, 2010. Effect of cold on protein, proline, phenolic compounds and chlorophyll content of two pepper (*Capsicum annuum* L.) varieties. Gazi Univ. J. Sci., 23: 1-6.

19. Khalid, K.A.,. 2006. Influence of water stress on growth, essential oil and chemical composition of herbs (Ocimum sp.).. Inter. Agrophys., 20: 289 –296

20. Locher, R., P.M. Suter, R. Weyhenmeyer and W. Vetter,. 1998. Inhibitory action of silibinin on low densitylipoprotein oxidation. Arzneimittelforschung., 48: 236-239.

21. Manivannan, P., C.A. Jaleel, B. Sankar, A. Kishorekumar, R. Somasundaram, G.M. Lashmana and R. Panneerselvam, 2007. Growth, biochemical modifications and proline metabolism in *Helianthus annuus* L. as induced by drought stress. Colloids and Surfaces B: Biointerfaces, 59(2): 141-149.

22. Ngouajio, M., G. Wang and R. Goldy, 2007. Withholding of drip irrigation between transplanting and flowering increases the yield of field-grown tomato under plastic mulch. Agricultural water management, 87: 285-291.

23. Ozkur, O., F. Ozdemir, M. Bor and I. Turkan,. 2009. hysiochemical and antioxidant responses of the perennial xerophyte *Capparis ovate* Desf to drought. Environ. Exp. Bot., 66(3): 487-492.

24. Petropoulos, S.A., D. Daferera, M.G. Polissiou and H.C. Passam,. (2008). The effect of water deficit stress on the growth, yield and composition of essential oils of parsley.. Sci. Hort., 115: 393 -397.

25. Pandey, P.K., J.W. Maranville and A. Admou,. (2001). Tropical wheat response to irrigation and nitrogen in a Sahelian environment. I. Grain yield, yield components and water use efficiency.. Eur. J. Agron., 15: 93 -105.

26. Perez-Peez, J.G., J.M. Robles, J.C. Tovar and P. Botia,. 2009. Response to drought and salt stress of lemon Fino 49 under field conditions: Wa-

ter relations, osmotic adjustment and gas exchange. Sci. Hortic., 122: 83-90.

27. Ramadan, L.A., H.M. Roushdy, G.M. Abu Senna, N.E. Amin and O.A. El-Deshw, 2002. Radio-protective effect of silymarin against radiation induced hepatotoxicity. Pharmacol. Res., 45: 447-454.

28. Sangtarash, M.H., M.M. Qaderi, C.C. Chinnappa and D.M. Reid,. 2009. Carotenoid differential sensitivity of canola (*Brassica napus)* seedlings to ultraviolet-B radiation, water stress and abscisic acid. Environ. Exp. Bot., 66(2): 212-219.

29. Schwambach, J., C.M. Ruedell, M.R. de Almeida, R.M. Penchel, E.F. de Araujo and A.G. Fett-Neto, 2008. Adventitious rooting of *Eucalyptus glubus*× *maidennii* mini-cutting derived from mini-stumps grown in sand bed and intermittent flooding trays: a comparative study. New Forests, 36(3): 261-271.

30. Skottova, N., R. Vecera, K. Urbanek, P. Vana, D. Walterova and L. Cvak, 2003. Effects of polyphenolic fraction of silymarin on lipoprotein profile in rats fed cholesterol-rich diets.. Pharmacol. Res., 47: 17 -26.

31. Sobolova, L., N. Skottova, R. Vecera and K. Urbanek, 2006. Effect of silymarin and its polyphenolic fraction on cholesterol absorption in rats.. Pharmacol. Res., 53: 104 -112.

32. Soto, C., R. Mena, J. Luna, M. Cerbon and E. Larrieta et al.,. 2004. Silymarin induces recovery of pancreatic function after alloxan damage in rats.. Life Sci.,75: 2167 -2180.

33. Singh, R.P., G. Deep, M. Chittezhath, M. Kaur, L.D. Dwyer-Nield, A.M. Malkinson and R. Agarwal,. 2006. Effect of silibinin on the growth and progression of primary lung tumors in mice. J. Natl. Cancer Inst., 98: 846-855.

34. Tamayo, C. and S. Diamond, 2007. Review of clinical trials evaluating safety and efficacy of milk thistle (Silybum marianum L. Gaertn.). Integr. Cancer Ther., 6: 146-157.

35. Valliyodan, B. and H.T. Nguyen, 2006. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Curr. Opin. Plant Biol., 9: 189-195.

36. Verslues, P.E. and R.E. Sharp, 1999. Proline accumulation in maize (Zea mays L.) primary roots at low water potentials. II. Metabolic source of increased proline deposition in the elongation zone. Plant Physiology 119, 1349–1360.

37. Yin, C., X. Pang and Y. Lei,. 2009 Populous from high altitude has more efficient protective mechanisms under water stress than from low-altitude habitats: A study in greenhouse for cutting. Physiol. Plant., 137: 22-35.

Table 1 - Climatic characteristics of experimental farm

Longitude	Latitude	Mean precipitation	Mean humidity	Maximum temperature	Minimum temperature
Eastern 50˚,56’	Northern 35˚,46’	247.3 mm	53%	42	-20

Table 2 - Chemical and physical characteristics for soil of experimental farm (at the soil depth of 0 - 30 cm)

Silt %	Sand %	Clay %	Absorptive Potassium% (p.p.m)	Absorptive Phosphorus % (p.p.m)	Total Nitrogen %	Organic Carbon %	Water absorption %	EC (ds/m)	pH	Soil texture
26	38	33	287	18.93	0.11	4.22	58	1.04	7.2	Loamy-clay

Table 3 - Variance analysis results of Drought stress effects on active substance Silymarin and proline of Milk thistle

S.O.V	df	Square Means	
		Silymarin µg/g	Proline µg/g
Replication	3	76.4ns	96.6ns
Drought stress	3	185617.4**	72962.7**
Error	9	106.7	112.5
C.V%		3.6	3.9

ns and ** : Non significant ,and significant at the 1% levels of probability, respectively

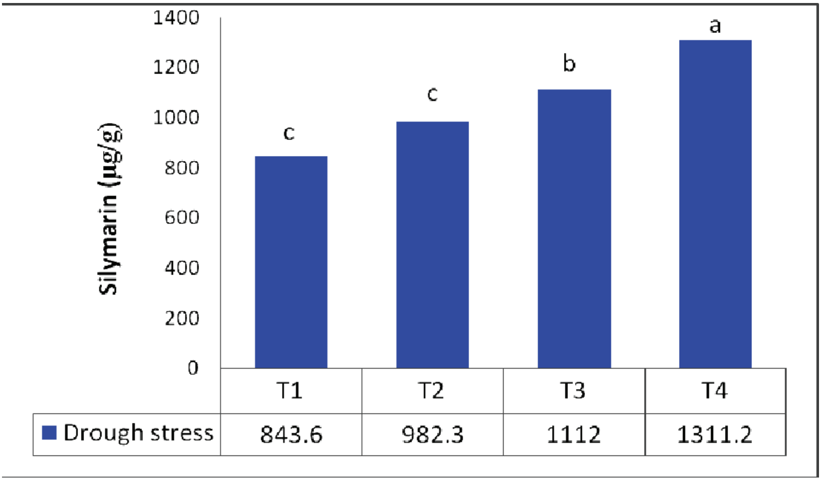

Fig 1 - Effect of drought stress treatments on the active substance silymarin. Values with different letters are statistically significantly different within 0.01 .

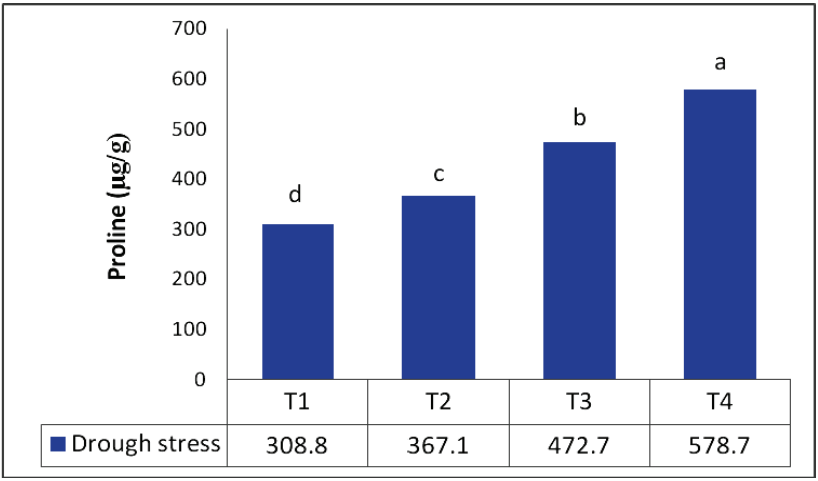

Fig 2 - Effect of drought stress treatments Proline. Values with different letters are statistically significantly different within 0.01 .