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IN VITRO EVALUATION OF ANTIOXIDANT POTENTIAL UNDER DROUGHT STRESS IN ENDANGERED *WITHANIA SOMNIFERA*

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ABSTRACT
The competence of any plant in regard to the antioxidant potential is of immense value.With this background the present investigation was carried on callus of different genotypes of *Withania somnifera* to check the antioxidant potential under drought stress as it is best expressed under such conditions. Genotypes responded positively in terms of increased antioxidant activity under stress. An enhancement of 213.51 and 200% in SOD activity was observed in case of genotype Nimitly after 15 and 30 days of stress respectively while of 24.30 to 37.37% for other genotypes after 15 days and 10.73 to 40.74% after 30 days. Best results in CAT activity were found at 1% PEG showing 274.88 > 199.85 > 155.58 > 66.68% enhancement in genotype Nimitly, J-20, Pratap and Chetak respectively after 15 days. PEG application also brought about an increment in non enzymatic antioxidant content. Here genotype Pratap found superior with maximum of 118.23 and 112.53% enhancement in Phenol and flavonoid content respectively. Increasing stress levels was also established as beneficial in improving ascorbate activity but it was found to be enhanced only up to 1% PEG level with a little increment of 5.97 to 12.72%. Conclusively *Withania somnifera* is a plant of higher antioxidant potential (either enzymatic or non enzymatic) and hence hold great medicinal importance even at callus level
Abbreviations : SOD - Superoxide dismutase, CAT- Catalase, PEG - Polyethylene glycol, ROS - reactive oxygen species, GAE - Gallic acid equivalent, 2,4-D - 2,4 dichloro phenoxy acetic acid, BAP-6 benzyl amino purine, PGRs-Plant growth regulators, DAI – Days after inoculation, DAT- days after treatment

INTRODUCTION

Herbal and natural products are on demand from centuries. Various plant parts such as leaves, bark, fruits, roots and seeds are used in treatment of various diseases (Kumar *et al.,* 2013). *With ania somnifera* has been considered medicinally important in terms of secondary metabolites and antioxidants from age to age. Despite its enormous therapeutic advantages, the annual production of this plant is not sufficient to meet the global requirement (Umadevi *et al.,* 2012) which influences the overall generation of antioxidants, the most important biochemical component of the plant. Antioxidants, scavengers of free radicals, consequently are very special group of nutritional supplements. Being strong reducing agents, it help to tie up free radicals and thus protect the body from their deleterious effects (Swapana *et al*., 2012). Level of antioxidants can also be enhanced by drought conditions as it is not always found detrimental for the biosynthesis of such compounds. Drop of water potential in plants due to drought develops a wide range of physiological and biochemical processes which help plants to cope up unfavorable conditions (Chaves *et al.,* 2003). In fact it is believed that "enhanced activity of antioxidants is directly correlated with oxidative injury promoted by drought".

In this regard, *In vitro* cultivation has emerged as one of the important tool to increase the proliferation of this plant as well as it will be easy to produce the drought tolerant and antioxidant rich plants by giving gradual stress through tissue culture as it require less water for it' s growth. Apart from this antioxidant can also be extracted even at initial callus level very easily. Also, very little attention has been drawn to study the alteration in antioxidant metabolism of various medicinal plants under water stress. With this background, the present study was carried out to characterize the enzymatic and non enzymatic antioxidants in callus of *W. somnifera* due to the illimitable therapeutic values and comparatively easy extraction of medicinally important compounds.

MATERIALS AND METHODS

The seeds of four genotypes of *W. somnifera* namely Jawahar-20, Nimitly, Chetak and Pratap were obtained from Central Institute of Medicinal and Aromatic Plant, Research Centre (CIMAP), Lucknow, India and sown in the garden section of Plant Physiology, College of basic Sciences & Humanities, Pantnagar. The young healthy leaves of all the genotypes were sterilized with 0.1% streptomycin followed by 0.3% bavastin and 0.1 % $HgCl_2$. This step was done with slight modification in the protocol adopted by (Singh *et al*., 2011). Sterilized leaves were cut in to small pieces and inoculated on MS media supplemented with various combination of 2,4-D, NAA and BAP (Adhikari and Pant, 2013; Singh *et al*., 2011). Cultures were kept in culture room at temperature of 25 ± 2^{o}C and 16h/8h (light/dark) photoperiod.

Leaf derived proliferated callus of all genotypes were

transferred on MS media supplemented with appropriate plant growth regulators and 1%, 2% and 3% PEG considering it as T1, T2 and T3 treatment. Media without PEG was taken as control.

Relative water Content

Relative water content (RWC) of drought stressed callus was estimated according to the method of Castillo (1996). Fresh weight (FW) of callus was measured and saturated in 100 ml distilled water for 24 h at 4°C in dark and their turgid weights (TW) were recorded. Thereafter, they were oven-dried at 65°C for 48 h and dry weights (DW) were recorded. RWC was calculated by using following formula:

$RWC\ (\%) = [(FW - DW) / (TW - DW)] \times 100$

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 (1977). 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. 1.5ml reaction mixture containing 50 μl of enzyme extract in the tubes was shaken thoroughly and illuminated with two 20W flourescent 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.

$Z = [(X - A)/X] \times 100$

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 (1976). 200mg root sample was homogenized with 10ml of phosphate buffer pH 6.8(0.1M) and 5ml portion was centrifuged at 2°C for 15min at 17000g. Clear supernatant was taken as an enzyme source. Reaction mixture consisted of 1ml of twice diluted enzyme extract + 1 ml of 300 μmol phosphate buffer (P^H 6.8) + 1ml of 100 μmol H_2O_2 (final volume 5ml with DW) was Incubated at 25°C for 1 min. Then reaction was stopped by adding 10ml 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 1mMol 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 *et al.,* 2006). 500 mg of root tissues were homogenized in 10 ml of 80% ethanol and centrifuged at 10000 rpm at 4ºC 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 *et al.,* 2003). Extraction procedure was the same for phenol as used in case of flavanoid. Aliquots (0.1 to 1 ml) were pipetted out from the prepared solution into the test tubes then the volume was made up to 3ml with distilled water and 0.5ml of Folin-Ciocalteau reagent was added. After 3 min, 2ml 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 Thimmaiah (1999). 0.5g 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 2ml 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 10μmol ascorbate. 5ml working standard was taken then 10 ml 4% oxalic acid was added. Titrated against dye and volume was recorded as V1

$$\text{Amount ascorbate (mg/100g sample)} = \frac{0.5\text{mg} \times v2(\text{ml}) \times 100\text{ml} \times 100}{v1(\text{ml}) \times 15\text{x wt .of sample}}$$

RESULTS AND DISCUSSION

Effect of PEG induced drought on callus growth

Preliminary examination of PEG induced stress was done on the basis of attributes such as size, color and texture of four weeks old callus. A marked difference was observed in all the genotypes. Large, glossy, nodular and white to pale colored calluses were found in PEG free medium while there were a transition to smaller size, dried, and brown to black colored calluses with increasing concentration of PEG (1- 3%). Polyethylene glycol (PEG) in the medium lowers the water potential and considered as the best known selective agent that increases osmotic pressure of the culture media (Abdel-Raheem *et al.,* 2007; Al-Taha, 2013). The browning of the callus cells was considered as an indicator of tissue culture intolerance to PEG induced drought (Fig 1). Surprisingly in between the black necrotic callus, whitish embryogenic mass of cells were observed which were taken as the tolerant cells and suggested as major adaptation against drought and inoculated again on the PEG induced stress media. Such type of conditions offers considerable opportunities for genetic improvement of plants by saving space and time through *in vitro* culture. These genetic changes occurs during the callogenesis phase of plant cells and considered a new source

Table 1: Fresh weight (g), dry weight (g) and relative water content (%) of callus in genotypes of *W. somnifera* grown for four weeks on MS medium supplemented with different concentrations of PEG (from 1 to 3%).

PEG Treatments	Jawahar 20			Nimitly			Chetak			Pratap		
	Fresh Weight	Dry Weight	Relative water content	Fresh Weight	Dry Weight	Relative water content	Fresh Weight	Dry Weight	Relative water content	Fresh Weight	Dry Weight	Relative water content
CONTROL	0.568±0.041	0.140 ±0.004	88.57 ±3.34	0.311±0.003	0.216 ±0.004	74.80 ±2.64	0.725±0.003	0.301 ±0.002	82.20 ±2.50	0.624 ±0.003	0.235 ±0.006	83.46 ±0.76
1%	0.234±0.003	0.118 ±0.002	64.44 ±1.74	0.193±0.005	0.035 ±0.003	62.90 ±1.91	0.442±0.002	0.195 ±0.003	73.68 ±3.04	0.293 ±0.006	0.128 ±0.001	66.81 ±2.73
2%	0.192±0.002	0.134 ±0.001	50.57 ±3.17	0.154±0.002	0.035 ±0.004	55.95 ±1.83	0.253±0.001	0.044 ±0.001	65.36 ±0.91	0.349 ±0.001	0.189 ±0.004	63.47 ±0.86
3%	0.165±0.003	0.142 ±0.003	38.47 ±1.30	0.147±0.002	0.120 ±0.002	32.46 ±2.97	0.248±0.005	0.190 ±0.003	36.17 ±4.30	0.260 ±0.003	0.183 ±0.002	52.10 ±2.17
SEm±	0.021	0.004	2.54	0.004	0.002	2.38	0.003	0.002	2.94	0.005	0.004	1.83
CD 5%	0.068	0.013	8.30	0.013	0.009	7.77	0.012	0.009	9.60	0.016	0.015	5.98

of changes intended to enrich the genetic resource for the improvement of plant species (Bouiamrine and Diouri, 2012). After four weeks observations of proliferated callus on stressed media were taken on the basis of growth and physiological parameters such as fresh weight, dry weight and relative water content (RWC). Fresh weight and RWC were found to be decreased with increasing concentration of PEG in all the genotypes. Nimitly showed minimum 52.73% reduction in callus fresh weight while minimum *i.e.* 37.57% reduction in relative water content was found in Pratap under maximum stress level when compared to control. On the basis of minimum reduction percentage in fresh weight and relative water content of these two genotypes may be considered as more tolerant against drought as compared to others. Reduction percentage for the fresh weight of callus and RWC in all other genotypes was 58.33% to 70.95% and 56.00% to 56.57% respectively (Table 1), indicating that callus have less ability to sustain water under stress conditions. Impact of PEG on culture media was also emphasized by (Abdel-Raheem *et al.,* 2007). He reported a significant decrease in callus fresh weight of tomato with increasing concentration of PEG (25-100g/L). In an another study, highest callus fresh weight and callus water content of sour orange fruits was found under PEG free media while lowest value was noticed for 8% PEG (Al-Taha, 2013). Such trend was not observed in case of dry weight of callus in all the genotypes. A reduction in DW was recorded up to T1 treatment while it was increasing after T1 in genotype J-20 with 1.43% increment. PEG-induced drought in the media produced substantial dehydration for tissues which may cause of the increase in dry weight of calluses**.** Similar findings were also obtained by (Sakthivelu *et al.,* 2008). They reported increased dry matter at 6% PEG level compared to control after six weeks treatment in callus of soybean cultivars. In Nimitly and Chetak, Dry Wt. was found to be decreased with increasing concentration of PEG up to T2. Genotype pratap showed less (22.12%) reduction than Chetak 36.87%. In case of Pratap, it was decreasing up to T1 treatment (Table 1). These results are in agreement with Bouiamrine and Diouri, 2012. They observed that increasing PEG in the medium significantly brings down the weight of callus of durum wheat (*Triticum durum Desf.)* and therefore the relative growth. However highest water content (88.75%) was recorded in the calluses from PEG-free media. It can be summarized that callus growth mainly depends on genotype, the type of tissue (juvenile or physiologically most active tissues give better callus formation), ratio of endogenous and exogenous plant growth regulators and conditions of growth medium (Adhikari and Pant, 2013).

Effect of drought on antioxidants

Drought tolerance or sensitivity of plants is well correlated with their antioxidant response. In general, tolerant varieties have a better capacity to protect themselves from drought induced oxidative stress by enhancing antioxidant activity. Tissue culture technology has major advantage over conventional method of propagation in terms of easy extraction of compounds from callus than from the plant parts.

Superoxide dismutase (SOD)

During the investigation a significant difference in SOD activity was observed in all the genotypes among treatments as well

Table 2: Superoxide dismutase and Catalase activity of callus in genotypes of *W. somnifera* grown for 15 and 30 days on MS medium supplemented with different concentrations of PEG (from 1 to 3%).

Genotype	PEG treatment	15 DAI		30DAI	
		SOD(Unit/mg protein)	CAT(Unit/mg protein)	SOD(Unit/mg protein)	CAT(Unit/mg protein)
J20	control	1.07 ±0.006	6.67±2.67	2.30 ±0.017	20.00 ±2.31
	1%	1.18 ±0.007	6.67±1.33	2.35 ±0.041	26.67 ±3.53
	2%	1.23 ±0.009	20.00 ±2.31	2.39 ±0.022	24.00 ±2.31
	3%	1.33 ±0.006	14.67 ±1.33	2.74 ±0.033	14.67 ±1.33
	SEm±	0.006	2.00	0.029	2.49
	CD 5%	0.020	6.51	0.096	8.13
Nimitly	control	0.37 ±0.002	10.67 ±1.33	0.63 ±0.033	24.00 ±2.31
	1%	0.49 ±0.012	16.00 ±2.31	1.47 ±0.005	21.33 ±1.33
	2%	0.58 ±0.007	33.33 ±1.33	1.67 ±0.025	28.00 ±2.31
	3%	1.16 ±0.004	40.00 ±2.31	1.89 ±0.007	33.33 ±3.53
	SEm±	0.008	1.88	0.049	2.49
	CD 5%	0.026	6.14	0.160	8.13
Chetak	control	1.06 ±0.005	16.00 ±2.31	1.89 ±0.005	25.33 ±1.33
	1%	1.12 ±0.007	25.33 ±3.53	2.04 ±0.024	16.00 ±2.31
	2%	1.18 ±0.008	26.67 ±1.33	2.15 ±0.008	10.67 ±1.33
	3%	1.33 ±0.001	9.33 ±1.33	2.66 ±0.013	6.67 ±1.33
	SEm±	0.006	2.30	0.013	1.63
	CD 5%	0.021	7.52	0.044	5.32
Pratap	control	0.99 ±0.004	12.00 ±2.31	2.05 ±0.023	20.00 ±2.31
	1%	1.12 ±0.004	17.33 ±3.53	2.17 ±0.016	13.33 ±1.33
	2%	1.28 ±0.005	30.67 ±1.33	2.18 ±0.023	12.00 ±2.31
	3%	1.36 ±0.005	12.00 ±2.31	2.27 ±0.012	5.33±1.33
	SEm±	0.004	2.49	0.017	1.88
	CD 5%	0.013	8.13	0.057	6.14

Table 3: Total phenol, flavanoid and ascorbic acid content of callus in genotypes of *W. somnifera* grown for 15 and 30 days on MS medium supplemented with different concentrations of PEG (from 1 to 3%)

Geno type	PEG trea tment	15 days after inoculation			30 days after inoculation		
		Total Phenol (μg/g FW)	Flavonoid (μg/g FW)	Ascorbic acid (mg/gFW)	Total Phenol (μg/g FW)	Flavonoid (μg/g FW)	Ascorbic acid (mg/gFW)
J20	control	200.00 ±3.85	113.48 ±0.15	79.76 ±1.19	164.44 ±2.22	107.85 ±0.39	84.52 ±1.19
	1%	240.49 ±3.70	168.74 ±0.53	84.52 ±1.19	223.56 ±1.74	156.30 ±0.30	110.71 ±2.06
	2%	310.44 ±1.46	187.26 ±0.15	79.76 ±3.15	313.78 ±0.80	175.70 ±0.90	88.10 ±3.15
	3%	358.89 ±3.49	231.26 ±0.53	61.90 ±2.38	371.78 ±1.90	221.48 ±0.39	72.62 ±1.19
	SEm±	3.27	0.39	2.14	1.74	0.54	2.06
	CD 5%	10.66	1.27	6.99	5.69	1.79	6.72
Nimitly	control	206.44 ±3.11	196.15 ±0.15	65.48 ±1.19	238.00 ±2.00	210.37 ±0.39	53.57 ±2.06
	1%	235.33 ±2.69	233.19 ±0.59	73.81 ±3.15	264.89 ±2.47	232.74 ±0.53	79.76 ±1.19
	2%	285.78 ±1.90	259.11 ±0.68	50.00 ±2.06	309.33 ±2.00	255.70 ±0.53	60.71 ±2.06
	3%	314.67 ±2.52	281.33 ±0.26	23.81 ±1.19	324.22 ±2.32	264.00 ±0.51	55.95 ±3.15
	SEm±	2.59	0.47	2.06	2.20	0.50	2.22
	CD 5%	8.45	1.55	6.71	7.19	1.63	7.25
Chetak	control	241.11 ±3.27	147.26 ±0.15	75.00 ±2.06	246.67 ±3.85	160.89 ±0.26	77.38 ±1.19
	1%	288.44 ±2.47	171.11 ±0.68	59.52 ±3.15	284.44 ±2.22	170.96 ±0.30	88.10 ±3.15
	2%	350.89 ±3.89	195.41 ±0.39	50.00 ±2.06	343.33 ±2.00	187.11 ±0.26	76.19 ±1.19
	3%	376.67 ±2.04	218.07 ±0.15	36.90 ±2.38	367.33 ±1.68	201.48 ±0.65	53.57 ±2.06
	SEm±	3.00	0.40	2.45	2.57	0.40	2.06
	CD 5%	9.80	1.32	8.00	8.39	1.30	6.72
Pratap	control	168.22 ±1.24	99.26 ±0.39	180.95 ±1.19	145.33 ±2.78	95.26 ±0.30	201.19 ±3.15
	1%	238.22 ±1.74	122.37 ±0.39	194.05 ±4.76	233.40 ±3.38	113.19 ±0.53	286.90 ±1.19
	2%	276.89 ±1.56	155.85 ±0.15	120.24 ±4.29	285.56 ±2.78	147.70 ±0.39	221.43 ±2.06
	3%	367.11 ±0.97	210.96 ±0.39	58.33 ±2.38	336.22 ±1.98	175.41 ±0.59	121.43 ±2.06
	SEm±	1.40	0.34	3.46	2.77	0.46	2.22
	CD 5%	4.58	1.12	11.30	9.04	1.53	7.25

as time duration. Enzyme activity was found to be increased with increasing drought in the media in all the genotypes namely J-20, Nimitly, Chetak and Pratap. Genotype Nimitly showed approximately 213.51% increase in SOD activity at maximum (3% PEG) stress in comparison to control after 15 days of inoculation while after 30 days it was 200% means it is decreasing with increasing time duration (Table 2). However other genotypes also showed an increment but the percentage

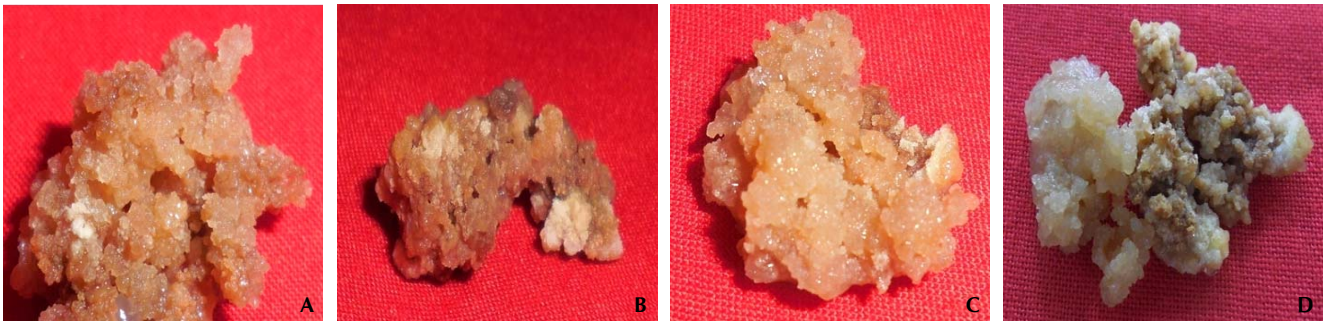

Figure 1: A, B, C, D showing tolerant callus cells in *W.somnifera* genotypes namely J-20, Nimitly, Chetak and Pratap respectively after 30 days of stress treatment under 3% polyethylene glycol (PEG)

was found to be less i.e. 24.30, 25.47 and 37.37 after 15 days and 19.13, 40.74 and 10.73 after 30 days of treatment in J-20, Chetak and Pratap respectively. Thus, genotype Nimitly showed greater capability of survival under stress condition. It is well documented that level of SOD may increase/decrease depending on the species and growth stage of plant and the degree of stress condition (Raychaudhuri, 2000). It catalyzes the dismutation of the superoxide free radical (generated during drought stress) to molecular oxygen & hydrogen peroxide and protects plants from oxidative stress (Alscher *et al.,* 2002; Xu *et al.*, 2013). Highest increase in SOD activity from 0.74 to 1.12 unit mg^{-1} protein min^{-1} was also reported in peanut cultivars under drought stress (Chakraborty *et al.,* 2015). Higher concentration of SOD in salt treated callus of *With ania somnifera* were also detected by using native polyacrylamide gel electrophoresis (Sabir *et al.,* 2012). In this way comparatively higher SOD activity in stressed callus underlines its suitability as antioxidant supplement.

Catalase

CAT is the major enzyme which is frequently used by the plants for the decomposition of hydrogen peroxide (H_2O_2). However H_2O_2 is the byproduct of plant metabolic pathway but it's concentration may increase due to environmental stresses (Shankhdhar and Shankhdhar, 2014). CAT is firstly discovered and characterized antioxidant enzyme (Garg and Manchanda, 2009) whose activity may get enhanced or reduced depending upon the intensity duration and type of stress (Han *et al.,* 2009). In our study CAT activity was found to be significantly different in all the genotypes for all the treatments as well as time duration. Genotypes were showing variation in CAT activity with respect to increasing conc. of PEG as well as increasing time interval. Enzyme activity was found to be increased up to T2 treatment in all the genotypes after 15 days of treatment except in Nimitly while after 30 days no such trend was observed. It was increasing up to 1% PEG for J-20 while decreasing for other genotypes at the same level (Table 2). Maximum i.e. 274.88% increase was recorded for Nimitly followed by J-20 (199.85%) and Pratap (155.58%) whereas minimum 66.68 % increase in CAT activity was recorded for genotype Chetak after 15 days of inoculation. An increase in CAT activity is generally positively related to the degree of drought experienced by plants (Sofo *et al.*, 2015).These findings are in agreement with the study of (Halime *et al.,* 2013). They carried out an experiment in plastic pots and plants of *Dracocephalum moldavica L.* were taken as sample. Drought stress was applied through measuring soil field capacity (FC) i.e. no stress (FC), moderate stress (2/3 FC) and severe stress (1/3 FC). Variation in catalase activities in plants under drought stress were observed in their work while maximum activity was recorded under severe drought stress i.e. 0.50 μmol H_2O_2/min and 0.55 μmol H_2O_2/min. in shoots and roots respectively. Enhanced SOD and CAT activity in lettuce under drought stress was also seen by (Al-Muhairi *et al.*, 2015).

Total Phenol content

Phenolic compounds constitute a large group of organic compounds that are widely distributed in plants and exhibit a broad spectrum of biological activities (Balasundram *et al.,* 2006). They have the ability to quench free radicals and their effectiveness which depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups. High molecular weight phenolics (tannins) have more ability to quench free radicals (Hagerman *et al.,* 1998).

Phenol content in all the genotypes differs significantly with stress duration as well as increasing level of stress. Increased phenol content was recorded with the increasing conc. of PEG in all the genotypes of *W. somnifera* in both the time intervals (Table 3). Pratap showed 118.23% and 131.35% phenol content at maximum (3% PEG) when compared with control after 15 and 30 days respectively followed by J-20 i.e. 79.45 % after 15 days and 126.09 % after 30 days of stress treatment. On the other hand Nimitly and Chetak Showed approximately 40% to 50% increment in total phenol content. The inferences drawn after calculating phenol content comply with the study of (Halime *et al.,* 2013). They showed variation in phenolic compounds of *Dracocephalum moldavica L* under drought stress and an increment was estimated at severe stress in shoots (64.81 mg/gFW) and roots (67.85 mg/g FW) when compared with no stress conditions.In an investigation it was reported that phenol content varied from the undifferentiated callus cells to the organized shoot tissue (Pathak *et al.,* 2012). Previous findings demonstrate that the total phenol content was widely distributed in different medicinal plants as well as their parts ranges between 1.21 to 135.56 mg of GAE/g. (Singh *et al.,* 2012). Considerably very high phenolic content in callus of Physalis peruviana L. after adding 50mM NaCl in the media

was also reported by (Jan *et al*., 2015).

Flavonoids content

Flavonoids act as scavengers of free radicals and also prevent their formation by chelating metals (Vaknin *et al.,* 2005). Present study showed a significant difference in flavonoids content at both time duration and increasing levels of PEG treatment in all the genotypes. Increased flavonoids content was recorded with the increasing conc. of PEG after 15 and 30 days of treatment in all the genotypes of *W. somnifera* (Table 3). Genotype J-20 and Pratap showed 103.78% and 112.53% increase in flavonoids content respectively after 15 days while other two genotypes i.e. Nimitly and Chetak showed 43.42 and 48.08% respectively. Similarly after 30 days genotype J-20 showed 105.35% whereas other genotypes showed 25.49 to 84.13% increment. According to the supportive findings flavonoids are rich in medicinal plants. It is a highly effective scavenger of free radicals, used for inhibiting various diseases associated with free radicals (Deepa *et al.,* 2009). Our results also seek support from the study of (Atanassova *et al.,* 2011), they estimated 48.86mg GAE/100g flavanoid content in lemon balm hence showing that herbs are the ancient source of medicine. (Yuan *et al.,* 2012) were used three months old plants of *Scutellaria baicalensis* (a Chinese traditional medicinal plant) and kept in two different soil water contents for 30,50 and 70 days. They showed significant increase in flavonoids content with increasing drought (30, 50 and 70 days of stress treatment). the effect of drought stress on flavanoid content of *Simarouba glauca DC* was also evaluated by(Awate and Gaikwad, 2014). Plants have provided the drought stress of 4,8,12 and 16 days while control plants were watered after every two days. They found 3 to 4 folds increase in flavonoids content than control plants. In the related study total phenolics and total flavonoid also found to be increased in plants of Cherry Tomato under stress, especially in those treated with high salt concentrations (Al Hassan *et al.,* 2015).

Ascorbate

Ascorbic acid, a major metabolite can act as cofactor for several enzymes as well as it has now been considered as a good antioxidant too. It is very helpful for plants to cope up the environmental stresses and plants with good ascorbic content are ultimately beneficial for human health (Smirnoff, 2005). Thus search of such plant species now has become a major consent for the researchers. During our investigation ascorbate content was found to be significantly different with time intervals as well as increasing stress levels in all the genotypes. Increased ascorbate content was observed up to 1% PEG in all genotypes (with a little increment of 5.97 to 12.72%) except in genotype Chetak after 15 days of treatment. In genotype Chetak ascorbate content was found to be decreased with increasing concentration of PEG which means this genotype is not adopting ascorbate accumulation for stress tolerance. This genotype also showed minimum % increase 13.85% after 30 days of treatment while other genotype ranged between 30.99% to 48.89%. Notably pratap showed much higher ascorbate content among all genotypes (Table 3).

Ascorbate is one of the most extensively studied anti-oxidant and has been detected in the majority of plant cell types, organelles and apoplast (Lawson *et al.,* 2003). The results of our study are supported by (Jaleel, 2009) in which stress treatment (10, 15 and 20 day interval drought) were given to the seedlings of *With ania somnifera* after 30 days of sowing. They observed increased ascorbic acid content with age of drought when compared with control while treatment wise it was increasing only up to 15 days interval drought from 8.91 to 10.01 mg/g dry weight. The consequential statements to be made after these observations are in direct agreement with the findings of (Singh *et al.,* 2012). They reported varying level of ascorbic acid in medicinal plants ranging from 10.20 to 118.36 mg/100g FW.

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