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Stress Induced Alteration in the Antioxidant Activity of In Vitro Adventitious Roots of Withania Somnifera (Genotype Jawahar 20)

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Abstract

The present study was aimed to characterize the enzymatic and non-enzymatic antioxidants in *in vitro* grown adventitious roots of *Withania somnifera* (genotype Jawahar 20) under PEG induced drought. Due to the illimitable therapeutic values of roots and the fact that "enhanced activity of antioxidants is directly correlated with oxidative injury promoted by drought", five major antioxidants (phenol, flavonoids, SOD, CAT and ascorbate) were evaluated. The roots of *Withania somnifera* were not able to enhance enzymatic antioxidants like SOD and CAT infact 19.28 to 28.54% and 63.89 to 89.48% reduction in SOD and CAT activity were measured with increasing stress duration. In contrast roots were efficient to enhance the phenols and flavonoids under drought with maximum i.e. 52.11% and 231.18% enhancement in phenol and flavonoid content respectively after 45 days of stress treatment. A sudden decrease in ascorbate content from 89.51 to 11.11 mg/g fresh weight and 123.46 to 12.35 mg/g fresh weight was also recorded with increasing concentration of stress after 30 and 45 days respectively, however not such reduction were observed after 60 days. The findings of study indicated that roots of *Withania somnifera* are rich source of phenolic compounds and their enhancement under drought make them capable to survive in stressful environment.

Keywords

Ashwagandha; Drought; *In vitro*; Antioxidants

Introduction

Ashwagandha (*Withania somnifera*), a short woody solanaceaous shrub has today been recognized potentially as one of the most valuable plants because of its great medicinal value and the ability to grow even in the most arid and nutrient-deficient soils [1]. Among all the plant parts, the roots of *W. somnifera* have turn into an area of interest for the researchers as well as the pharmaceutical companies due to their richness in secondary metabolites, having immense therapeutic values [2]. In Ayurveda, the roots of *W. somnifera* have been prescribed for gynaec disorders, bronchitis, arthritis, rheumatism, inflammation, fevers and skin diseases, etc. [3]. For ages ashwagandha has traditionally been believed to increase energy, youthfulness, vigour, endurance, strength, health, vital fluids, muscle fat, blood, lymph, semen and cell production. It also helps counter chronic fatigue, weakness, dehydration, bone weakness, loose teeth, thirst, impotency, premature aging emaciation, debility and muscle tension [4]. Despite its enormous therapeutic advantages, the annual production of this plant is not sufficient to meet the global requirement. The estimated production of ashwagandha roots in India is more than 1500 tonnes per year and the annual requirement is about 7000 tonnes, necessitating the increase in its cultivation and higher production [5,6]. The production should match the demands of ever increasing population. Contextually, *in vitro* culture system presents a suitable example to solve the tangle in the production of medically valuable compounds. The advantage of using root cultures over natively grown field plants is that they grow rapidly, are relatively easy to prepare and maintain a low level of variability and can easily be cloned to produce a large supply of experimental tissues [7].

Drought stress is a slow-onset but complex phenomenon posing a great challenge to the ecology. It can cause serious economic, social and environmental losses more than any other natural hazards in both developing and developed countries. This is actually due to the heavy losses caused to crop species under drought stress. One of the major factors leading to impaired plant growth and productivity under drought is the production of reactive oxygen species in organelles including chloroplasts, mitochondria and peroxisomes [8]. To protect themselves from reactive oxygen species, living organisms have developed several effective mechanisms. Enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, peroxidase, glutathione reductase, and monodehydro ascorbate reductase form a part of the antioxidant defense system designed to minimize the concentration of active species like superoxide and hydrogen peroxide radicals [9].

From the above literature, the importance of the ashwagandha roots and the role of secondary metabolites and antioxidants under drought stress are evident. It should thus be an endeavor of all researchers to enhance their production. Among abiotic stresses, drought is one of the burning issues and ashwagandha is found to grow in the regions which are drought prone as well. Hence, in the present investigation we came up with the idea to grow ashwagandha under *in vitro* drought stress. There are some reports which explain the antioxidant potential but one still unanswered question is the "alteration in antioxidant potential under drought or extreme limited conditions". Keeping the above view in mind, antioxidant potential has been checked under drought stress in *in vitro* roots of *W. somnifera.*

Materials and Methods

Sterilized leaf (size 0.5-2.0 cm) explants of genotype J-20 were carefully inoculated on MS medium supplemented with different concentrations and combinations of plant growth regulators i.e. 2,4-D (0.5 ppm) and NAA ranges between (0.2 – 2.0 ppm) for direct root induction. Initiated roots were sub cultured on media for proliferation and kept in culture room at temperature of 25 ± 2°C. Proliferated roots than transferred on basal media supplemented with 1%, 2% and 3% PEG considering it as T_1, T_2 and T_3 treatment, Media without PEG was taken as control.

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Relative growth rate (RGR)

The RGR was calculated according to the following formula [9]

$$RGR = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

Where, W_1= Dry weight of the whole root at start of the test period.

W_2= Dry weight of whole root at the end of the test period.

(t_2-t_1) = Period in days between initial and final observations.

Superoxide dismutase activity

SOD activity was determined by measuring its ability to inhibit the photochemical reduction of Nitroblue tetrazolium chloride (NBT) as described by Gianopolitis and Ries [10]. 0.2 g roots were homogenized in an ice cooled mortar and pestle by adding 4 ml ice cold extraction buffer and centrifuged at 16, 0000 g for 15 min at 4°C. The supernatant was used as crude enzyme extract for quantification of enzyme activity. 1.5 ml reaction mixture containing 50 µl of enzyme extract in the tubes was shaken thoroughly and illuminated with two 20W florescent tubes for 15 min. Then tubes were covered with a black cloth and the absorbance was recorded at 560 nm. Along with the reaction tubes one control (everything except enzyme) and one reference tube (immediately covered with a black cloth) was also set up. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of NBT photo reduction rate.

Z = [(X – A)/X] x 100

Total SOD unit = Z/50

Total SOD unit min^{-1} = Total SOD unit/15

Catalase activity

CAT activity was measured according to the method of Kar and Mishra [11]. 200 mg root sample was homogenized with 10 ml of phosphate buffer pH 6.8(0.1 M) and 5 ml portion was centrifuged at 2°C for 15 min at 17000 g. Clear supernatant was taken as an enzyme source. Reaction mixture consisted of 1 ml of twice diluted enzyme extract + 1 ml of 300 µmol phosphate buffer (pH 6.8) + 1 ml of 100 µmol H_2O_2 (final volume 5 ml with DW) was incubated at 25°C for 1 min. Then reaction was stopped by adding 10 ml of 2% H_2SO_4. Residual H_2O_2 was titrated with 0.01N $KMnO_4$ until faint pink colour persisted for 15 sec. Volume of $KMnO_4$ used was recorded. In control enzyme activity was stopped at 0 time. One unit of CAT activity is defined as the amount of enzyme which breaks down 1 mMol of H_2O_2 per min under assay condition.

CAT activity = Volume ($KMnO_4$) x 40 (extinction coefficient)

Flavonoid content

Flavonoid content in the sample was estimated according to the method of Ordonez [12]. 500 mg of root tissues were homogenized in 10 ml of 80% ethanol and centrifuged at 10000 rpm at 4°C for 20 min and then the supernatant was evaporated to dryness. The residue was dissolved in 5 ml of distilled water and then this solution was further used for the estimation of flavonoids. To 1.5 ml of sample solution, 1.5 ml of 2% $AlCl_3$ ethanol solution was added. The mixture was incubated for 1h at room temperature. After that the absorbance was measured at 420 nm. A yellow color indicated the presence of flavonoids. Flavonoids content was calculated as quercetin equivalent from the standard curve.

Total phenolic content

The phenolic content was estimated according to the method of Wolfe [13]. Extraction procedure was the same for phenol as used in case of flavonoid. Aliquots (0.1 to 1 ml) were pipetted out from the prepared solution into the test tubes then the volume was made up to 3 ml with distilled water and 0.5 ml of Folin-Ciocalteau reagent was added. After 3 min, 2 ml of 20% Na_2CO_3 solution was added to each test tube. Then the mixture was mixed thoroughly and the tubes were placed in a boiling water for exactly one min then cooled thereafter absorbance was measured at 650 nm against a blank. Total phenol was calculated from standard curve of catechol prepared by using different concentrations.

Ascorbic acid content

Ascorbic acid was estimated in roots according to the method of Sadasivam and Manickam [14]. 0.5 g sample was homogenized in 10 ml of 4% oxalic acid and centrifuged at 10000 rpm for 30 min. One ml of supernatant was taken and mixed with 2 ml of 4% oxalic acid. Reaction mixture was titrated against 2, 6 - dichloroindo phenol dye. Volume consumed for the titration was named as V2. Amount of ascorbate in sample was calculated by using standard solution of 10 µmol ascorbate. 5 ml working standard was taken then 10 ml 4% oxalic acid was added. Titrated against dye and volume was recorded as V1.

$$\text{Amount of ascorbate (mg/100g sample)} = \frac{0.5mg \times V2(ml) \times 100ml \times 100}{V1(ml) \times 15 \times wt.of\ sample}$$

Results and Discussion

In vitro root induction and effect of drought on growth

In vitro roots were initiated from the cut ends and margins of the leaf explants within two weeks of inoculation in culture medium without callus formation in genotype Jawahar -20. Root induction was evaluated on the basis of % root induction and no of roots/explants. 93.33 % roots were initiated when media was supplemented with 0.5 mg/l 2,4-D and 0.75 mg/l NAA. Maximum number of roots i.e. 25.00/explants were recorded for same concentration of PGRs while minimum i.e. 4.00 roots/explants was in the medium supplemented with 0.5 mg/l 2,4-D along with 2.00 mg/l NAA (Table 1). Synthetically-prepared auxins 2,4-D, NAA, IAA and IBA used in plant culture media metabolized rapidly within plant tissues as they tend to be denatured in media, so that they are useful for the developmental phases as rooting [15]. The advantage of using root cultures is that they grow rapidly and relatively easy to maintain, they showed low level of variability and can be cloned to produce in large amount for further experiments [7]. Hundred percent rooting with

Table 1: Effect of plant growth regulators on % root induction and number of roots /explant after 20 days of inoculation in *W. somnifera*.

S.No.	Treatments		Jawahar -20	
	2,4-D	NAA	% Root induction	No of roots/explant
1.	0.5	0.20	0.00 ± 0.00	0.00 ± 0.00
2.	0.5	0.50	45.56 ± 2.94	13.33 ± 2.03
3.	0.5	0.75	93.33 ± 3.85	25.00 ± 1.00
4.	0.5	1.00	61.11 ± 1.11	14.67 ± 0.88
5.	0.5	1.25	70.00 ± 3.85	11.00 ± 3.00
6.	0.5	1.50	78.89 ± 1.11	6.67 ± 0.33
7.	0.5	1.75	38.89 ± 2.94	4.00 ± 1.53
8.	0.5	2.00	17.78 ± 4.01	4.33 ± 0.88
SEM±			2.85	1.50
CD 5%			8.57	4.51

highest number of roots (18 ± 0.00) and root length (3.22 ± 0.24 cm) per shoot was reported in *Withania somnifera* when half strength MS medium with NAA (0.5 µM) was used [16].

Effect of drought on growth of roots

Roots of *Withania somnifera* were initiated and proliferated on PEG induced drought MS medium and evaluated morphologically in terms of proliferation with root length and growth of root hairs. Root length and root hair were found more in T1 (1% PEG) treatment as compared to control in all the time durations i.e., 30, 45 and 60 day after inoculation. In treatment T2 (2% PEG) and T3 (3% PEG), reduced root length and root hairs have been clearly seen with increasing concentration of PEG, proliferation rate was also less in comparison of control. Colour of the roots gets also changed from white to brown with increasing PEG in the media Photo Plate 1 (Figure 1). The enhancement in the root growth at initial stage of stress can be correlated with the slight drought tolerance which facilitates the capacity of the root system to extract more water from deeper soil layers [17].

33.33% increment in fresh weight (3.60g) and relative growth rate (0.12 g/g/days) with respect to control was observed during initial drought stress (T1) condition in the media after 30 days of inoculation while no significant difference in fresh weight and in RGR was found up to 60 days of inoculation (Figure 1 and Plate 1), indicates that during initial days, water deficit accelerate the root initiation growth as well. The ability of a plant to modify its roots to capture more water for transpiration may be an important mechanism to avoid drought stress. In addition, large root systems can maintain high water use efficiency under drought [18]. It would also be important to produce a large amount of biomass, which contributes to crop yield, using a low or limited amount of water [19]. As the results of this investigation it was found in a number of studies that at initial level of drought there was no or less effect on root growth but have decreased with further enhancement of PEG induced drought. In *P. Vulgaris* similar results were reported after 14th day of drought induction in the media [20]. Similarly it was reported that reduced fresh weight with increasing concentration of PEG (-0.1, -0.2, -0.3, -0.4MPa) in Black Cumin. They observed that maximum value corresponds to control [21].

Effect of drought on antioxidants of roots

Roots of *Withania somnifera* are rich source of phenolic compounds whose levels increased during stress conditions and prevent the tissues from oxidative damage of proteins, lipids and nucleic acids by scavenging the reactive oxygen species. However other enzymatic and non-enzymatic antioxidants may also involve in the protection of oxidative damage. In order to understand the role of other antioxidants under drought stress SOD, CAT and ascorbate were estimated in roots of *Withania somnifera* which were induced on PEG containing media [22]. SOD activity in roots of J-20 was decreased

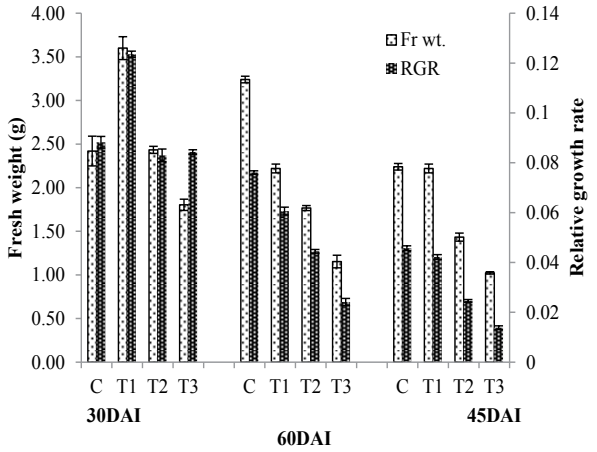

Figure 1: Effect of PEG induced drought on root Fresh weight (g) and Relative growth rate (g/g/days) after 30, 45 and 60 Days of stress treatments in *Withania somnifera*. T1 (1%PEG), T2 (2% PEG), T3 (3% PEG) and C (control).

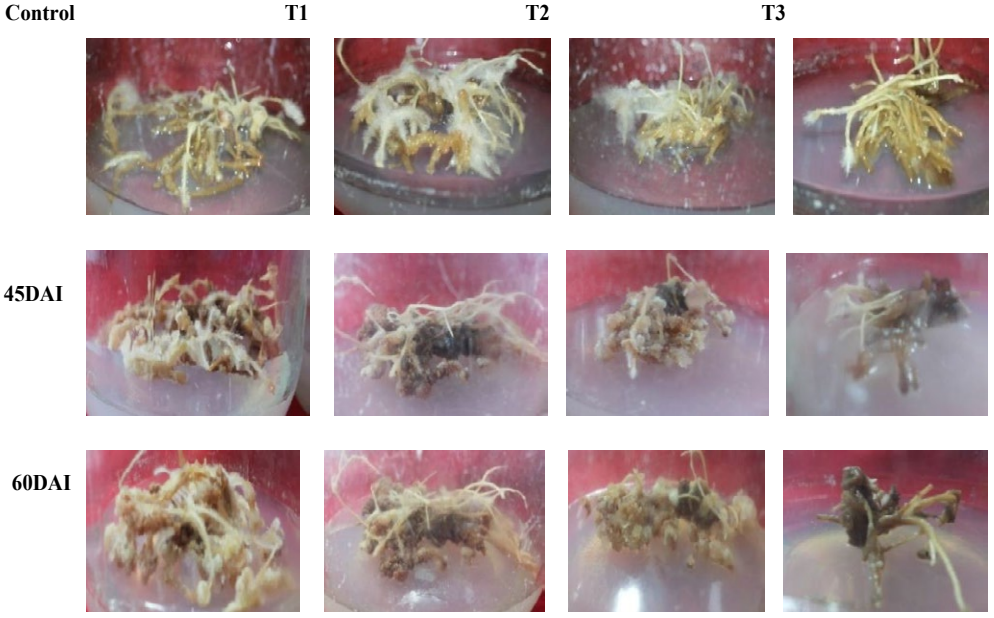

Plate 1: Effect of PEG induced drought on *in vitro* root morphology of *W. somnifera* under three time duration i e. 30, 45 and 60 days of stress. T1 (1%PEG), T2 (2% PEG), T3 (3% PEG) and C (control).

by 19.28% and 28.54% with increasing concentration of PEG after 30 and 45 days of stress treatment respectively while after 60 days it was increasing by 13.64% up to T1 treatment (Figure 2A). It is interesting to know that though there was a reduction in the SOD activity for initial days, the roots still survived and the level of SOD was increased after 60 days which means that the plant cells were able to increase the antioxidant level to cope up the cells from stress condition and also neutralized the effect of stress induced ROS. A little enhancement in SOD activity after 60 days of stress indicates the improved strategy of tolerance mechanism in root cells. Similar trend was found with CAT activity. It was also decreased with increasing conc. of PEG for all stress levels. Minimum % decrease (63.89%) was recorded after 30 days of stress treatment followed by 45 days (77.42%) while maximum (89.48%) was recorded after 60 days (Figure 2B). The root is the only organ of the plant that penetrates the soil and is, therefore, the only organ that is in direct contact with adverse stressors. Hence, the antioxidant level can be altered first in the roots [23].

In contrast to enzymatic antioxidants, *Withania somnifera* showed enhanced synthesis and accumulation of nonenzymatic antioxidants like phenol and flavonoids under drought. Phenol content in roots was significantly increased with increasing concentration of PEG in the media after 30 and 45 days of treatment but after 60 days it was increasing only up to T_1 level. Maximum phenol content (288 µg/g fw of root) was recorded after 45 days of stress followed by 30 days (244 µg/g fw of root) and 60 days (204 µg/g fw). On the other hand maximum % increment (52.11%) was recorded for 45 days followed by 60 days (37.63%) and 30 days (33.57%) (Figure 3A). Enhanced phenol content can be correlated with the improved stress tolerance mechanism. Researchers and food manufacturers have become more interested in polyphenols due to their potent antioxidant properties, their abundance in the diet, and their credible effects in the prevention of various oxidative stress associated diseases [24].

Flavonoids are the most abundant polyphenols in our diets [25]. In present study flavanoid content was also significantly increased with increasing concentration of PEG after 30 and 45 days of stress treatment while after 60 days such trend was not observed. Maximum % increase (231.78%) was recorded after 45 days followed by 30 days (228.06) while minimum (40.12%) was recorded after 60 days (Figure 3B). Flavonoids are a representative group of secondary metabolites that have recently been called 'specialized metabolites' because plants synthesize species-specific metabolites [26]. Our results seek support from the study of Basu, [27] they reported the significantly increased flavonoid content in three varieties of rice with the maximum increase in Pokkali (3.27 times) followed by Pusa Basmati and IR-29 (2.08 and 1.99 times, respectively). It was shown that *in vitro* roots of *W.somnifera* had higher content of total flavonoids (0.33 mg/g DM) than greenhouse materials (0.07 mg/g DM [28].

However ascorbate (vitamin C) is a major metabolite and antioxidant in most of the plant but our results indicate that it is species specific. In *in vitro* roots of *W.somnifera* ascorbate content was decreased with increasing concentration of PEG. Surprisingly a sudden decrease (89.51 mg/g fw to 11.11 mg/g fw of root) and (123.46 mg/g fw to 12.35 mg/g fw) was recorded in T1 treatment with respect to control after 30 and 45 days respectively. After 60 days of stress treatment such decrease was not recorded. Maximum % reduction was recorded after 30 days (96.89%) followed by 45 days (95.25%) and 60 days (78.37%) (Figure 3C). Among the non-enzymatic antioxidants, ascorbate is found to be one of the best characterized compounds, required for many key metabolic functions in plant cells [29]. Though ascorbate (AA) acts as an antioxidant, protecting cells against oxidative stress but here in case of ashwagandha, it was not found suitable for coping drought stress.

Conclusion

The present study is an effort to compile and update the knowledge regarding the effectiveness of antioxidants. It also explains

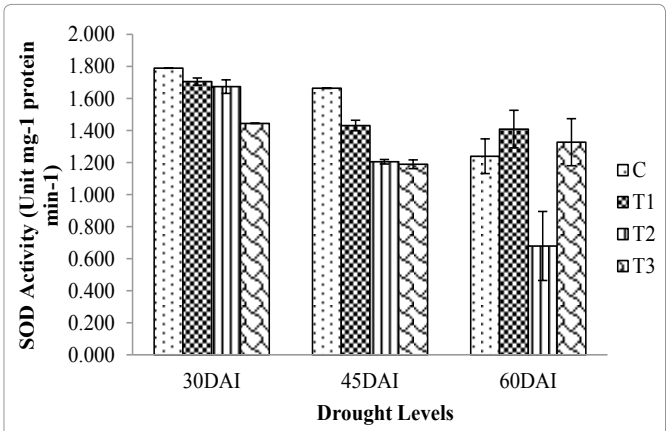

Figure 2 (A): Effect of PEG induced drought on activity of SOD in In vitro roots of W. somnifera. T1 (1%PEG), T2 (2% PEG), T3 (3% PEG) and C (control).

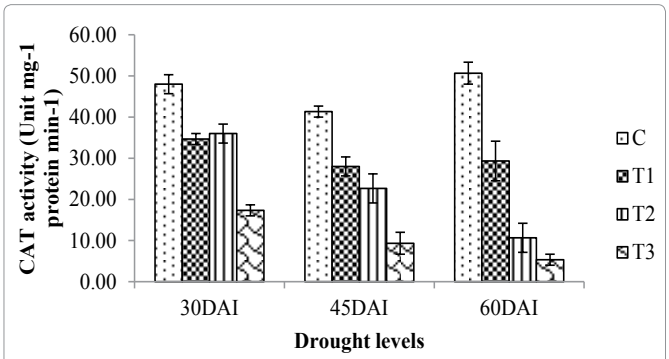

Figure 2 (B): Effect of PEG induced drought on activity of CAT in In vitro roots of W. somnifera. T1 (1%PEG), T2 (2% PEG), T3 (3% PEG) and C (control).

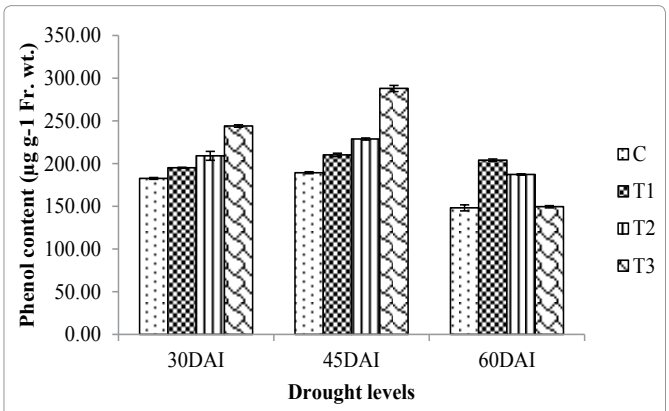

Figure 3 (A): Effect of PEG induced drought on total phenol content of roots of *W. Somnifera*. T_1 (1%PEG), T_2 (2% PEG), T_3 (3% PEG) and C (control).

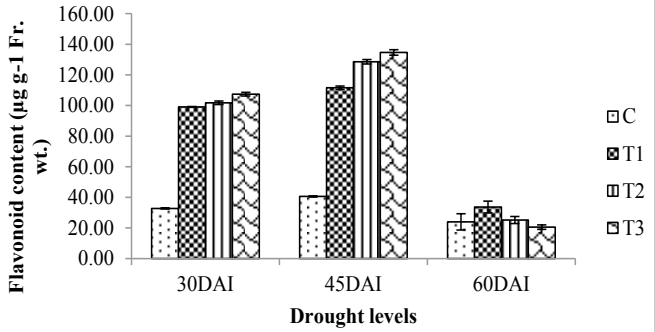

Figure 3 (B): Effect of PEG induced drought on total flavonoid content of roots of *W. Somnifera*. T_1 (1%PEG), T_2 (2% PEG), T_3 (3% PEG) and C (control).

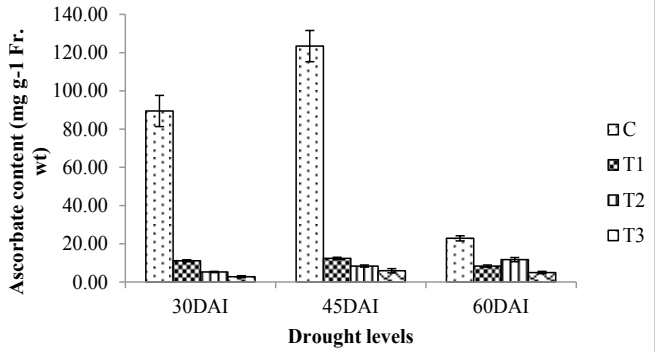

Figure 3 (C): Effect of PEG induced drought on ascorbate content of roots of *W. Somnifera*. T1 (1%PEG), T2 (2% PEG), T3 (3% PEG) and C (control).

that all the antioxidants do not work simultaneously against stress as their synthesis and concentration is species specific. Here higher accumulation of phenols and flavonoid is directly correlated with the stress tolerance in roots of *W.somnifera* while SOD, CAT and ascorbate were unable to cope up the tissues from drought.

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