Research Article

Oxidative Stress in Leaves, Stems and Roots of *Withania somnifera* on Copper Exposure

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ABSTRACT

The response to Cu^{2+} exposure (10, 20, 50, 100 and 200 mM as $CuSO_4.5H_2O$) on fifteen days post-emergent *Withania somnifera* L. Dunal growing under natural environment in pots *vis a vis* Cu accumulation and antioxidative activity was studied on leaves, stems and roots. The duration of the experiment was for 2 months (Mid August-October, 2013) and interval between successive exposures was 7 days. As compared to control the following results were obtained. Copper (Cu^{2+}) content in plant tissues increased markedly with increasing soil Cu^{2+} concentrations, and was highest in roots. Translocation factor (TF) and Bioconcentration Factor (BCF) values calculated were < 1, indicating a limited ability to accumulate and translocate Cu^{2+} by the plant. Hydrogen peroxide formation was highest in roots and displayed a progressive increase in response to Cu^{2+} exposure. With increasing Cu^{2+} proline (non-enzymatic antioxidant) content was highest in leaves however, proline accumulation followed the order: leaves (220.4 %) < stems (523.87%) < roots (534.47%). Activities of both superoxide dismutase (SOD) and catalase (CAT) increment were observed in order: leaves > stems > roots and reached a peak at 50 mM of Cu^{2+} exposure, whereas 100 mM and 200 mM Cu^{2+} in the soil of led to inhibition of activity. Stimulation of Ascorbate Peroxidase (APX) activity observed was in the order: roots > stems > leaves. This may possibly be due to rising H_2O_2 levels in the respective plant parts. The present study infers that *W.somnifera* as used as ayurvedic herbal treatment plant, has limited tolerance to Cu^{2+} toxicity and that this tolerance is dependent largely on the action of APX, rapid translocation of proline from leaves to the roots.

Keywords: Copper, Phytoextraction, SOD, CAT, APX, *Withania somnifera*.

INTRODUCTION

Copper, an essential micronutrient and a cofactor for certain enzymes *viz.* superoxide dismutase, catechol oxidase, L-ascorbate oxidase, polyphenol oxidase *etc.*, has an important role in maintaining metabolism of higher plants. Both the absence and excess of copper inhibits the plant growth and impairs important cellular processes. However, Cu^{2+} at high levels becomes strongly phyto-toxic to cells. It interferes with numerous biochemical and physiological processes including photosynthesis, pigment synthesis, nitrogen and protein metabolism, membrane integrity, enzyme activity[1] and eventually leading to increased generation of reactive oxygen species (ROS). Therefore, Cu induced production of ROS exceeds the plant's capacity in maintaining redox balance, and developed oxidative stress[2].

Commonly recognized ROS are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) originating from one, two or three electron transfers to dioxygen (O_2). Superoxide is the first reduction product of ground state oxygen, capable of both oxidation and reduction. In aqueous solution, O_2^- is moderately reactive, but this O_2^- is dismutated by SOD to a reactive H_2O_2[3].

Three types of SOD have been reported in plant cells - CuZn-SOD, Mn-SOD, and Fe SODs. In plants, Cu-Zn SOD is the most abundant of the three metallo-protein enzymes. Dismutation of superoxide anion by SOD in chloroplasts where it is largely produced is 10,000 fold faster rates than spontaneous dismutation[2]. Hydrogen peroxide is an electrically neutral ROS, but can pass through cellular membranes and reach cell compartments far from the site of its formation. Hydrogen peroxide produced reacts with Fe^{+2} and Cu^+ to produce highly toxic uncharged OH^- which can penetrate and damage biological membranes[4]. The main threat imposed by both superoxide anion and H_2O_2 lies in their ability to generate highly reactive hydroxyl radicals. Hydrogen peroxide however is rapidly converted to water by peroxidases and catalases, the activity of all these enzymes must be carefully calibrated.

A common response of plants to ROS, generated due to Cu^{2+} toxicity is activation of the antioxidant defense system wherein, variation in levels of ROS scavenging enzyme activity (*viz.* superoxide dismutase, catalase and ascorbate peroxidase) and non-enzymatic antioxidants (*viz.* proline, ascorbate, phenolic compounds, alkaloids) are observed.

The enhancement of antioxidant enzymes activity in Cu^{2+} exposed plants is normally associated with increased stress tolerance[5], suggesting that the activation of the antioxidant machinery is essential to mitigate the adverse effects resulting from metal-induced oxidative damage[2].

Furthermore, several nitrogenous metabolites, such as amino acids, particularly proline, is known to accumulate in plant tissues under Cu stress possibly to maintain redox homeostasis[6].

Nowadays, other than its role as an osmolyte, proline is considered a potent antioxidant that plants require to counteract the inhibitory effects of ROS[2]. This balance

between ROS generation and eradication determines the survival of the plant.

For centuries *Withania somnifera* Dunal, known as 'ashwagandha', is an important herb in the ayurvedic and indigenous medical systems of India[7]. Amongst other plants known for medicinal value, *Withania somnifera* (Family: Solanaceae) is important for its therapeutic potential. Ashwagandha is also used as an "adaptogen" helping the body cope with daily stress and as a general tonic. In view of its varied therapeutic potential, it is the subject of considerable modern scientific attention[8]. There is insufficient information on the status of antioxidants in plant organs exposed to copper. The present study was therefore, undertaken to determine the effects of copper induced phyto-toxicity on hydrogen peroxide production, proline accumulation and ROS scavenging enzymes *viz.* superoxide dismutase (SOD), catalase (CAT) and Ascorbate Peroxidase (APX), in *W.somnifera*.

MATERIALS AND METHODS

The seeds of *Withania somnifera* were obtained from CIMAP (Central Institute of Medicinal and Aromatic Plants), Lucknow, India. The seeds were grown (Mid August-October, 2013) in pots as described by Singh[9]. The plant parts separated washed patted dry and stored at -20 ˚C for further biochemical analysis. Dried root, stems, leaves and soil were used for estimation of Cu^{2+}. Double distilled water was used for preparation of all solutions and analyses.

Cu^{2+} Estimation

Soil

Soil sample was analysed using atomic absorption spectrophotometer (Perkin-Elmer 5000) according to the method of Pandey[10]. Soil samples (6.0 g) were oven-dried at 80 ˚C till constant weight. The dried soil was then ground finely and sieved. To the sieved soil samples (5.0 g) was added 20 ml of extracting solution (0.5N HCl: 0.025N H_2SO_4:1:1) and the mixture agitated for 15 minutes at low speed (200-250 rpm).

The extract was then filtered through Whatman no. 42 filter paper, and the final volume was made up to 50 ml with extracting solution. This solution was used for estimation of Cu^{2+}.

Plant Tissues

Copper content in plant tissues were also analysed according to Pandey[10]. Plant samples (leaves, stems and roots- 1.0 g fresh wt.) were oven-dried at 60 ˚C till constant weight. The dried samples were ground, added 10 ml of concentrated HNO_3 and left overnight. This mixture was heated carefully on a hot plate until the production of red NO_2 fumes ceased.

After cooling the solution added 2-4 ml of 70% $HClO_4$ and evaporated to ≥ 1.0ml over heat. The volume was made up to 50 ml with distilled water. This solution was used for Cu^{2+} estimation in a Perkin-Elmer 5000 atomic absorption spectrophotometer (AAS).

Determination of Hydrogen Peroxide (H_2O_2)

The procedure followed was given by Velikova[11]. For the estimation, 0.2 g fresh wt. acid (TCA) in an ice bath, and centrifuged at 12,000 rpm for 15 min at 4 ˚C. Supernatant aliquot (0.5 ml) was added to 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1.0 ml of 1 M KI, and absorbance was read at 390 nm. An extinction coefficient 0.28 $\mu M^{-1}cm^{-1}$ for H_2O_2 was used for calculations and the amount of H_2O_2 present expressed as µmol/g fresh wt.

Proline Estimation

Free proline accumulation was determined according to Bates[12]. Briefly, 0.5 g fresh wt. of tissue samples (leaves, stems and roots) were homogenized separately in 10 mL of 3% aqueous sulphosalycylic acid and filtered on Whatman no. 42 filter paper. Repeated the extraction and pooled the respective filtrates. To 2 ml of filtrate, added 2 ml of glacial acetic acid, 2 ml acid ninhydrin reagent and mixed. The mixture was heated at 100 ˚C for 1 hr on a water bath and the reaction terminated by placing the tubes on ice bath for 20 minutes. Added 4.0 ml of toluene and mixed vigorously for 20-30 sec. Aspirated the chromophore (toluene) layer and after reaching room temperature the absorbance read at 520 nm against a reagent blank. Proline concentration was determined from a calibration curve plotted and expressed as µmoles of proline/g fresh wt.

Enzyme Extraction and Assay

Enzyme extraction (SOD, CAT, and APX) was done following the modified method of Liu[13]. Tissue samples (leaves, stems and roots-0.5 g fresh wt.) of plants were ground with pinch of purified sand and homogenized in 4 ml of buffer containing 50 mM NaH_2PO_4 buffer (pH 7.0), 1mM EDTA-Na_2 and 2 % (*w/v*) polyvinylpolypyrrolidone (PVPP) at 4 ˚C .The homogenate centrifuged at 15,000 rpm for 30 min at 4 ˚C, the resulting supernatant was used as enzyme source. The amount of protein was determined according to the method of Lowry[14] using bovine serum albumin as standard.

Superoxide Dismutase (EC 1.15.1.1, SOD)

Superoxide dismutase (SOD) activity was measured using double beam UV-Vis spectrophotometer (Systronic) as described by Beyer and Fridovich[15] with slight modification. The assay mixture (3.0 mL) contained 25 mM potassium phosphate buffer (pH 7.0), 13 mM L-methionine, 75 µM nitrobluetetrazolium (NBT), 1 µM EDTA-Na_2, 2 µM riboflavin, distilled water and 50 µL enzyme extract. A tube without enzyme extract was taken as control. The reaction was initiated by addition of riboflavin and placing the tubes below a 30W fluorescent lamp light source for 15 min the temperature was at 25 ˚C. Reaction was stopped by switching off the light and the tubes covered with black cloth. A non-irradiated complete reaction mixture served as blank. Absorbance

was recorded at 560 nm and one unit of enzyme activity was defined as the amount of enzyme required to inhibit the photo-reduction of NBT by 50%.

Catalase (EC 1.11.1.6, CAT)

The activity of Catalase (CAT) was assayed according to the method of Chance and Maehly[16] with some modifications. A reaction mixture (3.0 mL) contained 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2, and 50 µL enzyme extract.

This mixture was incubated at 25 °C for 5 min. Catalase activity was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm for 5 min. An extinction coefficient 39.4 $mM^{-1}cm^{-1}$ for H_2O_2 was used for calculations and the enzyme activity expressed in units mg^{-1}protein.

Ascorbate Peroxidase (EC 1.11.1.11, APX)

Ascorbate Peroxidase **(**APX) activity was measured using the procedure of Nakano and Asada[17]. The reaction mixture (3.0 mL) contained 40 mM potassium-phosphate buffer (pH 7.0), 0.025 mM ascorbic acid, 15 µM H_2O_2 and 50 µl of enzyme extract. Hydrogen peroxide dependent oxidation of ascorbate was followed at 290 nm against the blank, for 5 min. An extinction coefficient 2.8 mM^{-1} cm^{-1} for ascorbate was used for calculations and the enzyme activity expressed in units mg^{-1} protein.

Statistical Analysis

All values reported were MEAN± STANDARD DEVIATION of three independent experiments.

RESULTS AND DISCUSSION

Copper Content

Copper (Cu^{2+}) toxicity thresholds vary greatly between plant species and affect tissues differently depending on metabolic requirements. Copper content in roots, stems and leaves of *W.somnifera* is given in Table 1.

Table 1: Estimated content of copper (mg/kg dry wt.) in leaves, stems, roots and soil media used to grow *W.somnifera*.

Treatments Cu^{2+} (mM)	Leaves	Stems	Roots	Soil
Control	12.52 ± 0.23	14.65 ± 0.17	27.30 ± 0.17	37.30 ± 0.41
10	30.06 ± 0.49	34.97 ± 0.18	66.30 ± 0.14	92.72 ± 0.28
20	42.40 ± 0.48	52.86 ± 0.28	102.72 ± 0.19	147.57 ± 0.29
50	62.77 ± 0.44	71.54 ± 0.38	160.64 ± 0.34	234.45 ± 0.34
100	79.25 ± 0.47	92.47 ± 0.26	218.60 ± 0.32	327.11 ± 0.48
200	98.20 ± 0.69	125.78 ± 0.98	301.97 ± 0.19	456.90 ± 0.29

*All values reported are MEAN± SD of three independent experiments.

The Cu^{2+} content varied from 30.06 ± 0.49 to 98.20 ± 0.69 mg/kg in leaves, 34.97 ± 0.18 to 125.78 ± 0.98 mg/kg in stems, 66.30 ± 0.14 to 301.97 ± 0.19 mg/kg in roots, in Cu^{2+} treated plants (10 mM to 200 mM). Similarly, Cu^{2+} of soil content which remained unavailable to plant tissues over the same concentration range increased from 92.72 ± 0.28 to 456.90 ± 0.29 mg/kg. We found that accumulation of Cu^{2+} in *W.somnifera* plant organs increased markedly with an increasing concentration of Cu^{2+} in soil, in the order: root>stem>leaf. Uptake of Cu as cupric ion (Cu^{2+}) by plants may be retarded as Cu^{2+} is strongly adsorbed in most soils.

Also due to preferential accumulation of Cu^{2+} in the roots there is possibly less translocation to plant aerial parts. It was reported that Cu^{2+} mainly accumulated in roots while a fraction of absorbed Cu^{2+} was transported to aerial parts[18]. Restriction on upward movement of Cu^{2+} from roots into aerial parts can be considered as factor in limited tolerance towards copper.

Other author also observed that copper content increased in four varieties of plants *Triticum aestivum* L, *Abelmoschusesculentus* L, *Basella alba* L, and *Solanum lycopersicum* L significantly with increasing Cu^{2+} concentrations[19].

Bioconcentration Factor (BCF) and Translocation Factor (TF)

A plant's ability to accumulate metals from soils can be estimated using the bioconcentration factor (BCF) and translocation factor (TF), which are defined as the ratio of metal concentration in roots, stems and leaves to that in soil and ability to translocate metals from the roots to the stems and leaves respectively.

By comparing the BCFs and TFs, we can compare the ability of a plant to take up metals from soils and translocate them to the aerial parts[20].

Table 2: Bioconcentration Factor (BCF) in leaves, stems, roots of *W.somnifera.*

Treatments Cu^{2+} (mM)	Leaves/Soil	Stems/Soil	Roots/Soil
Control	0.336 ± 0.0046	0.393 ± 0.0055	0.732 ± 0.011
10	0.324 ± 0.0061	0.377 ± 0.0026	0.715 ± 0.0023
20	0.287 ± 0.0035	0.358 ± 0.0026	0.696 ± 0.0026
50	0.268 ± 0.0025	0.305 ± 0.0020	0.685 ± 0.002
100	0.242 ± 0.001	0.282 ± 0.0012	0.668 ± 0.002
200	0.215 ± 0.0015	0.275 ± 0.0025	0.661 ± 0.001

*All values reported are MEAN± SD of three replicates.

Table 3: Translocation Factor (TF) in leaves and stems from roots of *W.somnifera.*

Treatments Cu^{2+} (mM)	Leaves/Roots	Stems/Roots
Control	0.459 ± 0.011	0.537 ± 0.0038
10	0.453 ± 0.007	0.527 ± 0.0036
20	0.412 ± 0.0042	0.515 ± 0.0025
50	0.391 ± 0.002	0.445 ± 0.0023
100	0.362 ± 0.0025	0.423 ± 0.0012
200	0.325 ± 0.002	0.416 ± 0.0032

*All values reported are MEAN± SD of three replicates.

Both BCF and TF were calculated according to the method of Yoon[20] to assess phytoextraction potential of *W.somnifera*. From Table 2, it can be deduced that in *W.somnifera*, BCF value decreased with increasing Cu^{2+} in soil, with control exhibiting the maximum value with increasing Cu^{2+} concentrations, BCF values were maximum in roots (0.715 ± 0.0023 to 0.661 ± 0.001) followed by stems (0.377 ± 0.0026 to 0.275 ± 0.0025) and leaves (0.324 ± 0.0061 to 0.215 ± 0.0015) respectively. On the other hand TF values given in Table 3 showed that the TF of Cu in stems and leaves from roots respectively decreased with the increasing Cu^{2+} concentrations in soil. Analogous to BCF results, highest values were observed in their respective controls. Translocation factor of Cu^{2+} in stems (0.537 ± 0.0038 to 0.416 ± 0.0032) were found higher than leaves (0.459 ± 0.011 to 0.325 ± 0.002).

The results emphasize that the Cu^{2+} accumulated in the roots of the plant, indicating a low degree of metal translocation into the aerial parts. The rate and extent of translocation of metals in plants depends on the metal and plant species. It was reported in hollyhock (*Althaea rosea* L.) that increasing Cu levels in soil significantly decreased the Cu^{2+} TF compared to the control plants[21]. In other report also found that TF value of Cu^{2+} decreased in leaves and stems of *Polygonum thunbergii* with increasing Cu in the medium[22], suggesting that at low applied external Cu concentrations, Cu^{2+} is rapidly absorbed and translocated. Whereas, at higher applied external concentrations, it is assumed that the uptake of Cu^{2+} is limited, therefore translocation is very low. Since TF values were < 1.0 in both stems and leaves, implying therefore, that roots are primary targets of Cu^{2+} accumulation, and transportation to the stems and leaves are limited.

Plants exhibiting TF and particularly BCF values <1, indicated that plant is not suitable for phytoextraction[23]. *W.somnifera* growing under Cu^{2+} stress of 10 mM to 200mM had limited ability of Cu^{2+} accumulation and translocation and is unsuitable for phytoextraction.

Hydrogen Peroxide Production

As a redox transition element, Cu can catalyze the overproduction of ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^•$), by Haber-Weiss and Fenton reactions[24].

Table 4: Changes in Hydrogen peroxide (H_2O_2) levels (µmol/g fresh wt) in leaves, stems and roots of *W.somnifera* exposed to different concentrations of $CuSO_4.5H_2O$.

Treatments Cu^{2+} (mM)	Leaves	Stems	Roots
Control	3.24 ± 0.104	5.07 ± 0.124	45.89 ± 0.107
10	4.05 ± 0.086	6.09 ± 0.166	46.82 ± 0.250
20	4.64 ± 0.083	6.79 ± 0.083	49.75 ± 0.107
50	5.83 ± 0.126	7.69 ± 0.086	58.32 ± 0.108
100	6.83 ± 0.104	8.41 ± 0.063	67.32 ± 0.179
200	8.45 ± 0.104	8.81 ± 0.063	71.11 ± 0.107

*All values reported are MEAN± SD of three independent experiments.

From Table 4, it is clear that application of Cu^{2+} concentrations (10 mM to 200 mM) resulted in a progressive increase in H_2O_2 production over controls. As compared to their respective controls maximum H_2O_2 production in roots was observed (46.82 ± 0.250 to71.11 ± 0.107 µmol/g fresh wt.) followed by stems (6.09 ± 0.166 to 8.81 ± 0.063 µmol/g fresh wt.) and leaves (4.05 ± 0.086 to 8.45 ± 0.104 µmol/g fresh wt.). This result is in agreement with the significant increase of H_2O_2 content in tomato roots and leaves under Cu^{2+} stress[25]. It is also reported in *B. juncea* and rice seedlings that treatment with Cu resulted in a general increase in H_2O_2 level in root tissues[26].

Proline Accumulation

Copper toxicity in plants leads to proline accumulation, as a general response to some types of abiotic stress. As shown in Table 5, with increasing Cu^{2+} concentrations, proline content increased concomitantly. Maximum proline content was found in leaves followed by roots and stems. However, on comparison with their respective controls accumulation of proline (10 mM to 200mM Cu^{2+} treatment) was in the order: leaves (44.00 to 220.4 %) < stems (16.39 to 523.87%) < roots (210.90 to 534.47%).

Table 5: Proline content (µmol/g fresh wt) in leaves, stems and roots of *Withania somnifera* exposed to different concentrations of $CuSO_4.5H_2O$.

Treatments Cu^{2+} (mM)	Leaves	Stems	Roots
Control	7.34 ± 0.077	0.86 ± 0.026	1.65 ± 0.026
10	10.57 ± 0.093	2.27 ± 0.026	5.13 ± 0.023
20	18.18 ± 0.079	3.63 ± 0.023	6.46 ± 0.023
50	20.51 ± 0.079	4.27 ± 0.027	8.71 ± 0.026
100	21.85 ± 0.065	4.45 ± 0.020	9.99 ± 0.023
200	23.53 ± 0.103	5.38 ± 0.019	10.46 ± 0.023

*All values reported are MEAN± SD of three independent experiments.

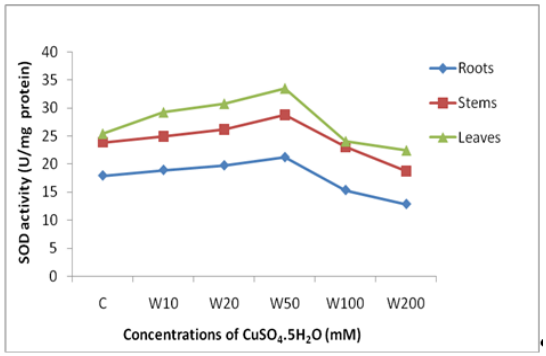
Since roots are in direct and constant contact with metal amended soil therefore, it was expected that proline content in roots would be very high. Our results indicate

that on exposure to Cu^{2+} highest proline content found in leaves; but rapid translocation from leaves caused more accumulation in roots. It has been relatively well established that amino acids (Glu, Asp and Gln) are transported from the shoot to the root *via* the phloem[27]. Long distance transport of proline through phloem vessels has been documented, and some proline transporters have been identified[28]. This is a first report on *W.somnifera* that indicates that proline generated in leaves may be transported from the shoot to the roots upon exposure to Cu^{2+}. Similar results have been observed in white clover (*Trifolium repens* L. cv. Huia) under water-deficit stress[29]. Also, it is observed that the proline requirement in barley (*Hordeum vulgare*) roots is possibly supported by phloem translocation from source leaves during water stress, and that HvProT, a proline transporter, facilitates proline uptake in the root cap or cortical cells of the apical region[30].

Proline accumulation observed may cause resistance to stress triggering increase in plant functions *viz.* osmoregulation, the protection of enzymes against denaturation, and the stabilization of protein synthesis. In addition to direct role, indirect roles of proline in scavenging ROS by enhancing plant antioxidant defense systems have also been reported.

Superoxide Dismutase Activity

The ability of plants to overcome heavy metal stress relies on the induction of antioxidant enzymes[31]. The effects of Cu^{2+} on the activity of antioxidant enzymes as well as the involvement of these enzymes in the defense of plant tissues against Cu-induced damage remain uncertain and vary from plant species, tissues analyzed, concentration, and duration of metal exposure[5]. In this study, three enzymes (SOD, CAT and APX) involved in the cellular antioxidant system were investigated. As superoxide dismutase (SOD) act as the first line of defense against ROS, catalyzing the disproportionation of O_2^- radicals into H_2O_2 and molecular oxygen which is then degraded to H2O by the action of catalase (CAT) and ascorbate peroxidase (APX).



*All values reported are mean of three independent experiments.

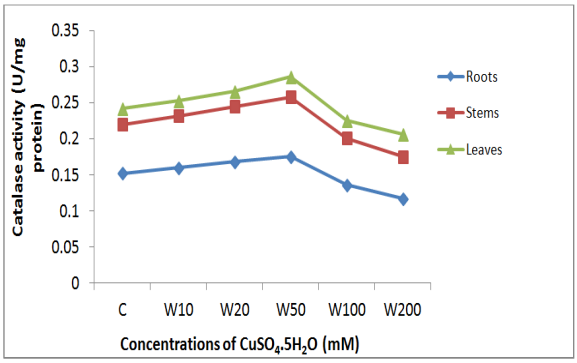
Figure 1: The activity of Superoxide dismutase (SOD) in leaves, stems and roots of *W.somnifera* under different concentrations of $CuSO_4.5H_2O$.

As shown in Figure 1, from 10 mM to 50 mM Cu^{+2} concentrations, SOD activity gradually increased by 15.02 to 31.83% in leaves, 4.46 to 20.56% in stems and 5.49 to 18.16% in roots as compared to their respective control. Exposure to low Cu^{2+} concentrations resulted in increment of SOD activity *viz.* leaves > stems > roots, and reached a peak at 50 mM Cu^{2+} treatment. However, higher treatments of Cu^{2+} led to inhibition of SOD activity from peak activity. At 100mM and 200Mm Cu^{2+} concentrations, SOD activity gets decreased by 5.27 to 11.68 % in leaves, 3.52 to 21.50 % in stems and 14.50 to 28.39% in root as compared to their respective controls. The results of this study regarding increased SOD activity in response to Cu^{2+} stress are in agreement with those obtained in oat[31] and *Prunus cerasifera* plantlets[32]. Studies on maize (*Zea mays* L.) also suggested that exposure to higher Cu^{2+} concentrations results in a significantly decreased SOD activity in stems and roots[33].

At lower Cu^{2+} concentrations, SOD activity may possibly increase due to direct effect of copper ions by participating in the dismutation reaction; and another reason may be due to an indirect effect mediated *via* increased level of O_2^- through partial reduction of O_2[34]. Copper could pass electrons to this molecular oxygen to generate superoxide through redox cycling *via* electron transport systems in mitochondria and chloroplasts, leading to enhanced SOD activity[35]. The inhibition of SOD activity at higher Cu^{2+} concentrations maybe due to two reasons (i) due to high alleviation of oxidative stress[26] causes oxidative damage to SOD and (ii) copper, induced altered SOD gene expression leading to depleted SOD production.

Catalase Activity

Catalase and APX both enzymes detoxify H_2O_2 to water and oxygen. According to Mittler[36], the different affinities of APX and CAT for H_2O_2 suggest that they belong to two different classes of H_2O_2-scavenging enzymes. Ascorbate peroxidase has a high affinity for H_2O_2 and is able to detoxify low concentrations, whereas catalase has a higher Vmax but a lower affinity for H_2O_2.



*All values reported are mean of three independent experiments.

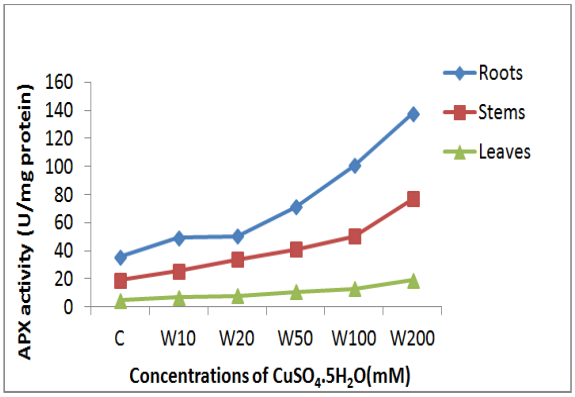
Figure 2: The activity of Catalase (CAT) in leaves, stems and roots of *W.somnifera* under different concentrations of $CuSO_4.5H_2O$. As shown in Figure 2 that in *W.somnifera*, catalase activity peaked at 50 mM Cu^{2+} treatment from

4.55 to 18.18 % in leaves, 5.46 to 17.27% in stems and 5.26 to 15.13 % in roots as compared to their respective controls. At higher Cu^{2+} treatments (100mM and 200Mm) inhibition of activity of CAT was 7.03 to 14.88 % in leaves, 8.64 to 20.46 % in stems and 10.53 to 23.03% in root; over respective controls. It was also reported in *Medicago sativa* that catalase activity increased at lower Cu concentrations, and then decreased with increasing Cu concentrations[37].

Increase in CAT activity at lower Cu^{2+} concentrations may be explained by an increase in its substrate, *i.e.* level of H_2O_2, and to detoxify this increased H_2O_2 level is an adaptive antioxidant mechanism of the plants[38]. Decline observed at higher concentration of Cu^{2+}, may be attributed to either to inactivation of enzyme by Cu-induced ROS generation[39], decrease in synthesis of enzyme, or alterations in the assembly of its subunits. Another reason for the decrease in CAT activity could be Cu^{2+} replacing Fe^{2+} in the enzyme[26]. Similar results were reported with CAT and SOD activities both in leaf and root of *in vitro* grown *Withania somnifera* L[39]. However, in contrast to their report we found CAT and SOD activities are higher in leaves than roots and stems under natural environment.

Ascorbate Peroxidase Activity

Ascorbate peroxidase reduces H_2O_2 to water; with the concomitant generation of monodehydroascorbate. Hydrogen peroxide generation is a systemic signal for the induction of APX[40].



*All values reported are mean of three independent experiments

Figure 3: The activity of Ascorbate Peroxidase (APX) in leaves, stems and roots of *Withania somnifera* under different concentrations of $CuSO_4.5H_2O$.

From Figure 3, it is clear that under this Cu^{2+} dose-response study, APX activity progressively increased in the order: roots > stems > leaves. Ascorbate Peroxidase activity was progressively and highly activated by Cu^{2+} exposure, exhibited a drastic increase over their respective controls with maximum activity being observed in 200 mM of Cu^{2+} treatment *viz.* 48.42% to 300.74% in leaves, 34.54% to 293.17% in stem and 38.73% to 287.31% in roots. The results are similar to reports of APX activity in jack bean[41] and rice plants[42] under copper stress.

As compared to the activities of SOD and CAT, the activity of APX increased with increasing levels of Cu^{2+} exposure. This suggests that APX mediated detoxicification of H_2O_2 is predominant *vis a vis* CAT in *W.somnifera* plant under Cu^{2+} stress.

CONCLUSION

The results indicate Cu accumulation in roots of *W.somnifera*. However it's translocation to stems and leaves is retarded. The BCFs and TFs values observed suggest that the plant is unsuitable for phytoextraction. Additionally, proline accumulation also appears to contribute to improved Cu tolerance with altered plant metabolism due to oxidative stress.SOD and CAT activities were inhibited at higher Cu^{2+} concentrations, though APX activity is highly stimulated. The present study infers that *W.somnifera* plants have limited tolerance to Cu^{2+} toxicity and that this tolerance is dependent largely on the action of APX, rapid translocation of proline to the roots and immobilization of excess Cu^{2+} in roots and its exclusion from the shoots. Proline content higher in leaves is possibly translocated to roots to combat Cu^{2+} related stress. High activity of APX in roots, indicate a co-relation between non-enzymatic (proline) and enzymatic antioxidant activity.

However, in addition to this study, further research on other enzymatic and non-enzymatic parameters in the natural systems on exposure to copper are needed, to understand better the tolerance mechanism along with biochemical detoxification strategies to improve medicinal value of *W.somnifera*.

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