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

DEEPIKA SHARMA*, NARENDRA KUMAR, VEENA PANDEY, S. C. SHANKHDHAR AND DEEPTI SHANKHDHAR
Department of Plant Physiology, College of Basic Sciences and Humanities,
G. B. Pant University of Agriculture and Technology Pantnagar - 263 145, Uttarakhand, INDIA
e-mail: khush19plant@gmail.com

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***Corresponding author**

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

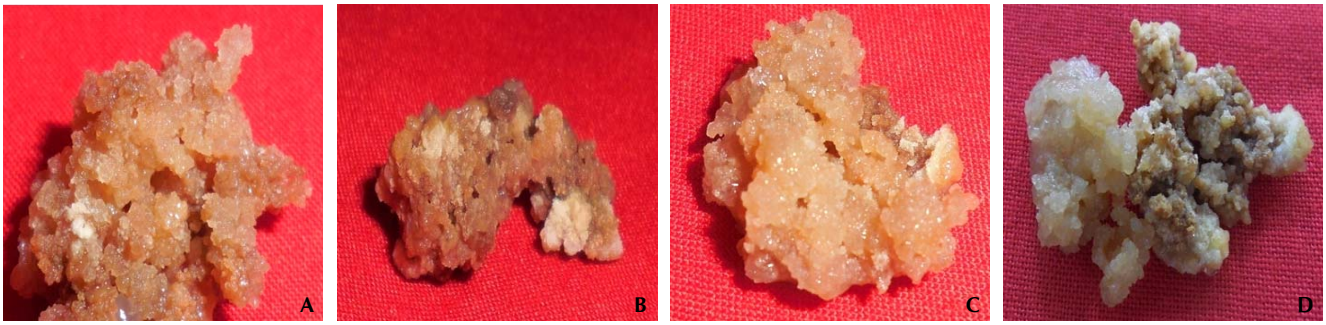

Figure 1: A, B, C, D showing tolerant callus cells in *W.somnifera* genotypes namely J-20, Nimitly, Chetak and Pratap respectively after 30 days of stress treatment under 3% polyethylene glycol (PEG)

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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Efficacy of auxins and gibberellic acid on floral attributes and essential oil per cent on German Chamomile (Matricaria chamomilla L.)

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Article

Efficacy of auxins and gibberellic acid on floral attributes and essential oil per cent on German Chamomile (*Matricaria chamomilla* L.)

Abhay Singh, Siddharth Shankar Bhatt, Yamuna Pandey, Narendra Kumar and Anupam Adarsh
Department of Horticulture, College of Agriculture, G.B. Pant University of Agriculture and Technology, Pantnagar - 263145, Uttarakhand, India
Department of Horticulture (Vegetable and Floriculture), B.A.U, Sabour, India

ABSTRACT

Chamomile (*Matricaria chamomilla* L.) is one of the important medicinal plants grown for its valuable flowers from which oil extracted with high pharmaceutical value. Plant bio-regulators can be effectively used to enhance both the vegetative as well as flowering attributes. Plants were foliarly treated with IAA, GA_3 and NAA at three concentrations (25, 50 and 100 ppm of each which improved floral parameters like days taken to first bud initiation, flower diameter (cm), number of flowers per plant and fresh and dry weight of flowers per plant (g). Among the different treatments, GA_3 at 100 ppm was found to be best treatment for most of the parameters *viz*. days taken to first bud initiation (15.53), flower diameter (2.57 cm), number of flowers per plant (268.93), fresh weight of flowers per plant (35.56 g) and dry weight of flowers per plant (6.96 g), whereas NAA at 100 ppm proved to be the best for stem diameter (6.76 mm). GA_3 @ 100 ppm was found to be the most effective for most of the vegetative and floral characteristics of chamomile cv. CIM Sammohak. **[Medicinal Plants 2015; 7(4) : 272-274]**

Keywords: Chamomile, medicinal plants, plant bio-regulators

Chamomile (*Matricaria chamomilla* L.) is an annual plant belonging to the Asteraceae family often referred to as the "Star among medicinal species" (Salmon, 2007). It is one of the important medicinal herb native to southern and eastern Europe (Reichling and Beiderbeck, 1991). Over 120 components have been identified in chamomile essential oil, while α-bisabolol, chamazulene, α- and β-bisabolol oxides, farnesene and α-bisabolonoxide, are the major important constituents (Jakovley *et al*., 1983). Chamomile is known to be its anti-inflammatory, anti-spasmodic, anti-bacterial and antiseptic properties (Franke and Schilcher, 2007). German chamomile are frequently used for digestive complaints and taken regularly to regulate the bowels. It contains an easily assimilable form of calcium and a tablespoon in a covered cup of boiling water with two slices of fresh ginger is very effective treatment for menstrual cramps and other pains and spasms (Tierra, 1999). The same tea may be used for minor digestive problems such as acid indigestion and gas.

The role of plant hormones in growth and flowering of medicinal plant have received considerable attention only recently, as they are of paramount importance in providing quick and often encouraging results. Plant bio regulators are chemicals that are designed to affect plant growth and/or development. Plant hormones are signal molecules produced within the plant, and occur in extremely low concentrations. Indole Acetic Acid (IAA) is one of the auxins biosynthesized within plant organs and affects many physiological processes, mainly required for cell-elongation.

It is a natural auxin, besides being involved in apical dominance, cell division and cell enlargement. On the other hand, another prominent phyto-hormone, Gibberellic acid (GA_3), has the potential control on growth and flowering process (Emongor, 2004). Naphthalene acetic acid belongs to the auxin group of bio regulators. They are characterized principally by their capacity to stimulate stem elongation

Corresponding author : Abhay Singh
e-mail : anupamadarsh111@gmail.com

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in excised stem. Little attempt has been made on effect of foliar spray of IAA, GA_3 and NAA on vegetative growth and flowering in chamomile. Therefore, the objectives of the present study aimed to reveal and compare the effect of these growth regulators (IAA, GA_3 and NAA) on vegetative growth and flowering of chamomile plant and to find out the most favourable treatment of these growth regulators.

The present investigation was conducted at "Medicinal Plants Research and Development Centre" Pantnagar, Uttarakhand. The experiment was plotted according to Randomized Block Design with three replicates per each treatment. The region is characterized by sub-tropical humid climate with dry hot summers, cold winters and intense rainy season. The planting of uniform sized seedlings was done in raised beds on November, 2012. The seedlings were transplanted at a spacing of 30 cm x 30 cm from plant to plant and row to row accommodating 36 plants per meter square area. The plants were watered immediately after planting and afterwards at weekly intervals during growing period.

Stock solution of known concentration was prepared only before use. The Plant bio regulators, IAA, GA_3 and NAA were first dissolved in a minimum volume of HCl and NaOH/ KOH of 0.1 N respectively then required volume was made up with distilled water to make a stock solution. Freshly prepared aqueous solution of IAA, GA_3 and NAA from stock solution were foliarly applied twice, 1st after one moth of sowing and 2nd spray 10 days after the 1st one. Wetting agent (Tapol, 1 m L^{-1}) was added to the freshly prepared solution of growth regulators before spraying on plant foliage till running (20 ml per plant) using plastic automizer. Three concentrations of IAA, GA_3 and NAA were 25, 50 and 100 ppm each growth regulator. In addition, untreated plants were spread with distilled water and wetting agent to serve as control.

Five samples were drawn from all treatments with three replications at full flowering stage. Plant height, number of branches per plant, plant spread, days taken to first bud initiation, flower diameter, number of flowers per plant and fresh and dry weight of flowers per plant were determined at full flowering stage.

Essential oil isolation

Isolation of essential oils was performed using hydrodistillation of dried sample of flower heads using a Clevenger-type apparatus over 3 hours. The oils were dried over sodium sulphate.

The data was subjected to statistical analysis using F test according to the procedure of Gomez and Gomez. Critical difference at 5% was calculated to compare the mean value of determined criteria of different treatment.

The perusal of data in Table 1 reveals that days taken to first bud initiation, flower diameter, number of flowers per plant and fresh and dry weight of flowers per plant of chamomile plants were found significantly maximum at 100 ppm GA_3 spray treatment as compared to other treatments. Early days taken for first flower bud initiation, with GA_3 might be due to the increase in endogenous gibberellic level in the plants as gibberellins are well known for inducing early flowering in several crop plants. GA_3 100 ppm also reduced the time required for flowering. Similar observations were made by Maurya and Nagda (2002**)** in gladiolus. The maximum number of flowers per plant (368.93) was recorded with GA_3 @ 100 ppm as compared to other treatments. The increased number of flowers per plant with GA_3@ 100 ppm (368.93) spray in comparison to control (334.40) might be due to enhance reproductive efficiency and photosynthesis in restructured plant type, produced maximum number of flowers per plant. The maximum flower yield by the application of GA_3 @ 100 ppm in Chamomile has also been reported by Fatma Reda *et al*. (2010). Meanwhile, the mean values of flower diameter varied from 2.26 cm to 2.57 cm. Among different treatments under study, chamomile showed maximum flower diameter (2.57 cm) at GA_3 100 ppm followed by GA_3 50 ppm (2.54 cm). Increase in flower diameter might be due to active cell elongation in the flower which resulted in increased flower diameter.

Gibberellic acid is also known to increase the sink strength of the actively growing parts. These findings are in conformity with those of Delvadia *et al*. (2009) in gaillardia. The fresh weight of flowers is related to number of flowers and diameter of flower (Table 2). The increase in number of flowers and flower diameter has directly influenced the fresh weight and dry weight of flowers. Similar findings were

Table 1: Effect of plant bio regulators on flowering of chamomile (*Matricaria chamomilla* L.)

Treatment	Days takes to first bud initiation	No. of flower per plant	Flower diameter (cm)
T_0 (Control)	19.86	264.46	2.26
T_1 (25 ppm IAA)	19.86	268.06	2.35
T_2 (50 ppm IAA)	19.33	285.46	2.40
T_3 (100 ppm IAA)	18.06	330.53	2.48
T_4 (25 ppm GA_3)	17.46	312.00	2.46
T_5 (50 ppm GA_3)	15.73	361.80	2.54
T_6 (100 ppm GA_3)	15.53	368.93	2.57
T_7 (25 ppm NAA)	19.53	275.53	2.39
T_8 (50 ppm NAA)	18.53	307.46	2.42
T_9 (100 ppm NAA)	16.66	334.40	2.52

Table 2: Effect of plant bio regulators on flower fresh weight, dry weight and essential oil percent of chamomile (*Matricaria chamomilla* L.)

Treatment	Fresh weight of flower (g)	Dry weight of flower (g)	Essential oil percentage (%)
T_0 (Control)	25.27	4.97	0.12
T_1 (25 ppm IAA)	25.85	5.15	0.14
T_2 (50 ppm IAA)	27.35	5.51	0.17
T_3 (100 ppm IAA)	31.66	6.38	0.20
T_4 (25 ppm GA_3)	29.90	5.95	0.23
T_5 (50 ppm GA_3)	35.17	6.95	0.24
T_6 (100 ppm GA_3)	35.56	6.96	0.26
T_7 (25 ppm NAA)	26.46	5.26	0.13
T_8 (50 ppm NAA)	29.86	5.93	0.15
T_9 (100 ppm NAA)	32.29	6.40	0.17

observed by Swaroop *et al.* (2007) in African marigold where gibberellins enhanced both fresh and dry weight of flowers.

Plant bio regulators also influenced the essential oil percentage of chamomile flowers. GA_3 100 ppm had tremendously increased the oil percentage (0.26%) from 0.16 per cent observed in control. Growth regulators can influence essential oil production through effects on plant growth, essential oil biosynthesis and the number of oil storage structures (Sharafzadeh and Zare, 2011). Literatures illustrated that exogenous applications of methyl-jasmonate can alter essential oil constituents of basil by gene regulation, promoting an increase in the number of transcripts of the enzymes linked to metabolic pathway of those compounds (Kim *et al.*, 2006; Li *et al.*, 2007). The application of growth regulators may affect essential oils due to their effects on enzymatic pathways of terpenoid biosynthesis (Sangwan *et al.*, 2001).

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Oryza Vol. 52 No.2, 2015 (123-130)

Effect of foliar application of boron on growth, yield, chlorophyll, amylose and nitrate reductase activity in rice

Narendra Kumar, Ritika Misra[1], SC Shankhdhar and Deepti Shankhdhar*

College of Basic Sciences and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar-263145,Uttarakhand, India

[1]M.J.P. Rohilkhand University, Bareilly, U.P, India

**Email: dshankhdhar@rediffmail.com*

ABSTRACT

A field trial was conducted during wet season 2010 to evaluate the effect of foliar application of boron (B) on the content of chlorophyll, amylose & boron, nitrate reductase activity and yield of rice genotypes namely, IET 20979, IET 21007, IET 21106, IET 21114, IET 21519, IET 21540 and Rasi. Boron was applied at 0.2- 0.8ppm at the time of anthesis. The results revealed that 0.4ppm foliar application of boron evoked best response from all genotypes in terms of total dry matter, biological yield, economic yield and harvest index. The content of chlorophyll, boron, amylose and nitrate reductase activity increased significantly in most of the genotypes as compared to the control. The study suggests that foliar application of boron may enhance performance of rice plant in terms of grain yield and quality.

Key words: *Oryza sativa L, growth, chlorophyll, amylose, boron*

Boron is an essential micronutrient for normal growth of higher plants. Boron deficiency in soil is widespread in several crop growing regions from tropical to temperate zones of the world (Shorrocks, 1997). It is required for better growth, yield of crop and plays important role in cell development and elongation, protein synthesis, pollen tube formation and pollen viability etc (Gupta and Solanki, 2013). Extent of boron deficiency is next to that of Zn. Boron deficiency reduces not only yield but also the quality of crops which include thinner stems, shorter and fewer tillers, and failure to produce viable seeds (Dell and Huang, 1997). The deficient boron level in soil limits plant growth or damage to the photosynthesis system and can causes severe physiological responses, such as the disruption in chlorophyll and auxin biosynthesis, leading to reduction in fruit or crop yield (Hirsch and Torrey, 1980). Boron is also involved in N_2 fixation, the activity of nitrogenase enzyme in boron-deficient cells is inhibited even when other metabolic processes like photosynthesis, respiration etc are not affected. It has been shown that short-term boron deficiency leads to a decline in root and leaf nitrate contents without affecting NR activity or the concentrations of other macronutrients such as magnesium, calcium, potassium or phosphate (Camacho-Cristóbal *et al.,* 2005). Acting on the membrane level, boron deficiency has been reported to inhibit membrane uptake of number of nutrients and also affects nutrient transport capacity due to lowering ATPase and NAD(P)H mediated redox activity (Jabeen and Ahmad, 2011). Boron deficiency affects the expression level of genes related to nitrogen metabolism, oxidative stress, boron uptake, and cell wall synthesis (Camacho-Cristóbal *et al.,* 2008).

Boron phytotoxicity also manifests in a broad range of physiological effects, including decreased shoot and root growth, root cell division and RNA content, reduced leaf chlorophyll, lower photosynthetic rates and stomatal conductance and decreased lignin and suberin levels (Roessner *et al.,* 2006). Leaf toxicity symptoms in barley are characterized by interveinal chlorotic or necrotic patches, generally at the margins and tips of

older leaves (Nable and Paull, 1991).

Boron can be provided to plants both by soil as well as foliar application. Foliar applied boron is believed to retain significant phloem mobility to flowering meristems. Thus, foliar sprays of boron provide not only a means to apply boron at a particular growth stage, but it also permits a rapidly-acting remedial action soon after the diagnosis of deficiency (Rashid *et al.,* 2004). Therefore, this study was aimed at characterizing the effect of foliar application of boron on various physiological and biochemical parameters in different rice genotypes to workout variability in yield and quality of grains for further improvement.

MATERIALS AND METHODS

A field experiment was conducted in three separate independent split plot design with three replications, 10 × 20 cm spacing with normal recommended package and practices that are being adopted during kharif season 2010 in Norman E. Borologue Crop Research Centre, G.B. Pant University of Agriculture and Technology Pantnagar, U.S. Nagar (Uttrakhand), India. Geographically, the site lies in Tarai plains about 30 km southwards of foothills of Shivalik range of the Himalayas at 29° N latitude, 79° 29' E longitude and at an altitude of 243.8 meter above the mean sea level and experiences humid subtropical climate with hot dry summers and cool winters. Winter season extends from November to March. The monsoon sets during second or third week of June and continues till September end. The soil of the transplantation site belonged to Typic Happlodoll with pH 8.0, EC 0.63, organic carbon 1.05% and 0.6 ppm boron content. It had gentle to moderate slope and was fine, loamy and mixed hyperthermic type. The seeds of seven rice genotypes, namely, IET 20979, IET 21007, IET 21106, IET 21114, IET 21519, IET 21540 and Rasi were obtained from the Directorate of Rice Research, Rajendranagar, Hyderabad, India. At anthesis boron was applied as foliar spray in the morning (10-11AM) with conc. 0.2 (T_1), 0.4 (T_2) and 0.8 (T_3) ppm while control was sprayed with water alone.

Total plant dry matter of three plant samples from each replication was recorded at active tillering and flowering by uprooting the plant and placing the samples in an oven at 65^0C for three days. Thereafter, weight of each sample was recorded. Each plant was uprooted from ground level at maturity and thereafter dried, the weight of intact plant was determined before threshing and the total weight of the above ground biomass was recorded and expressed as biological yield in tons/ha. Grain yield (economic yield) from each replication was recorded and finally expressed in tons/ha after harvesting.

Chlorophyll content was determined in fresh leaves at flowering by using method as described by (Hiscox *et al.,* 1979).

Nitrate Reductase (NR) activity was estimated *in vivo* in freshly harvested flag leaves at flowering by using method as described by (Hageman and Hucklesby, 1971).

Amylose content was estimated in rice grains by using method as described by (McCready *et al.,* 1950).

Boron content in shoots and grains at maturity was determined according to the method given by (Wolf, 1974).

The data recorded in triplicate was analyzed using ANOVA (analysis of variance) in accordance with using the SPSS-16 statistical package to quantify and evaluate the analysis of variation and means were tested using Duncan's test. The treatment means were compared at a significance level of 5% and the ranking of treatments denoted by alphabets.

RESULTS AND DISCUSSION

It is well known that rice is sensitive to micronutrient deficiency. Boron application and its deficiency lead to less growth and ultimately decreased yield. As it is associated with the development of cell wall and cell differentiation and hence, helps in root elongation, shoot growth and finally total dry matter of plant. In our investigation the total dry matter of rice crops at the time of flowering was recorded after foliar application of boron. A significant increase was found in total dry matter. The overall total dry matter was recorded maximum (853.09 g m^{-2}) at 0.8ppm boron level. A maximum 65.26% increase in total dry matter was recorded in IET 20979 however, in other genotypes it was ranges between 1.38-59.74% (Table 1). The improvement in total dry matter as a result of B application might be due to the enhanced photosynthetic and metabolic activity during flowering stage that leads

Table 1. Effect of different boron levels (0.2-0.8ppm) on total dry matter (g m^{-2}) at flowering in different genotypes of rice

Genotypes	Total dry matter (g m^{-2}) at Flowering stage			
	(0 ppm)	(0.2 ppm)	(0.4 ppm)	(0.8 ppm)
IET 20979	532.67^{d}	780.67^{bcd}	634.33^{c}	880.33^{b}
IET 21007	574.00^{d-}	556.67^{d}	909.00^{ab}	796.67^{bc}
IET 21106	677.33^{c}	882.33^{abc}	604.00^{c}	1082.00^{a}
IET 21114	851.67^{b}	723.67^{cd}	694.00^{bc}	734.00^{d}
IET 21519	1124.00^{a}	1073.00^{a}	736.67^{bc}	921.33^{a}
IET 21540	1095.33^{a}	966.67^{ab}	1006.67^{a}	911.00^{b}
Rasi	676.00^{c}	685.33^{cd}	916.67^{ab}	646.33^{d}

Means followed by a common letter in the columns are not significantly different

to an increase in various plant metabolic pathways responsible for cell division and elongation. Similarly, an application of boron also increased leaf area, which might be responsible for the increase in total dry matter. (Hatwar *et al.,* 2003). Boron deficient castor bean plants had less amount of dry matter per plant as compared to those treated with 0.27 mg/l of boron (Silva and Ferreyra, 1998). The external supply of boron in rice also improved the mean shoot dry weight significantly at 50-800 ng B mL^{-1} (Mehmood *et al.,* 2009).

The biological yield of rice genotypes was affected by the application of boron. At 0.4 ppm boron, maximum biological yield was found in genotype IET 21106 (20 tons/ha) with 13.68% increase as compared with control (Table 2). The biological yield increased due to the availability of boron which enhanced the plant height, number of tillers, leaf weight, weight and number of leaves per plant. Overall mean showed maximum economic yield (5.10 t/ha) at 0.4ppm boron. The maximum 11.6% increase in grain yield as compared with control was observed in IET 21106 (6.33 t/ha) at same level of boron (Table 2).

The harvest index indicates the total biomass at time of seed maturity. It depends upon economic and biological yield of the crop plants. When the grain and biological yield increase by the application of boron the harvest index ultimately increases. In our study, the effect of different boron levels on harvest index was also observed. There was not much difference at 0.2 and 0.4 ppm boron as harvest index was found to be 32.5% and 31.52% respectively. IET 21519 showed maximum (43.51%) and Rasi showed minimum (20.08%) harvest index at 0.4ppm level of foliar boron application. (Table 2). Similarly, Hussain and Yasin (2004) also observed that grain and straw yield was affected by 3 kg/ha boron fertilizers in BRRI Dhan 30 and that low grain and straw yield was observed in control condition. Application of 1.0 kg/ha of boron, reduced the panicle sterility, substantially increased grain yield in two cultivars of rice i.e. Basmati 385 and Super Basmati (25% and 20% over the control,

Table 2. Effect of different boron levels (0.2-0.8ppm) on total biological yield (t ha^{-1}), economic yield (t ha^{-1}) and harvest index (%) in different genotypes of rice

Genotypes	Biological yield (t ha^{-1})				Economic yield (t ha^{-1})				Harvest index (%)			
	(0 ppm)	(0.2 ppm)	(0.4 ppm)	(0.8 ppm)	(0 ppm)	(0.2 ppm)	(0.4 ppm)	(0.8 ppm)	(0 ppm)	(0.2 ppm)	(0.4 ppm)	(0.8 ppm)
IET 20979	13.33^{b}	14.00^{cd}	15.67^{cd}	13.33^{d}	4.33^{cd}	4.83^{ab}	4.83^{cd}	5.08^{b}	32.74^{b}	34.59^{a}	31.04^{a}	38.39^{a}
IET 21007	15.33^{ab}	14.67^{bcd}	18.67^{ab}	17.33^{a}	4.92^{bc}	5.50^{a}	5.67^{ab}	5.33^{b}	32.29^{b}	37.57^{a}	30.42^{bc}	30.84^{bc}
IET 21106	17.67^{a}	17.33^{a}	20.00^{a}	16.00^{ab}	5.67^{a}	5.17^{ab}	6.33^{a}	6.17^{b}	32.28^{b}	29.86^{a}	31.83^{a}	38.54^{a}
IET 21114	15.00^{ab}	16.00^{abc}	16.67^{bc}	16.00^{ab}	5.25^{ab}	5.08^{ab}	5.67^{ab}	4.50^{c}	35.10^{b}	32.10^{a}	34.14^{ab}	28.13^{bcd}
IET 21519	14.00^{b}	15.00^{abcd}	13.00^{d}	15.33^{bc}	4.08^{d}	4.33^{b}	5.50^{ab}	4.08^{d}	29.66^{ab}	28.94^{a}	43.51^{a}	26.71^{cd}
IET 21540	13.33^{b}	16.67^{ab}	14.67^{cd}	14.00^{cd}	3.94^{d}	5.25^{ab}	4.33^{c}	3.58^{e}	29.73^{ab}	31.60^{a}	29.61^{bc}	25.77^{d}
Rasi	14.00^{b}	13.00^{d}	16.67^{bc}	13.67^{d}	3.08^{e}	4.25^{b}	3.33^{d}	4.33^{cd}	22.42^{b}	32.84^{a}	20.08^{c}	31.73^{b}

Means followed by a common letter in the columns are not significantly different

respectively) (Rashid *et al.,* 2004; Dunn *et al.,* 2005). The straw yield of wheat was also increased by applying 2 to 4 kg Bha^{-1}. Boron deficiency in wheat causes relatively more reduction of straw than grain yield (Rashid *et al.*, 2011). The soil application was also effective in many studies. The cumulative effect of boron on rice grain yield was recorded by the application of 2 kg B ha^{-1} (Khan *et al.,* 2006). Boron influences the harvest index significantly with application of 2 kg B ha^{-1} at ray floret stage in sunflower at par with the same dose applied at button stage. The harvest index in sunflower was more than control in all the treatments of boron (Zahoor *et al.,* 2011).

The response of growth and yield attributes ultimately depends upon the photosynthetic rate which in turn is dependent on chlorophyll contents. Results of present study revealed that chlorophyll a, b and total chlorophyll of most of the rice genotypes increased in response boron. At the time of flowering, the total chlorophyll content was affected by different levels of boron. The overall mean of treatment showed maximum chlorophyll content (2.26 mg g^{-1} fr. wt) at 0.4ppm boron. In Rasi, 0.4 and 0.8 ppm of boron resulted the maximum total chlorophyll content (2.67 mg g^{-1} fr. wt) and minimum in IET 21540 (1.74 mg g^{-1} fr. wt) in the control (Fig. 1). It might be due to the optimum amount of boron availability. However, boron deficiency reduces the chlorophylls which results in reduction in the photochemical reactions and net photosynthetic rate. Excess amount of boron can damage the photosynthesis system and the ultra structure of chloroplast. These results are in agreement with the results of a study on super basmati rice, in which a significant increment in chlorophyll contents (a, b and total chlorophyll) was recorded in response to soil as well as foliar application of boron. The highest chlorophyll contents (a, b and total) was recorded in Zn + B at 6 + 3 kg/acre treated plants (Arif *et al.,* 2012). Similarly in case of broad bean, chlorophyll a, b and total chlorophylls increased by foliar application (Sharaf *et al.,* 2009). In another study, 10, 20 and 250 mg/ l boron and zinc spray on *Vigna sinensis* caused significant increase in the contents of chlorophyll a and b (Hassanein *et al.,* 2000).

The amylose content of rice usually ranges between 15-35%. However, in contrast to high amylose content rice, low amylose content rice shows high volume expansion and high degree of flakiness so intermediate amylose rice are preferred in most rice growing areas of the world (Fasahat *et al.,* 2012). Proper supply of essential nutrients help to maintain

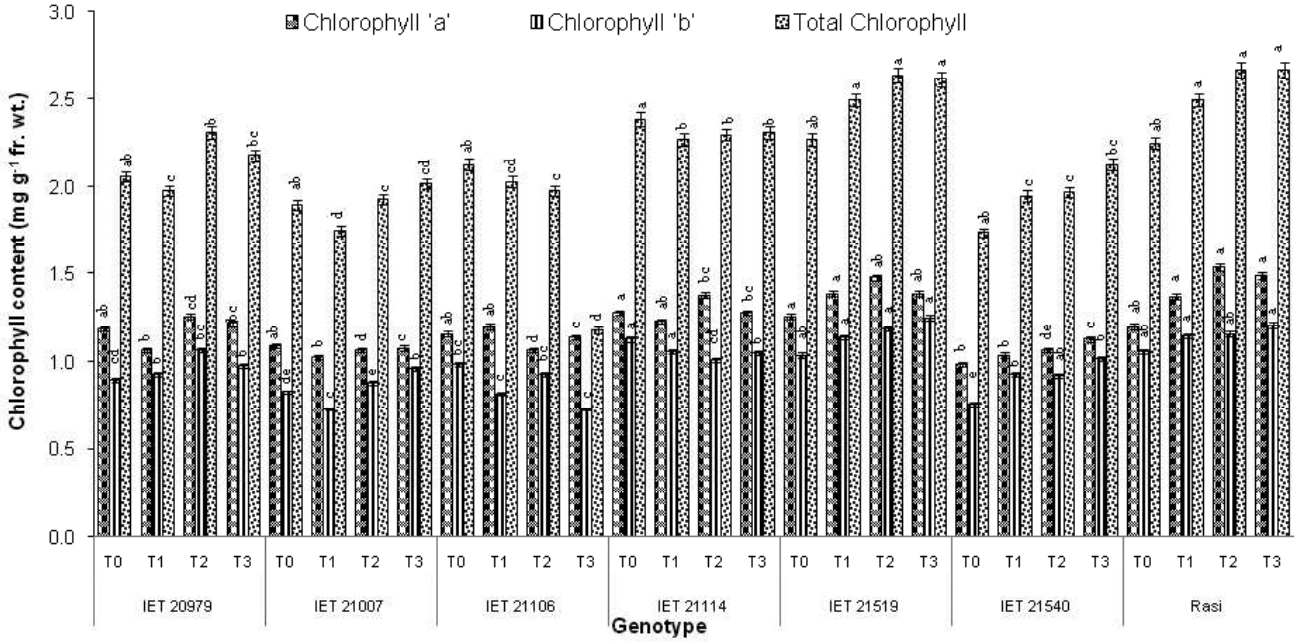

Fig.1. Effect of different B levels control (T_0) , 0.2 (T_1) , 0.4 (T_2) and 0.8ppm (T_3) on Chlorophyll content at flowering stage in different genotypes of rice.

the amylose-amylopectin ratio, boron may be one of such nutrient which may be used to maintain the percentage of amylose-amylopectin. In our study the amylose content was influenced by the foliar application of boron in all the genotypes and the overall mean of treatment showed that maximum amylose (17.88%) content in grains at 0.4 ppm level whereas, it was 14.96% in control. Maximum amylose content was recorded in IET 21106 and IET 21114 (17.88%) at 0.8ppm and minimum in IET 21007 (13.37%) in contro (Fig. 2). In all cases, amylose content increased with all the treatments; 0.4ppm boron was most effective to increase the amylose content. It might be due to the borate ion reacts chemically with the sugar molecule, and the resulting complex is transported across the membrane (Herrera-Rodriguez *et al.,* 2010). Actually syntheses of simple sugars or photosynthates are required for increasing the amylose, amylopectin and hence starch. Boron increases the rate of transport of sugars for the photosynthetic organs. However, boron does not play direct role in the synthesis of amylose or in activating the enzymes related to amylose synthesis (Lemoine *et al.,* 2013).

Nitrate assimilation in higher plants is greatly influenced by various essential nutrients and boron is one of them (Camacho-Cristobal and Gonzalez-Fontes, 1999). It has been shown that short-term boron deficiency lead to a decline in root and especially, leaf nitrate contents by decreasing NR activity (Kastori and Petrovic, 1989). In our investigation, the nitrate reductase (µmol NO_2^- g^{-1} fresh wt. h^{-1}) activity, measured at flowering, was affected by different levels of boron. Boron at 0.4 to 0.8 ppm was more effective in all the genotypes. Maximum NR activity was observed in IET 21007 (0.211 µmol NO_2^- g^{-1} fresh wt. h^{-1}) and minimum in IET 21540 (0.111 µmol NO_2^- g^{-1} fresh wt. h^{-1}) at 0.8 and 0.4 ppm boron respectively (Fig. 3). This might be due to the adequate and quick supply of boron through phloem tissues to the floral meristems which can influence the uptake and metabolism of nitrogen (Camacho-Cristobal and Gonzalez-Fontes, 2007). Interestingly, it was also reported that both a deficiency and high (toxic) levels of boron decreased the total N content and the activity of nitrate reductase and thus resulted in increased content of nitrates in roots and shoots of the sunflower plants. (Kastori and Petrovic, 1989). Similar to our study

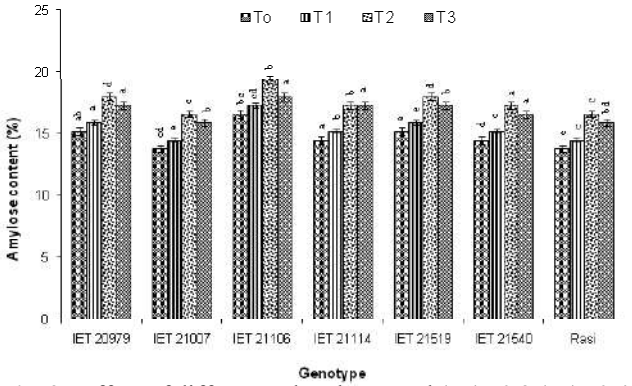

Fig. 2 . Effect of different B levels control (T_0) , 0.2 (T_1) , 0.4 (T_2) and 0.8ppm (T_3) on grain amylose content (%) in different genotypes of rice.

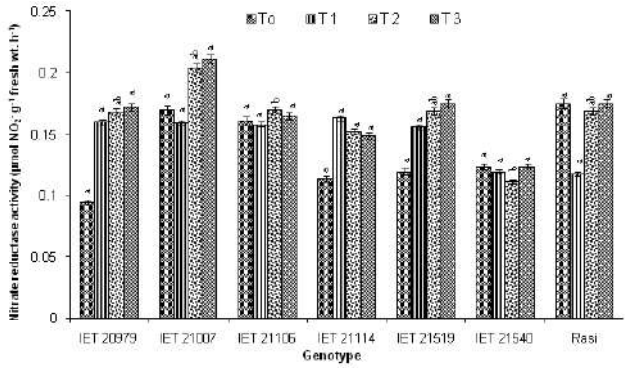

Fig. 3 . Effect of different B level on Nitrate reductase activity (µmol NO 2^- g^{-1} fresh wt.h^{-1}) at flowering stage in different genotypes of rice.

solution culture and pot experiment of *Brassica napus* indicated that nitrate reductase activity in the leaves of rape plants (Ningyou No. 7 and Ningyou No. 8) grown on nutrient solutions increased markedly with increasing boron concentration (from 0.025 micrograms B/ml to 0.1 micrograms B/ml). Nitrate reductase activity in leaves increased from the basal to the top parts of the plant when boron was supplied (Shen *et al.,* 1993). Although it cannot be discounted that excess boron concentration directly inhibits NR activity in root and leaf tissues of barley and wheat, it seems unlikely that it was the sole cause of the reduced NR activity observed in both species (Mahboobi *et al.,* 2002). Interactive effect of nitrogen and boron may also be responsible for such effects, since 2.5 to 40 mg kg^{-1} boric acid and 75 to 300 mg kg^{-1} N showed the

Table 3. Effect of different levels of boron (0.2-0.8ppm) on boron content (mg kg $^{-1}$) in rice grains and rice shoots in different genotypes of rice at maturity

Genotype	Boron content (mg kg^{-1}) in rice grains				Boron content (mg kg^{-1}) in rice shoots			
	(0 ppm)	(0.2ppm)	(0.4ppm)	(0.8ppm)	(0 ppm)	(0.2ppm)	(0.4ppm)	(0.8ppm)
IET 20979	12.84^{bc}	15.73^{a}	15.09^{a}	16.02^{a}	13.76^{a}	14.54^{c}	15.44^{a}	17.35^{b}
IET 21007	13.66^{abc}	15.42^{a}	16.46^{a}	17.12^{a}	15.59^{a}	15.56^{abc}	16.45^{a}	19.26^{ab}
IET 21106	15.36^{a}	15.94^{a}	16.87^{a}	17.66^{a}	15.55^{a}	16.93^{a}	17.46^{a}	19.50^{a}
IET 21114	14.64^{ab}	15.63^{a}	17.00^{a}	17.20^{a}	15.64^{a}	16.83^{ab}	17.37^{a}	19.16^{ab}
IET 21519	13.03^{bc}	14.73^{a}	16.84^{a}	17.50^{a}	14.75^{a}	15.50^{abc}	16.85^{a}	19.10^{ab}
IET 21540	14.24^{abc}	15.07^{a}	16.56^{a}	17.49^{a}	14.98^{a}	15.61^{abc}	16.00$_{a}$	18.81^{ab}
Rasi	13.39^{c}	14.77^{a}	16.16^{a}	16.72^{a}	14.70^{a}	15.35^{bc}	16.10^{a}	18.91^{ab}

Means followed by a common letter in the columns are not significantly different

interactive effect on growth in rice. Increasing level of nitrogen significantly affect the NR activity (Koohkan *et al.,* 2008).

Boron content in grains and shoots increased significantly with the levels of boron application in most of the genotypes. The overall mean showed that boron content in grains increased with the foliar spray of boron from 13.88 to 17.10 mg kg^{-1} irrespective of genotypes. The highest increase in boron content as compared to control was recorded in rice grains (4.47 mg kg^{-1}) and shoots (4.35 mg kg^{-1}) of IET 21519 at 0.8 ppm boron. However, at same treatment level, lowest increase (2.3 mg kg^{-1}) in grains of IET21106 and in shoots (3.52 mg kg^{-1}) of IET21114 was observed. In case of shoot, the overall mean ranged from15.0 to 18.87ppm boron irrespective of genotype and maximum 19.50 mg kg^{-1} was observed in IET21106 at 0.8ppm (Table 3). The absorption of boron is pH dependent and the uptake of boron in plants is found in the form of boric acid (Tanaka and Fujiwara, 2008). The soil or foliar application also increased boron content in different parts of rice plants. This is supported by Rashid *et al.,* 2004 who found that 1 kg boron ha^{-1} increased the concentration of boron in rice shoots as well as grains. Boron application also increased wheat grain yield significantly over check (No boron). The highest grain yield of 3490 kg ha^{-1} was recorded by the application of 1 kg B ha^{-1}, followed by 3287 kg ha^{-1} with application of 2 kg B ha^{-1} (Kizilgoz and Erda, 2010). From the present study it can be concluded that the effect of foliar application of boron on various physiological and biochemical parameters in different genotypes of rice showed genetic variability in their threshold limits of boron utilization i.e., most of the genotypes were influenced by 0.4 ppm boron however in some genotypes, biological yield was increased at 0.8 ppm boron but this increase in biological yield did not much influence grain yield which also showed that for better translocation of photosynthates from source to sink, 0.4 ppm of foliar applied boron seems to be sufficient for better yield.AcknowledgementsAuthors are thankful to DRR (ICAR), Hyderabad,India for extending financial support under the All India coordinated Rice Improvement programme.

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CHAPTER 15

Phytoremediation facilitating enzymes: an enzymatic approach for enhancing remediation process

Narendra Kumar[1], Neha Jeena[2], Narendra Kumar[3], Saurabh Gangola[4] and Hukum Singh[1]
[1]Forest Ecology and Climate Change Division, FRI, Dehradun, India
[2]Department of Biotechnology, Bhimtal Campus Kumaun University, Nainital, India
[3]Department of Botany, Govt. M. B. (P.G.) College, Haldwani, India
[4]Department of Microbiology, Crop Production Division, ICAR-VPKAS, Almora, India

15.1 Introduction

Soil pollution is an important environmental problem that has increased in recent years and can cause damage to human health, soil productivity, and ecosystem and ecological health (Conesa, Evangelou, Robinson, & Schulin, 2012). The main reason for soil contamination is linked with anthropogenic activities, such as increasing industrialization (Jaak, Marika, Mikk, Hiie, & Jaanis, 2015). There are a number of methods for removing pollutants from soil as well as water. Phytoremediation is one method to remediate environmental adulteration. Phytoremediation is a bioremediation application in which there is microbial removal of contaminants from soils, sediments, groundwater, and surface water creating less hazardous substances. Various reports have been related to the involvement of organisms such as animals and plants for detoxifying pollutants through bioremediation. For example, genetically, engineered plants (*Arabidopsis thaliana*) have been used to absorb arsenic pollutants. These have two bacterial genes of which one gene converts arsenate into arsenite and the second binds to the arsenite and is finally stored in the vacuoles. In addition, different reactions that have roles in remediation of contaminants by plants include phytoextraction, phytostabilization, phytotransformation, phytostimulation, and phytovolatization (Leung, 2004). Bioremediation plays an important role in the microbial activities which enzymatically convert

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pollutants to nonhazardous products. However, the detoxification of the pollutants will proceeded efficiently only if there are favorable conditions for the growth and activity of the microorganism. Specific bacteria are involved in the removal process of organopollutants, depending on the various types of intra- and extracellular enzymes (Madadi & Abbas, 2017).

15.2 Rhizoremediation

Rhizoremediation is the degradation process of contaminated soil in the rhizosphere through microbial activity. The rhizosphere is related to the root and surrounding soil surface. There are three zones of the rhizosphere as follows (Pinton, Varanini, & Nannipieri, 2001) (Fig. 15.1):

1. Endorhizosphere: part of the root tissue (endodermis and cortical layers).
2. Rhizoplane: surface of the root where microbes adhere with soil particles. It has an epidermis layer, a middle layer cortex, and a polysaccharide layer.
3. Ectorhizosphere: the zone where the roots are adjacent to the soil surface.

In the rhizosphere microorganisms are considered as plant growth-promoting organisms which enhance plant growth directly or indirectly. These are symbiotic relationships in which plant roots provide a recess for the growth of the microbes and where a number of microbes act as the biocatalyst to eliminate pollutants. The interaction of plants and rhizobial microbes leads to the removal of harmful pollutants from the soil. The

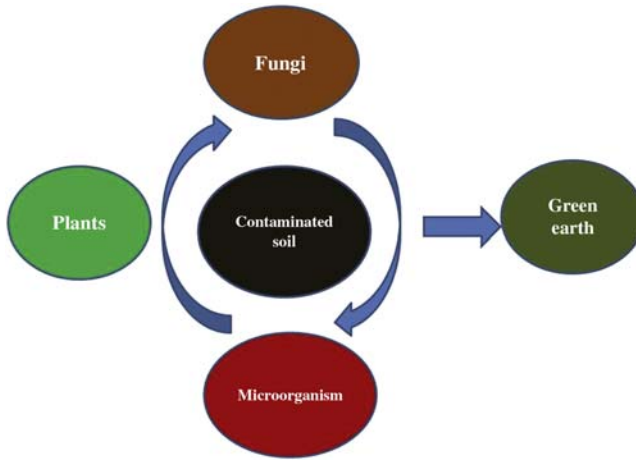

Figure 15.1 The process of bioremediation.

process of transforming contaminated waste into nonhazardous waste is brought about by rhizomicroflora and is known as rhizoremediation. It is a part of phytoremediation and is a clean and green phenomenon suitable for large or small sites with low to moderate pollutant levels (Zhuang, Chen, Shim, & Bai, 2007).

15.3 Rhizofiltration

In an aquatic environment the toxic metals and other contaminants are remediated using plant biomass by a phytoremediation approach, that is, rhizofiltration. This is similar to the concept of phytoextraction, which does not involve remediation of soil contamination but plays a direct and important role in the removal of groundwater contamination. In this process plants are situated where they absorb contaminating metals and concentrate them in roots and shoots. Agricultural runoff and industrial discharge can be treated by rhizofiltration (Yadav, Siebel, & Bruggen, 2011; Yan-de, Zhen-li, & Xiao-e, 2007).

15.4 Rhizostimulation

It was observed that appropriate microorganisms already exist in soil but are not able to remediate pollutants at the required rate. This may be due to the consequences of suboptimal pH, limited availability of inorganic nutrients, absence of organic cosubstrates, compaction, anaerobiosis, etc. Established bioremediation strategies recognize these constraints and include plowing, irrigation, fertilization, manuring, and liming, all of which will stimulate the indigenous microflora and accelerate degradation. In rhizostimulation either the native plant species are exploited or new ones are sown which are suited to the soil type and climate (Kuiper, Bloemberg, & Lugtenberg, 2001).

Microorganisms have the ability to degrade hydrocarbons, with the exception of complex polyaromatic hydrocarbons which are not fully degraded. This is because of soil conditions which are unsuitable for the enzymatic degradation of hydrocarbon, some authors have reported the following criteria:

1. Soil moisture should be 30%.
2. pH of soil varies from 6.5 to 8.
3. Oxygen content ranges between 10% and >30%.
4. Soil type should be low clay.

In addition, if hydrocarbon biodegradation is from *psychrophilic* environments, due to the microbial activity and hydrocarbon solubility rate, the atmospheric temperature increases causing a decreased biodegradation rate (Das & Chandran, 2011; Pelletier, Delille, & Delille, 2004; Vidali, 2001).

15.5 Soil enzymes facilitating rhizoremediation

Enzymes act as biocatalysts and are involved in metabolism as well as biochemical reactions. Certain enzymes are used for treating environmental pollutants and act as organic catalysts in various processes as described subsequently.

15.6 Oxidoreductase

These enzymes participate in the oxidation reaction which reduced substrates by transferring oxygen from molecular oxygen by utilizing FAD/NADH/NADPH as cofactors. Oxidoreductases follow the reactions $A^- + B \rightarrow A + B^-$ where the oxidant is A and the reductant is B. The toxicity of these pollutants can be remediated by various bacteria as well as higher plants and is driven by enzyme oxidoreductases. By transferring the electrons from the substrate to the compound it helps to break chemical bonds and finally the contamination is oxidized to harmless organic compounds. Lignin decomposition in a soil environment with humification of phenolic substances occurs through this enzyme. In addition, it can also detoxify xenobiotic compounds through the processes of binding to humic substances and polymerization. Most plant families such as fabaceae and solanaceae release oxidoreductases, which are involved in the detoxification process in soil (Husain, 2006; Vidali, 2001).

Various microbial enzymes (namely, oxygenases, reductases, dehalogenases, dehydrogenases, and hydroxylases) play an important role in remediation of environmental pollutants which have been distributed in the biosphere due to anthropogenic activities. Among these enzymes, oxygenases are important enzymes because they are primarily involved in the initial process of degradation and catalyze and degrade the aromatic compounds. They catalyze by the addition of oxygen atoms into the substrates. Two major classes of oxygenases have been identified: monooxygenases (addition of one oxygen molecule) and dioxygenases

(addition of two oxygen molecules) (Arora, Srivastava, & Singh, 2010; Karigar & Rao, 2011).

15.7 Microbial monooxygenases

In substrate there is incorporation of a single oxygen molecule with the help of enzyme monooxygenases. On the basis of the cofactors it is categorized into two subclasses: (1) flavin-dependent monooxygenases and (2) P450 monooxygenases (e.g., CYP102 from *Bacillus megaterium* BM3). The prosthetic group of the first subclass is flavin, which is activated by the coenzymes NADP or NADPH, while the second subclass contains heme. Monooxygenases are cofactor-dependent biocatalysts involved in bioremediation. Some enzymes are cofactor-independent and perform their activity with the molecular oxygen only. A number of processes, such as desulfurization, denitrification, nitrification, ammonification, dehalogenation, transformation, hydroxylation, and aromatic and aliphatic biodegradation are regulated by enzyme monooxygenases, for example, ActVAorf6 monooxygenase, TcmH, etc. Methane monooxygenase enzyme is the best characterized as playing a role in the degradation of hydrocarbons (Lock, Nichol, Murrell, & Smith, 2017; Sirajuddin & Rosenzweig, 2017; Syed, Porollo, Miller, & Yadav, 2013; Urlacher, Lutz-Wahl, & Schmid, 2004).

15.8 Microbial dioxygenases

These are multicomponent enzyme systems which link molecular oxygen to the substrate. They degrade the aromatic compounds that cause serious environmental damage. On the basis of the mode of action of the enzyme it can categorized into two subclasses: hydroxylation and cleavage dioxygenases. The hydroxylation enzyme catalyzes the addition of two oxygen atoms into the substrate, whereas the cleavage enzyme catalyzes an aromatic ring typically carrying two or more hydroxyl groups. The cleavage dioxygenases are further classified into two groups, intradiol (ortho cleavage and utilize FeIII) and extradiol (meta cleavage and utilize FeII and MnII). These enzymes are involved in degrading aromatic molecules in the environment. They are soil bacteria which are involved in the transformation process by changing aromatic precursors into aliphatic products (Fulekar, 2017; Muthukamalam, Sivagangavathi, Dhrishya, & Rani, 2017; Xenia & Refugio, 2016).

15.9 Microbial laccases

These are the oxidases of blue multicopper which act as biosensors which catalyze substrate oxidation. Most of these microorganisms have the potential to synthesize intracellular laccases and extracellular laccases involved in the oxidation of aminophenols, lignins, ortho and paradiphenols, polyamines, polyphenols, aryl diamines, etc. Laccases decolorize and detoxify industrial effluents and help in wastewater treatment. They act on highly environmental contaminant, phenolic and nonphenolic organic compounds and are valuable for the textile, pulp, and paper industries. In addition, they are directly involved in the degradation of xenobiotics and bioremediation. In addition, thermophilic laccase enzyme, which was isolated from desert plant species such as *Cereus* spp. and *Opuntia* spp., has shown thermophilic properties and is utilized in the pulping industry (Nigam, 2013; Viswanath, Rajesh, Janardhan, Kumar, & Narasimha, 2014).

15.10 Microbial peroxidases

Peroxidases (EC 1.11.1.7) are widely distributed in nature. These enzymes are produced by a variety of sources including plants, animals, and microbes. They are produced from different sources including fungi, bacteria, yeast, and *Cyanobacteria*. These microbial enzymes are involved in the degradation of environmental contaminant, feedstock of animal, and raw materials for the chemical, agricultural, and paper industries, textile dye degradation (Table 15.1), paper/pulp industry for lignin degradation, dye decolorization, sewage treatment, and also as biosensors. In plants, they are involved in lignin synthesis, cell wall formation, metabolism of auxin, cell elongation, and defense mechanism. They are also subdivided into heme and nonheme proteins. Hemeperoxidases are divided into two major groups which include prokaryotes as well as eukaryotes (Bansal & Kanwar, 2013; Falade et al., 2016). Peroxidases are again subdivided into three classes based on the comparison.

1. Class I: This is an intracellular enzyme which has ascorbate peroxidase, yeast cytochrome c peroxidase, as well as catalase peroxidases.
2. Class II: This is involved in the lignin degradation of wood which consists of fungal peroxidases, namely, manganese peroxidase (Mnp) and lignin peroxidase.
3. Class III: This category of enzyme is involved in the formation of cell wall and lignifications and contains plant peroxidases, namely, horseradish, barley, or soybean (Falade et al., 2016).

Table 15.1 Microbial peroxidase(s) and their applications.

S. no.	Type of peroxidase	Type of microorganism	Microorganism	Application
1.	Peroxidase	Bacteria	*Escherichia coli*	Dye degradation
2.	Peroxidase	Bacteria	*Bacillus* sp. F31	Dye degradation
3.	MnP, LiP	Fungi	*Coriolus versicolor* (L.) *Quel., Tyromyces albidus* (Schaeff.) *Donk*, and *Trametes gallica*	Biodelignification
4.	LiP	Bacteria	*Citrobacterfreundii* (FJ581026) and *Citrobacter* sp. (FJ581023)	Black liquor (pulping by product causes serious environmental problems)
5.	LiP	Yeast	*Candida krusei*	BV extensively used in human and veterinary medicine as a biological stain and in various commercial textile processes
6.	LiP	Bacterium	*Pseudomonas desmolyticum*	Diazo dye Direct Blue-6
7.	Mn-peroxidase	Bacterium	*Pseudomonas* sp.	Malachite green, a widely used recalcitrant dye, has been confirmed to be carcinogenic and mutagenic against many organisms
8.	LiP	White-rot fungi	*Pleurotus ostreatus*	Remazol brilliant blue R (artificial dye)
9.	Peroxidase	Bacterium	*Pseudomonas* sp.	Congo red decolorization
10.	LiP isoenzymes (LiP 4.65, LiP 4.15, and LiP 3.85)	Fungus	*Phanerochaete chrysosporium*	Azo, triphenyl methane, heterocyclic, and polymeric dyes
11.	Peroxidase	Bacterium	*Clostridium bifermentans*	Reactive azo dyes
12.	Versatile peroxidase	Fungus	*Thanatephorus cucumeris*	Anthraquinone dye reactive blue 5
13.	DyP-type peroxidases	Fungi	*Auricularia auricula-judae*	High-redox potential dyes
14.	Extracellular LiP	Bacteria	*Bacillus* sp.	Navy blue 2GL-azo dye
15.	DyP	Fungi	*Pleurotus ostreatus*	Azo dyes

BV, Basic violet 3; *DyP*, dye-decolorizing peroxidases; *LiP*, lignin peroxidase; *Mnp*, manganese peroxidase.

15.11 Classification of peroxidases

1. *Microbial lignin:* during secondary metabolism these heme proteins are secreted by white-rot fungus and degrade the cell wall of plants containing lignin.
2. *Microbial Mnps:* these are extracellular heme enzymes. In a multistep reaction they catalyze the oxidation of Mn^{2+} to Mn^{3+} oxidants. A reduced form of Mn enhanced the production of Mnps and the oxidant acts as a precursor for phenolic compound oxidation.
3. *Microbial versatile peroxidases:* these helps to oxidize methoxybenzenes, Mn^{2+}, phenolic compounds, and dimers of lignin. Therefore they are required for the removal of environmental contaminants (Karigar & Rao, 2011).

15.12 Hydrolytic enzymes for bioremediation

Besides the abovementioned enzymes, some hydrolytic enzymes are also important in the bioremediation of soil pollutants. Water is contaminated by industrial waste, petroleum waste, and degraded by some hydrolytic enzymes such as proteases, lipases, pullulanases, amylases, and xylanases. These enzymes have a diverse role in different areas including in different industries and biochemical sciences.

15.13 Lipases

Lipase is present in plants and animals as well as microorganisms, and plays an important role in lipid degradation. It acts on the soil organic contaminants and helps in the reduction of pollutants from the contaminated soil; the reactions include esterification, aminolysis, hydrolysis, etc. It is an indicator to degrade hydrocarbon pollutants from soil. Lipase has various applications in the cosmetic, food, paper, pulp, food and chemical industries, etc. but the cost of production is restrictive (Nigam, 2013; Okino-Delgado et al., 2017).

15.14 Amylases

Alpha amylases are an extracellular enzyme which breaks the α-1,4-glucosidic linkage in starch molecules and produces oligosaccharides. β-Amylase also breaks the second α-1,4-glycosidic bond of maltose and is synthesized in plants as well as bacteria. Amylases are significant enzymes for their

Table 15.2 Application of amylases in different industries (Singh, Mittal, Kumar, & Mehta, 2016).

S. no.	Industry	Applications
1.	Food	Starch liquefaction, in corn syrups; in baking, to increase the shelf life of breads
2.	Detergent	Starch-based stain removal
3.	Paper	In viscosity reduction, drainage improvement
4.	Textile	Warp sizing of textiles fibers
5.	Biofuel	Bioalcohol production
6.	Pharmaceutical	Digestive aid
7.	Bioremediation	Bioremediation of vegetable wastes
8.	Leather	Fiber splitting

specific use in the industrial starch conversion process. These enzymes act particularly on disaccharides (sucrose) and polysaccharides (starch) and are categorized in the glycoside hydrolases group. Application of these enzymes has been established in the starch liquefaction, paper, food, sugar, and pharmaceutical industries (Table 15.2; Gopinath et al., 2017).

15.15 Proteases

These are a group of hydrolytic enzymes which cleave peptide bonds that occur within the primary structure of polypeptides and other proteins as well. They are well known industrial enzymes, with a key role in different industries, for example, pharmaceutical, textile, feed food, and can be extracted from plants, animals, and microorganisms. Protease provides potential applications for the management of waste from some processing industries and household activities. In addition, it is also helpful in poultry processing industries (Bhunia and Basak, 2014).

15.16 Pullulanase

α-Dextrin 6-glucanohydrolases or pullulanases is synthesized from several microorganisms such as *Klebsiella* spp., *Bacillus* spp., and *Geobacillus stearothermophilus*. Due to its specific enzymatic action on pullulan, particularly in the specific linkages (α-1,6 linkages), it is very popular. It is very important for its action as a bioprocessor of starch. Pullulanase enzyme has a wide role in genetic engineering and some industrial uses

(Karigar & Rao, 2011; Lee, Jang, & Chung, 2017). It has five groups which were described earlier, as follows:

1. pullulanase type I;
2. hydrolase type III;
3. neopullulanase;
4. amylopullulanase; and
5. isopullulanase.

15.17 Enzyme kinetics in phytoremediation

Phytoremediation is the most widely recognizable name techniques used for cleaning polluted waste waters, soils, and sediments using plants. Various kinds of pollutants, such as petroleum products and solvents, are degraded more rapidly through plants. Various processes are known to influence the oxidation of pollutants, transportation of oxygen, evapotranspiration, and uptake of chemicals through plants. However, pollutants are directly metabolized either by plants or by the indirect action of microbial activity (Trapp, Ucisik, Romano, & Larsen, 2007).

For removal or degradation processes, plants are involved in a number of mechanisms to remove both organic or inorganic pollutants from contaminated environments, such as by heavy metal precipitation, rhizofiltration, absorption by roots, phytoextraction—accumulation and extraction of organic or inorganic pollutants in leaves and roots, phytodegradation—complex organic molecules degraded in CO_2 and H_2O and their incorporation into plant tissues, rhizodegradation or plant-assisted bioremediation—stimulation of fungal and microbial oxidation by exudates and root enzymes in the rhizosphere and phytostabilization—adsorption and precipitation of pollutants (Rao, Scelza, Scotti, & Gianfreda, 2010).

A synergic interaction occurs between plant roots and microorganisms in the soil environment. Microorganisms possess a potent catabolic pathway to degrade pollutants from soil while plants do not have complete degrading catabolic pathways. However, plants with a specific gene added have the additional ability of pollutant degradation, and so the efficacy of phytoremediation can be directly enhanced. In addition, some genes from the roots directly stimulate the rhizodegradation of polluted environments (Abhilash, Jamil, & Singh, 2009).

15.18 The kinetics of plant enzymes

Enzymes catalyze chemical reactions in a process referred to as enzyme kinetics. In this, the rate of reaction can be measured, as can the different

conditions of the reaction. This plays an important role in the measurement of the activity of enzymes, and also metabolism and measurement of the drug effect. Enzymes can manipulate the function of a particular substrate and act as a protein. A series of enzymatic reaction substrates can bind to the active site of the enzyme so that it can be transformed into a specific product (Biasutti, Albuin, Silber, Correa, & Lissi, 2008).

Plant enzymes kinetics can be explained by Michaelis–Menten kinetics, for example, if the substrate concentration is low, the degradation is considered first order, while it is restricted at higher substrate concentrations. According to Monod kinetics, bacteria can be utilized as substrate for growth and at higher availability substrate growth is better. However, it was observed that bacteria have limited degradation ability if the substrate concentration is very low which can prohibit the biodegradation of contaminated sites. Therefore combing plants and bacteria can be used to overcome environmental pollutant problems (Trapp et al., 2007).

15.19 Growth and metabolism kinetics in plants

Xenobiotic metabolism is found in the plant cells, which play a vital role in detoxification of toxic compound. Xenobiotics, for example, herbicides, cytochrome P450 monooxygenases, and glutathione *S*-transferases (GST) are the most important enzymes (Pflugmacher & Schroder, 1995). The enzyme P450 is catalyzed mainly by phase I transformation reactions, such as hydroxylation, but also sulfoxidation, and N- and O-dealkylation. Phase 2 GSTs are also responsible for the conjugation reaction, which has a very large role in the detoxification of herbicide reactions for plants. Phase III plant xenobiotics are responsible for storage of insoluble solutes in the cell wall and compartmentation and storage in the vacuole of plant cells, respectively (Komossa, Langebartels, & Sandermann, 1995).

However, the growth rates are linked to the exponential growth phase of plants including the mass, volume, size, and ripening which decrease simultaneously.

Xenobiotics are not used as substrate by plants. The growth of plants is generally not affected by exposure to xenobiotics. Michaelis–Menten kinetics elaborates the metabolism kinetics of enzymatic reactions (Cornish-Bowden, 1995).

Therefore xenobiotics depends on the rate of the enzymatic reaction, the mass of plant, and the uptake frequency of xenobiotics by the plant (Fig. 15.2).

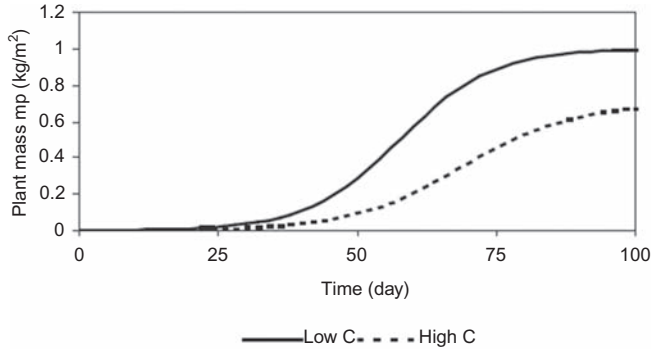

Figure 15.2 An example of plant growth and reduced growth, due to the toxic impact of chemicals (Trapp et al., 2007).

15.20 Metabolism kinetics of bacteria

Bacteria are heterotrophic organisms and require organic substances such as xenobiotics to feed on. These substrates are utilized as nutrient sources by degrader bacteria. A wide range of enzymes are release by the bacteria, which can chemically alter the xenobiotics. Xenobiotics can therefore be used as electron acceptors/donators and also as energy sources (Schlegel & Zaborosch, 1993).

Therefore bacterial growth depends on substrate availability. Thus growth and decay of the bacteria can be explained by Monad kinetics.

$$\frac{dB}{dt} = \frac{\mu_{max} \times C \times B}{K_s + C} - k_{death} \times B$$

where B is bacterial mass, μ_{max} is maximal growth rate, C is the substrate concentration, and K_S is the half-growth concentration. When the rate of death is higher than that indicated by the probability, the growth curve for bacteria is negative.

Elevated xenobiotic pollution was degraded more rapidly and more completely than where there was only a low amount of contamination (Fig. 15.3). Fig. 15.3 shows the effect of bacterial xenobiotics for two initial concentrations ($C = 1$ and 3 mg/L, respectively). All other parameters remained unaffected or unchanged (Fig. 15.3).

15.21 Hyperaccumulator plants

Soil contaminated with heavy metal poses a serious problem for all animals, including humans. Plants able to hyperaccumulate specific metals with good cleanup ability have existed over last two decades. These plants

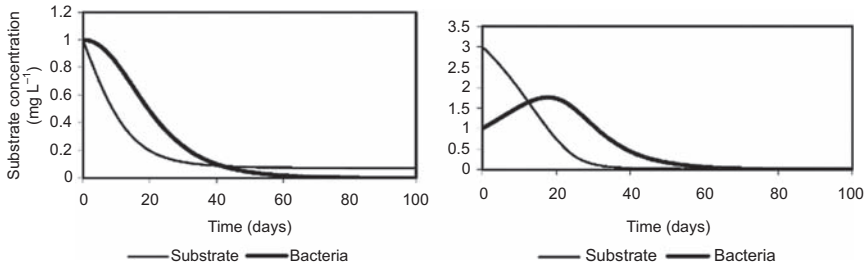

Figure 15.3 Bacterial population *B* and substrate concentration *C* at low (left) and high (right) initial substrate concentrations (Trapp et al., 2007).

are directly involved in the remediation of pollutants from soils and this process is known as phytomining. In addition, several studies have reported some heavy metals as essential nutrients that can also be used in food fortification. Plants belonging to a family with potential to grow on toxic metal-polluted soils with accumulation in their aerial organs without causing phytotoxicity are called "hyperaccumulator plants." These plants have specific threshold values for hyperaccumulation of each toxic metal above which they may be subject to phytotoxicity. Based on this criterion hyperaccumulator plants can tolerate concentrations of >10 mg/g (1%) Mn or Zn, >1 mg/g (0.1%) Se, Cr, As, Co, Tl, Pb, Ni, and >0.1 mg/g (0.01%) Cd in their soft organs, without any phytotoxic damage (Verbruggen, Hermans, & Schat, 2009).

15.22 Differences between hyperaccumulators and nonhyperaccumulators

There are three basic hallmarks of nonhyperaccumulating taxa that distinguish them from hyperaccumulators:

S. no.	Hyperaccumulators	Nonaccumulators
1	Heavy metal uptake enhancement	Do not or only very slow take up heavy metals
2	Faster root-to-shoot translocation	Do not or only very slowly translocate heavy metals
3	Greater ability to detoxify	Do not detoxify
4	Sequester heavy metals, especially in leaves, at concentrations 100- to 1000-fold higher	Heavy metals accumulate in root or ground organs of the plant
5	Reliance on hypertolerance	Low level of tolerance for heavy metals

15.23 Effect of heavy metals on physiological responses of plants

Heavy or toxic metals such as Cd, As, Hg, Pb, or Se, are nonessential elements, whereas Zn, Mo, Cu, Mn, Co, and Ni are essential elements which are essential to plant growth, development, and metabolism. Increased concentrations of the latter elements up to supraoptimal values can lead to poisoning. Alterations to enzymes can change the physiology of plants by blocking the metabolically active substrate at the cellular or molecular level, and to overcome this inactivation there is a requirement for the essential elements followed by alterations to the integrity of the membrane which can cause heavy metal phytotoxicity. One of the most common consequences observed during this activity is the enhanced production of a toxic form of oxygen (reactive oxygen species) due to interference with the movement of electrons in the membrane of the chloroplast (La Rocca, Andreoli, Giacometti, Rascio, & Moro, 2009; Pagliano et al., 2006). This further increases the cell exposure toward oxidative stress, causing lipid peroxidation, biological macromolecule deterioration, dismantling of membrane, leakage of ions, and DNA-strand cleavage.

15.24 Common defense mechanism for heavy metals in plants

Different defense mechanisms are used in plants to control the accumulation, uptake, and translocation of toxic elements. Some of the most common strategies are listed here:

- Interruption in apoplastic movement, blockage of the movement of heavy metals in the roots of plants by complexing them with amino acids, organic acids, or metal-binding peptides, etc. (Hall, 2002). This interruption of the movement of heavy metals toward the roots to young plants protects young tissues and photosynthetically active cells from heavy metal toxicity.
- Oxidative stress is counteracted by enhancements to cell antioxidant systems (Sgherri, Cosi, & Navari-Izzo, 2003).

15.25 Molecular basis of heavy metal accumulation

At a molecular level a number of genes are responsible for the process of hyperaccumulation in plants (Rascio & Navari-Izzo, 2011). The HA

hyperaccumulation gene is present in more than 400 plant species. The HA gene is found in plants of the *Brassica* family as well as in the model organism *Arabidopsis*. Its presence and the expression level of the particular gene provide information about the potential to remove toxic metals, namely, As, Co, Fe, Cu, Cd, Pb, Hg, Se, Mn, Zn, Mo, and Ni. This capacity for hyperaccumulation mainly depends on environmental exposure and the expression of members of the ZIP gene family, although some studies have illustrated a partial role for other environmental factors. Expression of the HA gene is dependent on its upregulation and presence in particular plant species. The main strategy involved in the transportation of toxic metals is proteins coded by genes in the ZIP family. Proteins coded by ZIP gene family encode heavy metals, such as Mn, Cd, Zn, and Fe transporters. The ZIP gene family also has another important function in the transportation of Zn to metalloproteins (Poschenrieder, Tolrà, & Barceló, 2006). It has been demonstrated that an inherited capacity for hyperaccumulation was found in plants of the Bassicacease family, namely, *Thlaspi caerulescens*, and the ZTP family was identified as having high sequence similarity to other Zn transporters. Some other gene families, such as ZNT and ZTP (Zn transporters), have been reported to cause heavy metal hyperaccumulation (Persans, Nieman, & Salt, 2001). It was also reported that there was chronic expression of ZNT1 and ZNT2 alleles in hyperaccumulating species (Assunção et al., 2001).

15.26 Conclusion

Phytoremediation is a process to degrade or remediate soil and water pollutants with the combined action of plants and microbes. Enzymes are involved in catalyzing the rate of the reaction and this enzymatic action is more effective and more popular for the oxidation of toxic pollutants from contaminated environments. Different pathways present in microbes and plants can affect an important role in remediation. In addition, many plants act as hyperaccumulators for the removal of specific industry-based toxic metals from polluted environments. This process is advantageous because of the low cost of processing, publicly accepted techniques, and the capacity to degrade heavy metal toxic elements. In addition to the advantages of phytoremediation, there are some disadvantages such as phototoxicity, and geological and climatic limitations.

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IN VITRO EVALUATION OF ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANTS IN *Rauvolfia serpentina* (VAR. CIM-SHEEL) UNDER PEG INDUCED DROUGHT

NARENDRA KUMAR*, DEEPIKA SHARMA, SIDDHARTH S. BHATT#, S. C. SHANKHDHAR AND DEEPTI SHANKHDHAR

Department of Plant Physiology, College of Basic Sciences and Humanities,
G.B. Pant University of Agriculture & Technology, Pantnagar - 263145, Uttarakhand, INDIA
#Department of Horticulture,
College of Agriculture, G.B. Pant University of Agriculture and Technology, Pantnagar - 263 145 (Uttarakhand) INDIA
e-mail: narendrap1987@gmail.com

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***Corresponding author**

ABSTRACT

An experiment was performed to evaluate enzymatic and non enzymatic antioxidants in leaf derived callus of *Rauvolfia serpentina* var. Cim-sheel, a perennial medicinal shrub from family apocynaceae, under poly ethylene glycol (PEG 6000) induced drought. As it has a great potential to induce the synthesis of secondary metabolites and antioxidants under stressful environment. Large, fragile and creamy white callus (grown in 2.0 mg l^{-1} 2, 4 D and 0.5 mg l^{-1} BAP) were inoculated on PEG induced drought MS medium for further study. Its proliferated callus responds positively and enhanced antioxidants level with increasing drought in media. Three concentrations of PEG (10, 20 and 30 gl^{-1}) along with 2, 4 D (2 mg l^{-1}) and BAP (0.5 mg l^{-1}) were used in media and data were taken after 15, 30 and 45 days of inoculation. Enhanced stress declined relative water content in the range of 55.69% to 22.37 % while the level of antioxidants like SOD and CAT was elevated in the range of 15.38% to 73.24 % and 14.29% to 100 % respectively with increasing concentration of PEG. Ascorbate and flavonoids content also enhanced 25% to 54.55% and 9.92% to 55.70 % with increasing drought and age of callus.

INTRODUCTION

Plants encounter several abiotic stresses as drought, salinity, high/low temperatures, heavy metals and nutrient deficiency *etc*. in the natural environment that affects their growth and development. Drought is one of the major stresses amongst all. During drought plants respond by inducing several morphological, physiological and metabolic responses (Fang and Xiong, 2015). To cope with the water stress condition plant having different adaptations and acclimations mechanism such as drought avoidance, drought escape and resistance against dehydration of the protoplast. So plant has multiple mechanisms to respond the water stress for enhancing drought stress tolerance (Deikman *et al.,* 2012, Juenger, 2013). The relative water content (RWC) consider is a important physiological marker parameter and indicator for drought stress in plants, because it shows the balance between the water supply to the plant leaf and rate of transpiration (Lugojan and Ciulca, 2011).

The responses of plant against drought are depending on the inheritance strategies of species (Cruz de Carvalho, 2008). Prolonged drought stress results in oxidative damage (oxidation of lipids, modification in proteins and mutations in DNA) of plants due to the over production of reactive oxygen species (ROS) like the singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$). Due to partial reduction of O_2 under stress the equilibrium between generation and scavenging of ROS in plants is disturbed (Smirnoff, 1993). The extent of oxidative stress in a cell could be determined by the amounts of ROS like superoxide, H_2O_2, and hydroxyl radicals *etc*.

In order to control the ROS levels and protection of the cell from oxidative damage, plants have great potential to induced non enzymatic and enzymatic antioxidants under stress (Ahmed *et al.,* 2012). Enzymatic antioxidants include superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase and non enzymatic antioxidants such as phenolic, flavonoids, ascorbic acid, glutathione, carotenoids, proline and tannins (Chand and Dave, 2009). Superoxide dismutase (SOD) is a metalloenzyme which catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2 and O_2. Catalase is a tetrameric heme-containing enzyme that dismutate H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification under stress conditions (Ahmad *et al.,* 2008; Gill and Tuteja, 2010; Karuppanapandian *et al.,* 2011). The synthesis and accumulation of non enzymatic antioxidants may be due to two reasons first is genetically make up of plant which give rise innate ability to produce phytochemicals for proper physiological processes and to protect themselves against biotic and abiotic stresses. The second cause is that to synthesize reductant phytochemicals which have natural tendency to respond atmospheric stress condition. The low

molecular weight antioxidants like glutathione and ascorbate in cytosol and chloroplast stroma used NADPH as an electron donor (Alscher *et al.,* 1997). Ascorbate is formed during the aerobic metabolism which further reacts rapidly with ($O_2^{-\cdot}$) and singlet oxygen (chemically) and H_2O_2 (enzymatically) with an enzyme ascorbate peroxidase to neutralize the influence of toxic substrate, it also helps to regenerate the antioxidants pigments such as carotene, xanthophyll and vitamins E (Meyer and Hell, 2005; Noctor *et al.,* 2012).

Medicinal plants have great importance of secondary metabolite. World's two third plant species have medicinal importance, almost all of these medicinal plants have great antioxidant potential (Krishnaiah *et al.,* 2011). *Rauvolfia serpentina* (Apocynaceae) *is* a woody perennial medicinal shrub. Due to rich source of secondary metabolite it is used to prevent various diseases like mental disease, epilepsy, sleeplessness and several other diseases. Every part of plant contains a lot of important secondary metabolite like alkaloids reserpine, ajmalicine, ajmaline, serpentina, yohmibine and lot of antioxidants.

In vivo propagation of this plant is difficult due to production of large number of non viable seeds, low seed germination and low vegetative propagation rate (Mitra, 1976; Nayar, 1956; Dutta *et al.,* 1962), however it grows well in dry soils. It is reported in various cases that antioxidants potential is increased in stressful environment. So it may be potential source for production of alkaloids and antioxidants under drought stress. Investigation of antioxidant potential in drought is difficult under field conditions, so the plant tissue culture techniques can perform under aseptic and controlled stressful environment to the plant tissue from cell level. The plant tissue culture allows various opportunities to study the unique and complex responses of plants against environmental stresses very easily (Sakthivelu *et al.,* 2008, Lokhande *et al.,* 2011). Thus the present study was aimed to investigate various enzymatic and non enzymatic antioxidants potential under PEG induced drought in *Rauvolfia serpentina.*

MATERIALS AND METHODS

In vitro cultures

The root and stem cuttings of *Rauvolfia serpentina*, (variety Cim-Sheel) were obtained from Central Institute of Medicinal and Aromatic Plant, Research Centre (CIMAP), Pantnagar, (Uttarakhand) and propagated in the garden section of the department of Plant Physiology, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture & Technology, Pantnagar.

The healthy leaves of *Rauvolfia serpentina* were surface sterilized with tween-20 for 15 min. Thereafter explants were treated with 0.3% bavastin, 0.1% streptomycin, 0.2 % mercuric chloride and finally 70 % ethanol for 5 min, 2 min, 1 min and 30 sec respectively. The explants were cut in to small pieces (0.5-1.0 cm^2) and inoculated on (Murashige and Skoog, 1962) media supplemented with different concentration of plant growth regulators ranging from 0.5- 2.0 mg l^{-1} BAP and 0.5-2.5 mg l^{-1} of 2,4-D. Culture bottles were transferred to the culture room at 25ºC, with 16/8 hour light and dark periods with 3000 lux light intensity.

Leaf derived callus of were transferred to MS media supplemented with 2.0 mg l^{-1} of 2,4-D and 0.5 mg l^{-1} BAP (selected on the basis of % callus induction) for proliferation. Drought stress was given to proliferated callus by supplementing different concentrations of PEG (1,2,and3%) along with 2,4 D (2 mg l^{-1}) and BAP (0.5 mg l^{-1}) and media without PEG was used as control. Different observations were taken after 15, 30 and 45 days after inoculation.

Estimation of relative water content

Relative water content (RWC) of leaf derived callus under stress was estimated according to the method of (Barrs and Weatherley, 1962). Fresh weight of callus was measured and immediately placed in distilled water for 4 hours at room temperature and their turgid weight was recorded. Thereafter they were kept in an oven at 60ºC for 24 hours and measure the dry weight of samples. RWC was calculated by using the formula given below and expressed in percentage.

RWC (%) = [(FW-DW) / (TW-DW)] x 100

Antioxidant enzyme assay

Superoxide dismutase (SOD)

The enzymatic antioxidants like SOD activity was determined by taking 0.2 g callus homogenized in ice containing bucket with pre chilled pestle and mortar by adding 4 ml extraction buffer and centrifuged 16000g for 15 min at 4ºC. Supernatant was taking as a crude enzyme extract for quantification of SOD. 1.5 ml of reaction mixture possessing 50 µl enzyme in test tube shaken and illuminated with 20 W fluorescent light for 15 min. After this tubes were cover with a black cloth and taking absorbance at 560 nm for control everything were added except enzyme along with the reaction tubes and taking one reference tube covered immediately with black cloth and incubate for 15 min. 1 unit of Superoxide dismutase activity measure as amount of enzyme require to cause 50 per cent inhibition of Nitroblue Tetrazolium Chloride (NBT) photo reduction rate described by (Gianopolitis and Ries, 1977).

Z = [(X – A)/X] x 100

Total SOD unit = Z/50

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

Catalase (CAT)

Catalase activity was measured by method given by (Kar and Mishra, 1976). 0.2 g of callus was homogenized in pre chilled pestle and mortar and adding 10 ml of phosphate buffer 0.1 M at 6.8 pH and centrifuged at 17000 g for 15 min at 2ºC. The supernatant was taken for enzymatic activity. After that prepare reaction mixture by taking 1 ml of twice diluted enzymatic extract, 300 µM phosphate buffer (6.8 pH) and 100 µM H_2O_2 and making final 5 ml volume with double distilled water. Incubated at 25ºC for 1 min and stop the reaction by 10 ml 2 % H_2SO_4. Reaction mixture titrate with 0.01N $KMnO_4$ until faint pink colour persisted for 15 sec than record the volume of $KMnO_4$. For control, reaction stopped at 0 time. 1 unit of catalse activity is defined as the amount of enzyme breaks down 1mM of H_2O_2 min^{-1}.

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

Flavonoids

Estimation of flavonoid content performed by the method

(Ordonez *et al.,* 2006). 0.5 g of callus homogenized with 10 ml 80 % ethanol than centrifuged 10000 rpm for 20 min at 4ºC. Supernatant was collected and kept for dryness in hot water bath. Residue was dissolved in 5 ml of double distilled water than solution was prepared for estimation of flavonoid content. After that reaction mixture was prepare by the mixing of 1.5ml sample +1.5 ml 2 % $AlCl_3$. That mixture incubated for 1h at room temperature and taking absorbance at 420 nm yellow colour appears in the presence of flavonoid. Calucaltion of flavonoids was done by making the standard curve of quercetin.

Ascorbate

500 mg callus was homogenized in 10 ml 4% oxalic acid than centrifuged at 10000 rpm for 30 min. The supernatant was for estimating ascorbate content. Reaction mixture was prepared by mixing 1ml of supernatant + 2ml of 4% oxalic acid. That mixture titrated against 2, 6 - dichloroindo phenol dye (DCPIP) and record the V_2 volume which was consumed in titration. The amount of ascorbate in sample is calculated by using the standard of 10 μM ascorbate. 5ml working standard + 10 ml 4% oxalic acid was added then titrated against DCPIP dye and record V_1 volume according to the method given by (Thimmaiah, 1999).

Amount ascorbate(mg/100g sample) = 0.5mg x V_2 (ml) X 100ml X 100/ V_1 (ml) X 15 X wt. of sample

The biochemical and enzymes analysis were carried out with three replications in each treatment. The statistical analysis was done by using the analysis of variance for completely randomized design (CRD) with means being tested at $P > 0.05$ using STPR software designed at the Department of Mathematics, Statistics and Computer Science, CBSH, G.B. Pant University of Agriculture and Technology, Pantnagar, India.

RESULTS AND DISCUSSION

Callogenesis in drought stress

To induce the callus, leaf explants of *R. serpentina* were cultured aseptically *in vitro* on MS medium supplemented with 3.0% sucrose (w/v) and 0.7% agar (w/v) in different concentration of plant growth regulators such as 2, 4-D (0.5-2.5 mg l^{-1}) and BAP (0.5- 2.0 mg l^{-1}). Calluses were initiated very well in 2.0 mg l^{-1} 2, 4-D and 0.5 mg l^{-1} BAP and recorded maximum 90% callus induction frequency (Table 1). To proliferate callus further subcultures were made on MS medium with same concentration of plant growth regulators. After 15-20 days of sub culturing excellent loose textured fragile calluses were obtained. The major portion of callus observed creamy white (Photo plate 1). Since callus is an unorganized mass of the cells whose formation is controlled by growth regulators present in culture medium.

To study the effect of drought on *in vitro* antioxidant levels, proliferated calluses of *R. serpentina* were transferred on media containing 1, 2 and 3% PEG (polyethylene glycol) with appropriate concentration of plant growth regulators. PEG was used to induce water stress in *in vitro* cultures. It is a high molecular weight, non-penetrating, non ionic, inert osmotic agent that lowers the osmotic potential of nutrient solutions, it is non phytotoxic. PEG stimulates water stress in cultured plant cells in the same way it does in the cells of intact plants (Ruf *et al.,* 1967; Kaufman and Eckard 1971; Lawlor, 1970).

Preliminary examination was done on the basis of attributes such as size, color, texture as well as relative water content of stressed callus. Large, fragile, nodular and creamy calluses were obtained in PEG free medium while increasing concentration of PEG (1- 3%) in the media turned callus to blackish brown color. Appearance of drought tolerant white embryogenic cells patches between blackish brown calluses was the indication of adaptation against drought (Photo plate 2). Different plant growth regulators such as cytokinin (CK), auxin, gibberellic (GA), brassinosteroids and jasmonic acid (JA) are very important for adaptation under changing environment (Wolters and Jurgens, 2009). A laboratory experiment conducted on wheat seedling treated were 20% PEG-6000 to induce osmotic stress and exogenously apply cytokinin (BAP and TDZ) which increase its endogenous level of cytokinin via up regulating and down regulating expression of IPT and CKX gene respectively under PEG induced osmotic stress conditions (Nagar *et al.,* 2015).

Relative water content of stressed callus

RWC is considered as a marker to determine the effect of drought stress in plants. Drought stress causes a significant loss in RWC of plants tissue. During investigation, relative water content of controlled and treated callus was determined after 15, 30 and 45 day of inoculation. It was decreased with increasing concentration of PEG in the media with increasing time interval. After 45 days of inoculation, maximum decline in RWC (22.37%) was found in 3% PEG containing media as compare to control (55.69%). While 32.64% and 27.12% decline was found after 15 and 30 of inoculation when compared with control (46.62% and 50.60%) respectively (Figure 1). Relative water content is the appropriate determination of water status in terms of the physiological consequence of cellular water deficit.

The major decline in RWC was also observed in pot experiment when drought stress was given to the *Allium cepa* var. aggregatum in different days interval such as 50, 65, 80, and 95 days after sowing (DAS). Considerable decline observed

Photo Plate 1 : Leaf derived callus initiation of *R. serpentina* on MS mediun supplimented with 2,4-D (2 ppm) & BAP (0.5 ppm) (A) after 5 days of inoculation (B &C), after 15 days of inoculation (D&E) Subcultuing of callus in same MS medium after 22 days to inoculation.

Table1: Percent callus induction in leaf of *Rauvolfia serpentina* (variety Cim-sheel) on MS medium supplemented with different concentration of 2, 4 D and BAP

S .No.	2,4-D (ppm)	BAP(ppm)	Cim -sheel	
			Days to callus induction	% Callus Induction
1.	0.5	0.5	34.33 ± 0.88	15.56 ± 1.11
2.	0.5	1.0	34.66 ± 0.33	21.11 ± 2.93
3.	0.5	1.5	34.00 ± 0.33	17.78 ± 1.11
4.	0.5	2.0	33.00 ± 0.57	16.67 ± 1.92
5.	1.5	0.5	31.00 ± 0.57	22.22 ± 2.22
6.	1.5	1.0	31.00 ± 1.15	24.44 ± 2.22
7.	1.5	1.5	30.00 ± 0.57	23.33 ± 0.57
8.	1.5	2.0	27.60 ± 0.33	68.89 ± 2.22
9.	2.0	0.5	22.90 ± 0.49	90.00 ± 1.92
10.	2.0	1.0	24.00 ± 0.57	78.89 ± 2.93
11.	2.0	1.5	28.00 ± 0.57	45.56 ± 2.93
12.	2.0	2.0	32.00 ± 0.57	22.22 ± 2.22
13.	2.5	0.5	32.33 ± 0.33	18.89 ± 1.11
14.	2.5	1.0	33.66 ± 0.33	21.11 ± 1.11
15.	2.5	1.5	34.00 ± 1.15	20.00 ± 1.92
16.	2.5	2.0	34.33 ± 0.33	17.78 ± 1.11

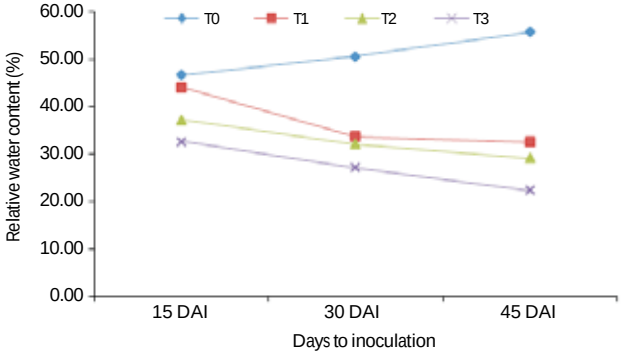

Figure 1 : Effect of different concentration of PEG on relative water content (%) in callus of *Rauvolfia serpentina* (variety Cim sheel) after 15,30 and 45DAI

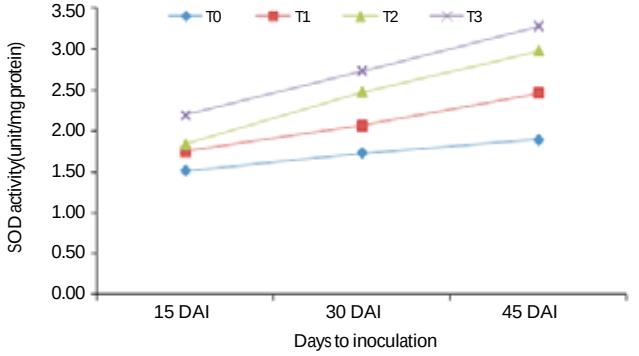

Figure 2 : Effect of different concentration of PEG on superoxide dismutase activity (unit/mg protein) in callus of *Rauvolfia serpentina* (variety Cim sheel) after 15,30 and 45DAI

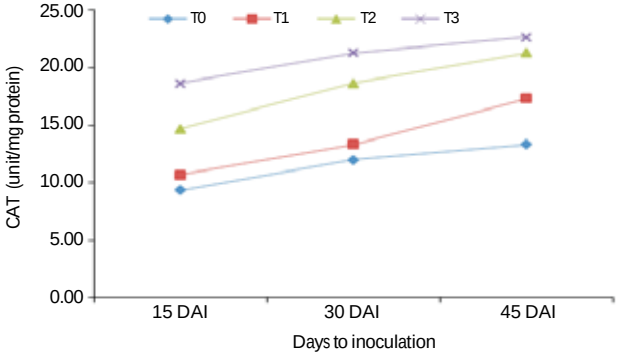

Figure 3: Effect of different concentration of PEG on Catalase activity (unit/mg protein in callus of *Rauwolfia serpentina* (variety Cim sheel) after 15,30 and 45DAI

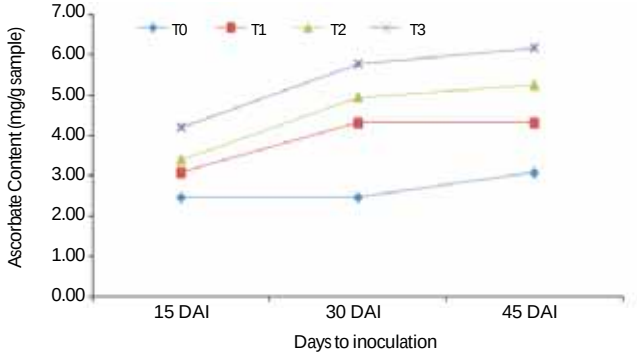

Figure 4 : Effect of different concentration of PEG on Ascorbate Content (mg/g sample) in the callus of *Rauvolfia serpentina* (variety Cim sheel) after 15,30 and 45DAI

in different growth parameter like relative water content etc. as compared to unstressed plants (Ahmad and Murali, 2015). In an another study seeds of 4 varieties of *Lycopersicon esculentum* cultivars Deep-953, 327, 326 and Aditya-955 were germinated in petri dishes, by treating with 15% PEG up to 7 days regularly along with control. Drought stress significantly affects the water status of the plant, resulting major decline in relative water content was observed in Deep-953 (Mendhulkar and Nisha, 2015).

Effect of drought on antioxidants

Various enzymes as superoxide dismutase (SOD)and catalase (CAT) and non enzymatic metabolites as ascorbate and flavonoids were estimated in stressed and non stressed callus

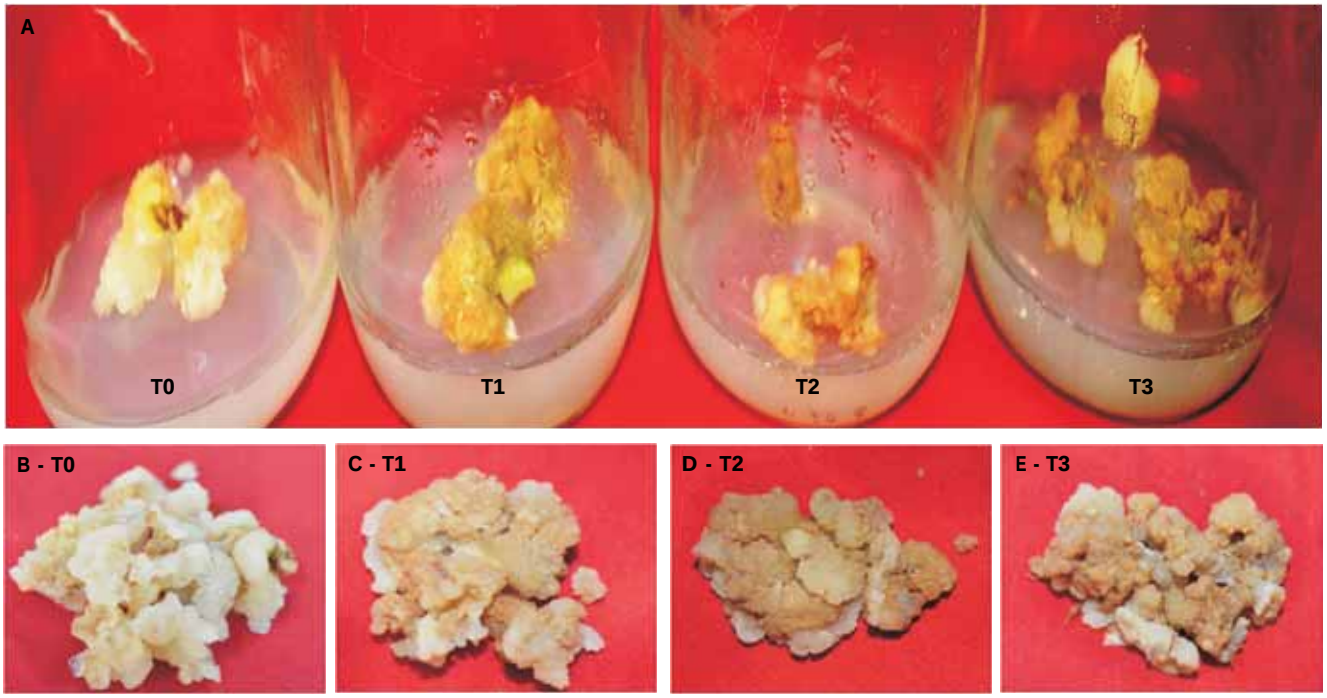

Plate2 : Effect of different concentration of PEG (T0, T1, T2 & T3) on proliferation of *Rauvolfia serpentina* (A) 30 days after inoculation and (B, C, D &E) is T0, T1, T2 & T3 respectively, 45 days after inoculations.

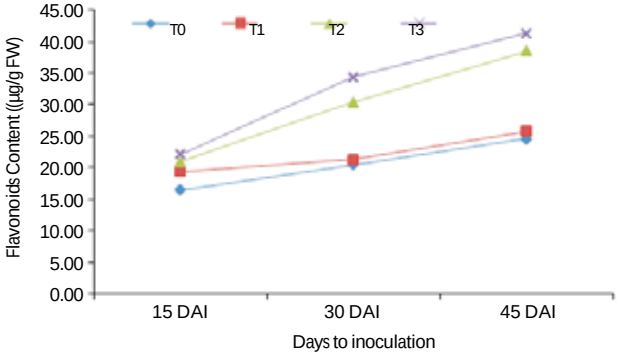

Figure 5: Effect of different concentration of PEG on Flavonoids content (µg/g FW) in the callus of *Rauvolfia serpentina* (variety Cim sheel) after 15, 30 and 45DAI

of *R. serpentina,* they operate defense system in the cell and play important roles when cells are exposed to various kind of oxidative stress in plants (Sharma *et al.,* 2012).

Superoxide dismutase

SOD activity was enhanced gradually with increasing concentration of PEG (1-3%) in the media and time intervals i e., 15, 30 and 45 days after inoculation. The maximum SOD activity (2.19, 2.37& 3.27unit/mg protein) was recorded in the callus developed on 3% PEG while minimum (1.51, 1.73 and 1.89 unit/mg protein) in PEG free media after 15, 30 and 45 days of inoculation, respectively.

SOD activity was enhanced 15.38, 21.30 and 44.97 % after 15 days of inoculation, 19.49, 43.09 and 57.76 % after 30 days of inoculation and 30.07, 57.32 and 73.24 % after 45 days of inoculation in 1,2 and3% PEG in the media, respectively when compared with corresponding controls. The activity was also increased with age of callus as 15, 30 and 45 days after inoculation even in controlled callus tissue. In controlled (PEG free) condition it was increased by14.24 % after 30 days of inoculation and 24.47 % after 45 days of inoculation when compared with callus of 15days. Similarly 26.67% and 95.47 % enhancement was found in 1 % PEG treated callus, 102.28 and 169.07 % in 2 % PEG treated callus after 30 and 45 days of inoculation respectively when compared with the SOD activity of 15 days callus in all the cases which indicates that age of tissue is also a factor for variation in antioxidant activity (Figure 2). Superoxide dismutase are the major enzymatic scavenger and it provide defense at first under stressful environment (Jaiswal *et al.,* 2014). Therefore, it showed clearly that PEG induced drought stress enhancing the level of superoxide dismutase in callus. The several studies also revealed the enhanced stress tolerance by the production of SOD and other antioxidants. Superoxide dismutase is metalloprotein catalyzing the dismutation of the superoxide free radical ($O_2^{\bullet-}$) to molecular oxygen and H_2O_2, protects plants from oxidative stress and considered as first line defense against ROS (Alscher *et al.,* 1997). A similar trend of enhancing antioxidants was found in another medicinal plant *Withania somnifera* (known for great antioxidants potential.). In this case antioxidants metabolism of *Withania somnifera* studied under different regimes such as 10, 15 and 20 days interval drought (DID) water stress in a pot experiments. The level of superoxide dismutase and other antioxidants was enhancing under 10, 15 and 20 days intervals irrigation in pots (Jaleel, 2009).

Catalase

The maximum catalase (18.67, 21.33 and 22.67unit/mg

protein) activity was recorded in callus proliferated on 3% PEG containing media, while minimum (9.33, 12 and 13.33 unit/mg protein) in control after 15, 30 and 45 days of inoculation respectively. It was increased by 14.29, 57.14 & 100 % after 15 days of inoculation, by 11.11, 55.56 and 77.78 % after 30 days of inoculation and 30, 60 and 70 % after 45 days of inoculation in 1-3% [1] PEG in the media respectively when compared to control. Catalase activity was also seems to be affected by age of callus as 15, 30 & 45 days after inoculation even in without PEG treated callus considering as control, it was increased by 28.57 % after 30 days & 42.86 % after 45 days of inoculation when compared with 15 days callus. Activity of catalase was also increased in all the treatment with respect to age of callus. 25 & 62.50 % enhancement was found in 1 % PEG treated callus, 27.27 & 45.45 % in 2 % PEG treated callus while 14.29 and 21.43 % in 3 % PEG treated callus of 30 and 45 days respectively when compared with 15 days old callus in all the case which strengthen the statement that age factor of tissue is also a cause of variation in antioxidant activity (Figure 3). In the same laboratory calluses of four genotypes of *W. sominefera* namely J- 20, Nimitly, Chetak and Pratap were also tested for antioxidant potential under drought. All the genotypes showed increased catalase activity with increasing concentration of PEG and age of callus as compare to the control (Sharma *et al.,* 2016).

The catalase is ubiquitous tetrameric heme-containing enzyme, breaks the H_2O_2 into H_2O and O_2, however the intensity, type and duration of stress may vary the activity of catalase Actually hydrogen peroxide is a toxic waste material of metabolism, involved in many stress situations and generated rapidly in various organelles of the cell. This compound is converted immediately into less dangerous chemical products by the enzyme catalase and helps in the protection of the cells from highly active molecules. (Shankhdhar and Shankhdhar, 2014).

Ascorbate

During the course of this study, the level of ascorbate in callus tissues was also evaluated which was found to be increased with increasing drought in the media and time duration also. The highest content of ascorbate (6.17 mg g^{-1} sample) was observed in 3% PEG after 45 days of inoculation followed by 5.77 and 4.20 mg g^{-1} sample after 30 and 15 days of inoculation respectively. In normal condition the level of ascorbate was recorded minimum (2.47 mg g^{-1} sample) after 15 days of inoculation followed by 2.47 and 3.09 mg g^{-1} sample after 30 and 45 days of inoculation, respectively. Ascorbate in tissues was increased by 25, 37.50 and 70 % after 15 days of inoculation, 75, 100, 133.75 % after 30 days of inoculation and 40,70 and 100 % after 45 days of inoculation in 10, 20 and 30 g l^{-1} PEG in MS medium respectively when compared with control. Like others, ascorbate content was also varying with age of callus. No change in ascorbate content in the callus tissues was found after 30 days of inoculation while increased by 25% after 45 days of inoculation as compared to 15 days. However it was increased by 40% in 10g l^{-1} PEG after 30 and 45 days of inoculation. Similarly 45.45 and 54.55 % increase in 2% PEG treated callus and 37.50 and 47.06 % in 3% PEG treated callus of 30 and 45 days respectively as compared to 15 days. Thus results indicate that age of tissue is also an important factor to vary the antioxidant compounds (Figure 4). In a pot experiment two sets (first set was watered daily as control and second set withhold water imposed to water stress of 10, 15 and 20 days interval) were used to study the ascorbate content in two varieties *viz*. alba and rosea of *Catharanthus roseus.* The level of ascorbate enhanced positively in both the variety under drought stress condition as compare with control (Jaleel *et al.,* 2008).

Ascorbate is low molecular weight, most abundantly present compound in the plant system which acts as antioxidant during the course of stressful environment. It prevents plant systems by oxidative damage which is generally created by enhanced ROS. As it is a very good donor of electrons that's why able to protect the membrane by scavenging $^1O_2$ and $OH^{\cdot}$ and regenerating the tocopherol from tocopheroxyl radicals (Noctor and Foyer, 1998; Sharma *et al.,* 2012). Ascorbate works alone and together with glutathione and several other enzymatic antioxidants to detoxify the $O_2^{\cdot -}$ and $OH^{\cdot -}$ (Asada, 1999).

Flavonoids

Maximum flavonoids content (41.33µg g^{-1} FW) in callus tissues was recorded after 45 days of inoculation followed by 30 and 15 days of inoculation (34.37 and 22.07 µg g^{-1} FW), respectively in 3% PEG while lowest was recorded (16.44, 20.44 and 24.59 µg g^{-1} FW) under controlled condition after 15, 30 and 45 days of inoculation respectively. It was increased 18.02, 27.03 and 34.23 % after 15 days of inoculation, 4.35, 48.55 and 68.12 % after 30 days of inoculation while 4.82, 56.63 and 68.07 % after 45 days of inoculation in 10, 20 and 3% PEG in the media, respectively when compared with control. Varying levels of flavonoid with age of callus in both control and treated callus strengthen the statement that antioxidants are enhanced with age of tissue. 9.92 and 20.83% enhancement was found in 1% PEG treated callus after 30 and 45 days of inoculation when they were compared with callus of 15 days. No changes were found in 2 and 3 % PEG treated callus (Figure 5). Enhanced flavonoids in old age callus and stressed might be due to scavenging property of flavonoid as it scavenges ROS by transferring electrons to hydroxyl, peroxyl and peroxy nitrite radicals. Basically, scavenging property and metal chelating ability of flavonoids are depend on configuration, substitution and total number of hydroxyl groups presents in rings in flavonoids. The B ring hydroxyl configuration consider as a most significant determinant of scavenging of reacting oxygen species (ROS) (Kelly *et al.,* 2002; Pandey *et al.,* 2012).

In a field experiment 5 water treatments (95 to100%, 80 to 85%, 65 to 70%, 50 to 55% and 35 to 40%) were given to *Glechoma longituba* (Nakai), perennial medicinal plant and the field capacity (FC) was used to evaluate the influence of water deficiency on total flavonoid and other physiological growth parameter. Highest flavonoid content was recorded in 80 to 85% water treatment as compare to control (Zhang *et al.,* 2012). The same enhancing trend of flavonoids was also revealed in the cell suspension culture of *Glycyrrhiza inflata* Batal. The cells were cultured in liquid MS medium along with plant growth regulators having 5-30% poly ethylene glycol (PEG) at 25 ± 1ºC. Cells in the liquid medium without PEG consider as control. Resulting drought stress enhancing the

biomass production 27.1 g l^{-1} and flavonoid 151.5 mg l^{-1} which was 2 and 1.5 fold of the control respectively (Yang *et a*l., 2007).

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Research Article

A SCITECHNOL JOURNAL

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

Sharma Deepika[1], Kumar Narendra[1], Misra Ritika[2], Shankhdhar SC[1] and Shankhdhar Deepti[1]*

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.

***Corresponding author:** Shankhdhar Deepti, Department of Plant Physiology, GBPUA&T, Pantnagar, Uttarakhand, India, E-mail: dshankhdhar@rediffmail.com

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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

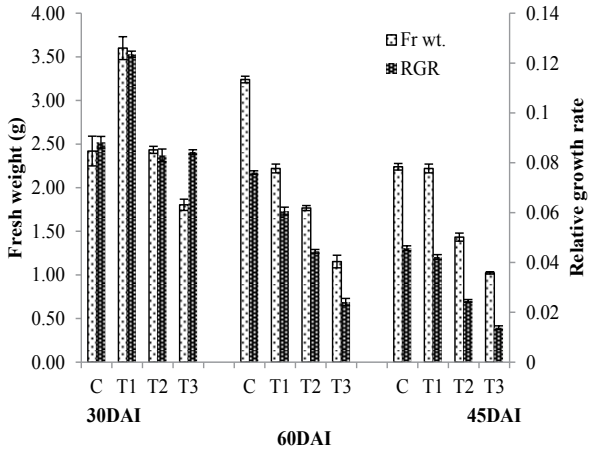

Figure 1: Effect of PEG induced drought on root Fresh weight (g) and Relative growth rate (g/g/days) after 30, 45 and 60 Days of stress treatments in *Withania somnifera*. T1 (1%PEG), T2 (2% PEG), T3 (3% PEG) and C (control).

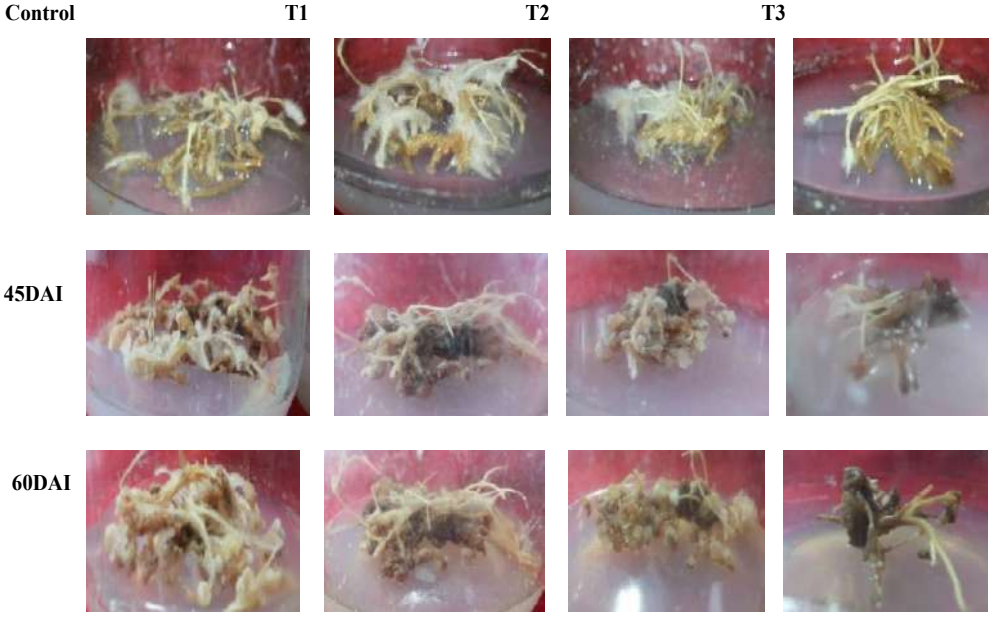

Plate 1: Effect of PEG induced drought on *in vitro* root morphology of *W. somnifera* under three time duration i e. 30, 45 and 60 days of stress. T1 (1%PEG), T2 (2% PEG), T3 (3% PEG) and C (control).

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

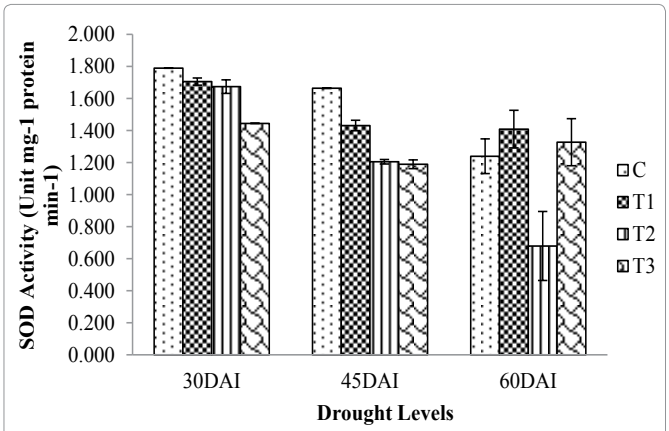

Figure 2 (A): Effect of PEG induced drought on activity of SOD in In vitro roots of W. somnifera. T1 (1%PEG), T2 (2% PEG), T3 (3% PEG) and C (control).

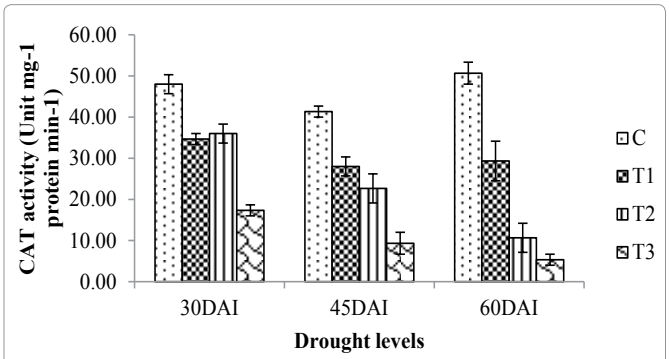

Figure 2 (B): Effect of PEG induced drought on activity of CAT in In vitro roots of W. somnifera. T1 (1%PEG), T2 (2% PEG), T3 (3% PEG) and C (control).

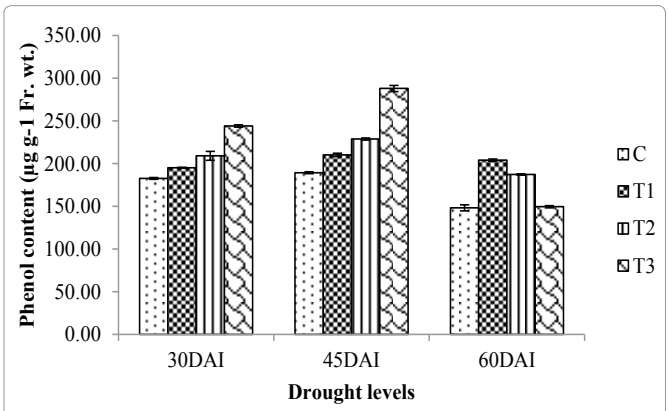

Figure 3 (A): Effect of PEG induced drought on total phenol content of roots of *W. Somnifera*. T_1 (1%PEG), T_2 (2% PEG), T_3 (3% PEG) and C (control).

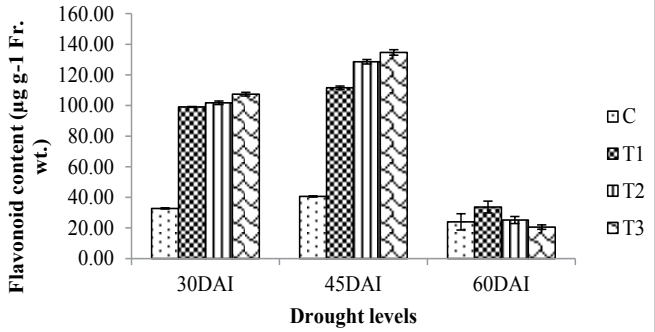

Figure 3 (B): Effect of PEG induced drought on total flavonoid content of roots of *W. Somnifera*. T_1 (1%PEG), T_2 (2% PEG), T_3 (3% PEG) and C (control).

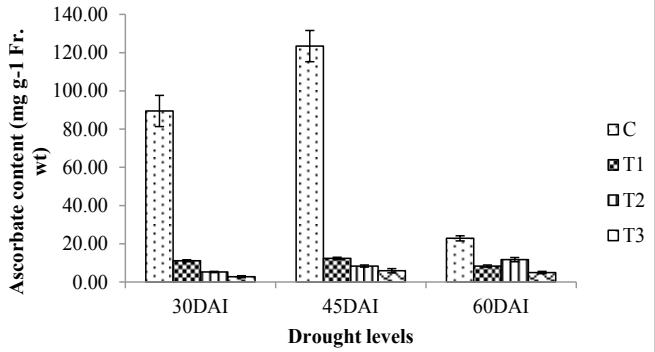

Figure 3 (C): Effect of PEG induced drought on ascorbate content of roots of *W. Somnifera*. T1 (1%PEG), T2 (2% PEG), T3 (3% PEG) and C (control).

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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Author Affiliations

Top

[1]*Department of Plant Physiology, GBPUA&T, Pantnagar, Uttarakhand, India*

[2]*M.J.P. Rohilkhand University, Bareilly, U.P, India*