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Effect of Salinity Stress on Germination, Tolerance and Antioxidant Response in Arabidopsis Thaliana Overexpressing Cu/Zn-SOD

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Abstract: **Salinity negatively affects plant growth and causes significant crop yield losses world-wide. Susceptibility or tolerance of plants to high salinity is a coordinated action of multiple stress responsive genes, which also interacts with other components of stress signal transduction pathways. In plants, Cu/Zn superoxide dismutase (Cu/Zn-SOD), ascorbate peroxidase (APX), and catalase (CAT) are important scavengers of reactive oxygen species (ROS) to protect the cell from damage. In the present study, we isolated Cu/Zn-SOD from high altitude plant of western Himalaya namely, *Potentilla atrosanguinea* and overexpressed it under CaMV 35S promoter. The aim of the present study was to unravel whether the overexpression of antioxidant gene has any effect on the germination and tolerance levels in transgenic under salinity stress. Transgenic Arabidopsis overexpressing Cu/Zn-SOD showed tolerance to salt stress and improved germination rates that could have been due to the higher expression levels of the genes encoding enzymes of the antioxidant pathways. We propose that Cu/Zn-SOD overexpression elevates transcript levels of antioxidant related genes, leading to enhanced antioxidant activity. The resultant increase of antioxidant activity improves abiotic stress tolerance.**

Keywords: **Arabidopsis thaliana, Salinity stress, Cu/Zn-SOD, Antioxidant, Germination.**

1. INTRODUCTION

Agriculture is severely affected by the presence of salinity in the soil all over the world [1]. Salt stress influences many physiological, biochemicals, cellular, and molecular processes of a plant [2]. To limit the effect of salt stress, plants have developed defence mechanisms [3; 4]. Enzymes of antioxidant pathways play a key role and protect the plant cells from oxidative damage by scavenging of free radicals [5]. Expression of various genes encoding enzymes of antioxidant pathways is enhanced during salt stress conditions, and transgenic plants overexpressing many of the genes of these pathways are tolerant to salt stress [6; 7; 8; 9; 10; 11; 12].

These reactive oxygen species (ROS) scavenging antioxidative mechanisms include specific antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase, catalase (CAT), and some other low-molecular-weight antioxidants [13; 14; 15]. Some studies have demonstrated that the antioxidant enzymes comprise several isoenzymes located in different cellular compartments of higher plants, such as the cytosol, chloroplasts, microbodies, and mitochondria, indicating their important roles in controlling cellular ROS levels in multiple stress responses [16]. Two

key ROS antioxidant enzymes in the chloroplast are SOD and APX. SOD first catalyzes the dismutation of two O_2^- into O_2 and H_2O_2, and then APX uses ascorbate as an electron donor to reduce H_2O_2 to water [14; 17].

Plants overexpressing these scavenging enzymes had been engineered with the goal of enhancing protection against stresses. In most cases, these transgenic plants exhibited increased tolerance to various stresses, such as cold, extreme temperature, salinity and other stresses [18; 19; 20; 21; 22; 23; 24; 25]. Thus, previous attempts to produce stress-tolerant plants have mainly focused on the manipulating a single scavenging enzyme or APX in combination with other antioxidants. In this study, we investigate the effects Cu/Zn-SOD overexpression on stress tolerance and germination rates of transgenic. Cu/Zn-SOD (from high altitude plants *Potentilla atrosanguinea* (which grows at daytime air temperatures of 3–10°C in Lahaul and Spiti districts of Himachal Pradesh) was over-expressed under control of the CaMV35S promoter. The transgenic plants expressing Cu/Zn-SOD exhibited higher tolerance to salinity stresses than wild type (WT) plants. These results indicated that Cu/Zn-SOD can scavenge H_2O_2 which may help Arabidopsis withstanding stresses, information that could guide the development of transgenic plants for increased stress tolerance

2. MATERIALS AND METHODS

Development of Transgenic lines and PCR confirmation:

Plasmid construction and transgenic plant development Previously, a full length complementary DNA (cDNA) of Potentilla atrosanguinea copper-zinc superoxide dismutase (Cu/Zn-SOD), which retains catalytic activity in the presence of NaCl [26], was overexpressed in Arabidopsis thaliana as described by Gill et al. [11]. Briefly, coding nucleotide sequences was amplified using the gene specific primers with incorporated NcoI and BglII restriction sites at the tails. PCR products were cloned into a cloning vector pGEMT easy (Promega) and then subcloned into binary plant vector pCAMBIA1302 under the cauliflower mosaic virus 35S promoter. The prepared plasmid construct was mobilized into Agrobacterium tumefaciens strain GV3101 and used for plant transformation. Arabidopsis plants (5weeks old) were infected with the A. tumefaciens via vacuum infiltration method [27] and grown in the greenhouse. The collected seeds were screened on Murashige and Skoog [28] medium supplemented with 20 µg ml−1 hygromycin. Homozygous (T3 generation) transgenic Cu/Zn-SOD lines (S26 and S15) were screened for integration of genes in the host genome, using gene specific primers with PCR conditions as mentioned in Table 1.

RT-PCR Expression Analysis:

Total RNAwas isolated from transgenic and the wild-type Arabidopsis plants using Total RNA Extraction Kit (Real Genomics). One microgram of total RNA was used for oligo (dT) primed first-strand cDNA synthesis in 20-µl reaction using Superscript III reverse transcriptase (Invitrogen). This cDNAwas used in 27-cycle PCR using gene specific primers for Cu/Zn-SOD gene. Constitutively expressed 26S rRNA gene was amplified simultaneously in 27 cycles to ensure equal amounts of template cDNA used.

SOD enzyme activity assay:

Total SOD activity was estimated as intensity of nitro blue tetrazolium (NBT) reduction using spectrophotometer as described earlier [29]. Briefly, leaf samples (100mg) were homogenized in a precooled mortar in homogenizing buffer containing 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.5 % (v/v) Triton-X100, and 10 % (w/v) PVPP in 50 mM phosphate buffer pH 7.8. The homogenate was transferred to 1.5-ml microfuge tube and centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatant was collected, and total SOD activity was estimated. The total SOD activity was measured by adding 5-µl enzyme extract to a reaction mixture (200 µl) containing 1.5 µm riboflavin, 50 µm NBT, 10 mM DL-methionine, and 0.025 % (v/v) Triton-X100 in 50 mM phosphate buffer. One unit of enzyme activity was defined as the amount of enzyme required for 50 % inhibition of NBT reduction per min at 25 °C. Specific activity of SOD was calculated accordingly. Protein content was estimated according to the dye-binding method of Bradford using BSA as standard.

Evaluation of salt stress tolerance:

Seeds of transgenic lines (T3) overexpressing Cu/Zn-SOD (S26 and S15) and WT were grown in MS medium for 10 days and transplanted thereafter to the mixture of vermiculite/peat moss/perlite (1:1:1) in the greenhouse under a 16-h light and 8-h dark cycle at 20±1 °C. For stress treatment, 21-day-old seedlings of WT and transgenics were supplemented with desired concentration of NaCl (0, 50, 100, and 150 mM). Three biological replicates were collected from each sample at respective time points after salt stress.

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Estimation of electrolyte leakage, relative water content, total soluble sugars, and proline content

Electrolyte leakage was measured using an electrical conductivity meter as described by Lutts et al. [30]. Relative water content (RWC) was measured according to Barrs and Weatherley [31]. Total soluble sugar (TSS) content was determined by anthrone method. Free proline content was estimated using the acid ninhydrin method described by Bates et al. [32].

In-situ ROS staining:

In situ ROS staining was done in accordance with Beyer and Fridovich [33], on the basis of the principle of NBT (nitroblue tetrazolium) reduction to blue formazan by O_2^-. The intracellular concentration of ROS (O_2^-) was directly proportional to the development of intensity of blue color in the leaves. Briefly, leaf tissue was detached from the wild type and transgenic plants and vacuum infiltrated with 10 mM sodium azide (NaN3) in 10 mM potassium phosphate buffer for 1 min. The infiltrated leaf tissue was incubated in 0.1% NBT (nitroblue tetrazolium) in 10 mM potassium phosphate buffer; pH 7.8 for 30 min. The stained leaf tissue was boiled in acetic acid:glycerol:ethanol (1:1:3) solution to remove other pigments and the stain content was visually documented under Carl-Zeiss Stereo DiscoveryV12 with Axiovision software. This experiment was repeated three times from three biological replicates.

Microscopy:

Explants were collected at 0, 1, 2, 3, and 4 weeks from initiation and evaluated using scanning electron microscopy (SEM) and light microscopy (LM). Samples were fixed in formalin, glacial acetic acid, and 50 % ethyl alcohol (FAA) (1:1:18) at room temperature. Samples were subsequently dehydrated in a tertiary butyl alcohol series, embedded in paraffin (melting point 58–60 °C), and 8–10-mm thick sections were cut using a Finesee microtome. Sections were stained with 1 % safranin in water and with 4 % fast green in clove oil for 4 h and for 30 s, respectively. These were mounted in Canada balsam and examined using bright field microscope (Zeiss LSM510 meta GmbH, Germany) equipped with a Zeiss Axiovert 100 M inverted microscope.

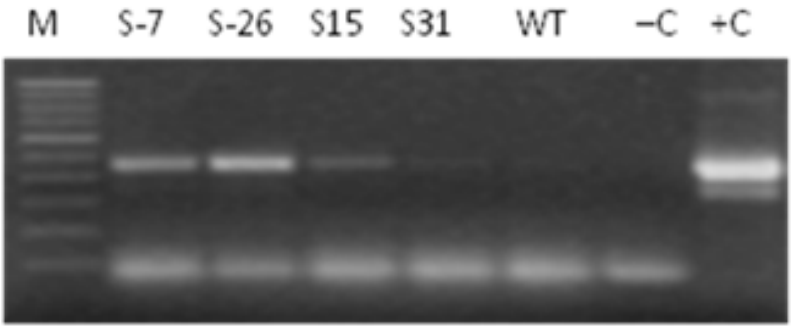
Statistical analysis:

All experiments were conducted with at least three independent repetitions in triplicate. All values are shown as the mean±standard deviation. The statistical analysis was performed using Statistica software (v.7). The statistical significance between the mean values was assessed by Analysis of Variance (ANOVA) applying Duncan's multiple range test (DMRT). A probability level of $P \leq 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

Transgenic lines exhibit improved salt stress tolerance:

Confirmation of transgenic was done with RT-PCR analysis (Table 1) of the four single copy inserts was performed with WT as negative control (Fig.1.). The order of expression of the four transgenic single copy insert lines (S7, S15, S26 and S31) in the case of *Cu/Zn-SOD* was S26>S7>S15>S31. Transgenic lines S15 and S26 were used for further studies.



Figurel. PCR amplification of cDNA with *Cu/Zn-SOD* specific primer of single copy inserts lines. M- 100bp ladder, S7, S15, S26 and S31 (*Cu/Zn-SOD* single copy insert transgenic lines), WT- WT control, -C is the negative control and +C is a positive

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Table 1: Primer sequence, PCR conditions, and amplicon size for the Cu/Zn-SOD and 26S rRNA (reference gene) used for semiquantitative PCR

Genes	Sequence 5' to 3'	PCR Conditions	Amplicon Size (bp)
AtSOD	F: TGCCATGGCGAAAGGAGTTGCAG R:ATAGATCTGCGCCCTGGAGACCAATGATG	94 °C, 4 min; 94 °C, 1 min, 56 °C, 30 s, 72 °C, 1 min, 27 cycles; 72 °C, 7 min	456
AtAPX	F: ATAGATCTGATGGCTGCACCGATTGTT R: TAAGTAGTCTTCATCCTCTTCCGGATCTC	94 °C, 4 min; 94 °C, 1 min, 57 °C, 30 s, 72 °C, 1 min, 27 cycles; 72 °C, 7 min	861
26S rRNA	F:CACAATGATAGGAAGAGCCGAC R:CAAGGGAACGGGCTTGGCAGAATC	94 °C, 4 min; 94 °C, 1 min, 57 °C, 30 s, 72 °C, 1 min, 27 cycles; 72 °C, 7 min	534

Improved germination rates, growth and development in transgenic under salinity stress

Germination and seedling development of Cu/Zn-SOD transgenic lines (S15 and S26) were compared with WT at 0, 50, 100, 150 and 200 mM concentration of NaCl in the MS0 medium (Fig. 2.). A significant difference was observed in the rate of germination on 3rd, 4th and 5th d (Fig. 2C and D.). At 150 and 200 mM NaCl stress the final percent germination was significantly more in transgenics than in the WT. At 150 mM NaCl stress, the germination of WT was only 25% with visibly small size of the cotyledons and less root growth, whereas the germination of S15 and S26 transgenic lines was 65 and 79% respectively (Fig. 2D.) and with comparatively large size cotyledons and more root growth (Fig. 2.). At 200 mM level of stress seeds of S15 and S26 achieved 20% germination whereas for the WT no germination was recorded (Fig. 2D.). The To confirm whether the *Cu/Zn-SOD* overexpressing transgenic lines have enhanced NaCl tolerance during early seedling establishment, the germinated seeds were allowed to grow until 10 d after germination (Fig. 2.).

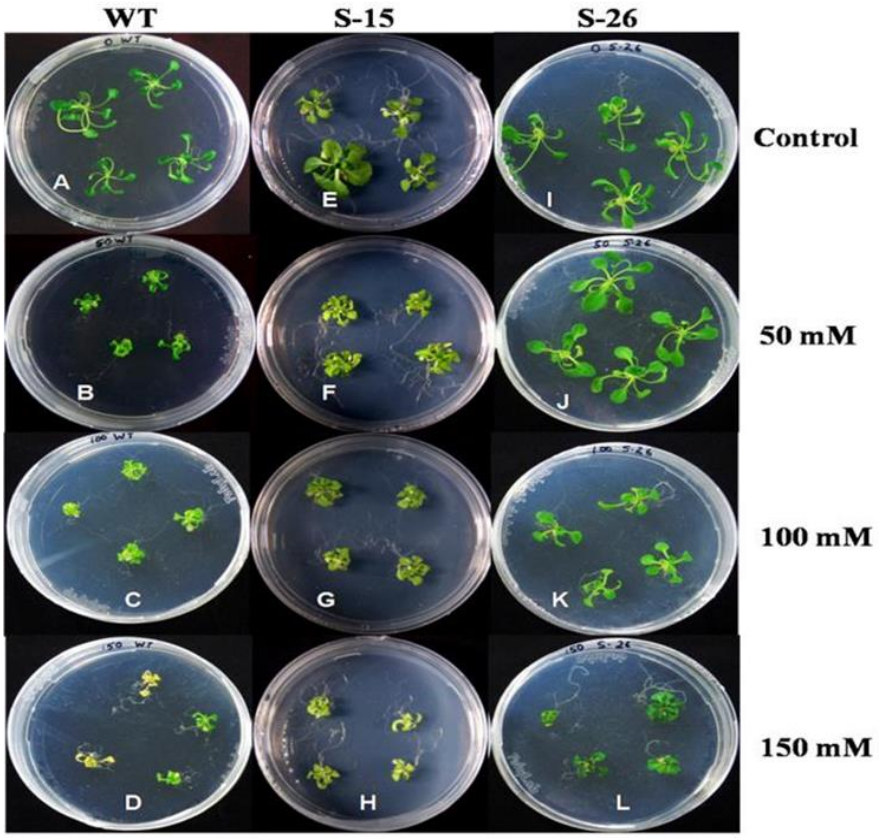

Figure 2. Growth assessment of plants at 3 week stage on MS medium supplemented with 0, 50, 100 and 150 mM NaCl. A-D are WT seedlings, E-H are transgenic line S15 and I-L are transgenic line S26

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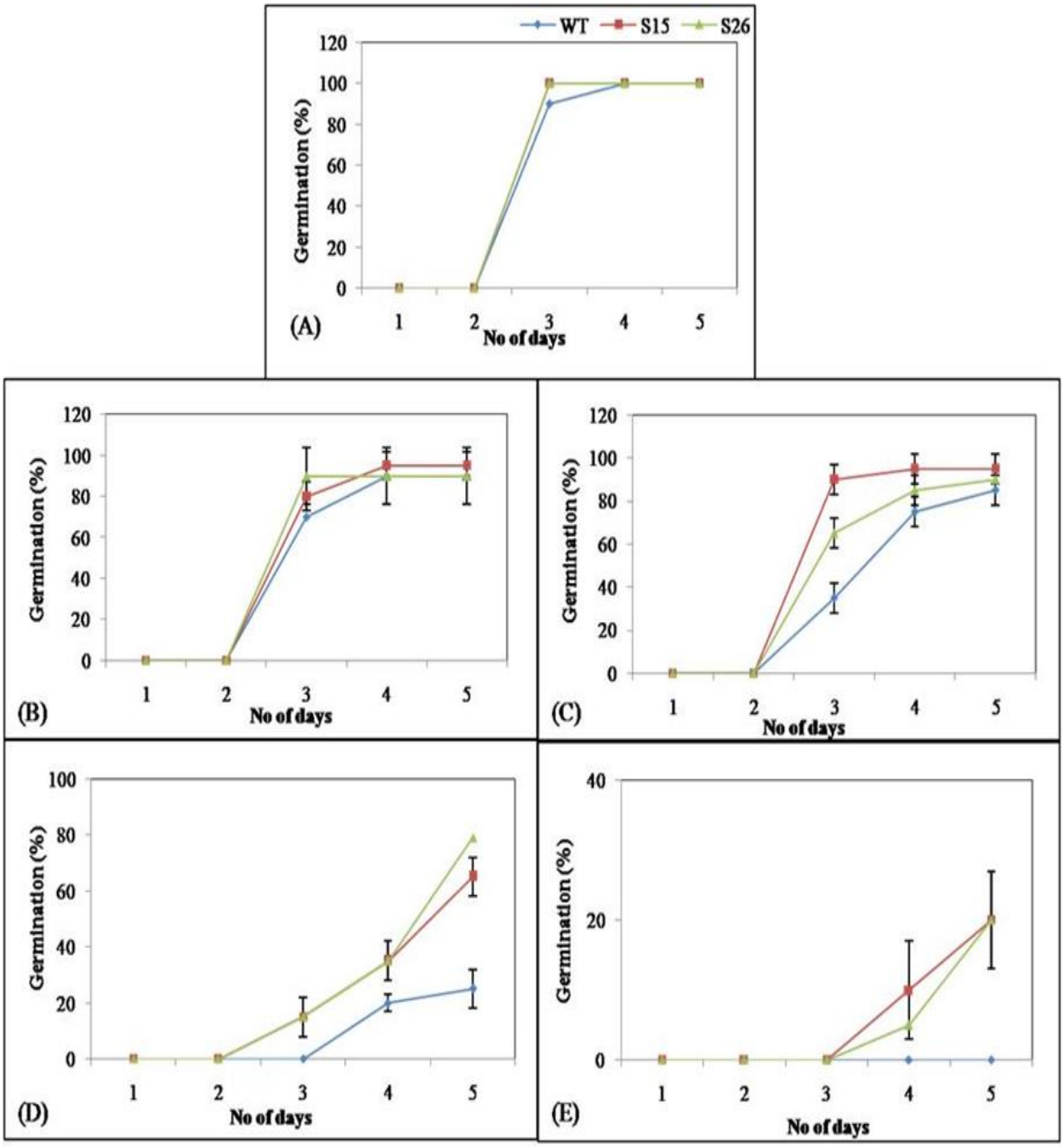

Figure 3. Effect of salt stress on germination of *Cu/Zn-SOD* transgenic plants. Rate of seed germination on (A) 0 mM; (B) 50 mM; (C) 100 mM; (D) 150 mM and (E) 200 mM NaCl. Germination was recorded from 3rd d onwards. Errors bars represent ±SE.

Cu/Zn-SOD Expression levels and activity at various Stages of Salinity Stress:

Arabidopsis transgenic plants over expressing Cu/Zn-SOD under the control of CaMV35 S promoter were generated as reported earlier (Shafi et al. 2015). RNA was isolated from the experimental lines (WT, S15 and S26) under various levels of stress (0, 50, 100 and 150 mM) and expression level of the transgene (Cu/Zn-SOD) along with that of the native genes (*At* Cu/Zn-SOD*)* encoding antioxidant enzymes were studied (Fig. 4.). WT plants did not show any amplification with Cu/Zn-SOD whereas the level of gene expression in transgenic S15 and S26 was upregulated with the increase in the level of stress (Fig. 4.). Both the transgenic lines exhibited different level of gene expression which may be due to the positional effect of insertion. The expression level of the native At Cu/Zn-SOD gene did not show much change in expression at different levels of stress.

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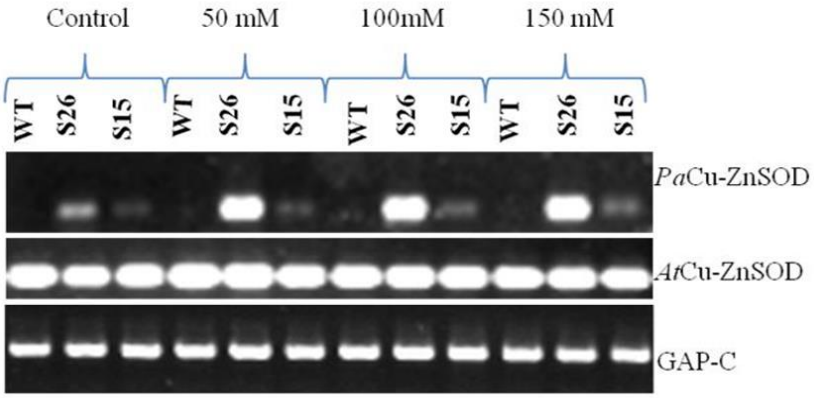

Figure 4. Expression analysis of AtSOD, AtAPX at callus stage (CS), Shooting stage (SS) and Rooting Stage (RS). 26S rRNA was used as the loading control.

Superoxide dismutase enzyme activities were estimated from the leaf samples of WT and transgenic plants at different time points during salt stress. As expected total SOD activity was significantly higher in transgenic plants when compared to WT plants even under normal growth conditions, whereas 1.6, 1.2 and 1.3 folds increase in SOD levels was observed in SOD (S26, S15) plants, respectively throughout salt stress (Fig. 5.). Abrupt elevation of these enzyme activities were recorded during the initial till 100 mM salt stress in WT and all the transgenic lines and consequently a gradual decrease was recorded at 150 mM, after which the minimal levels were maintained to sustain the growth and development of plants (Fig. 5.).

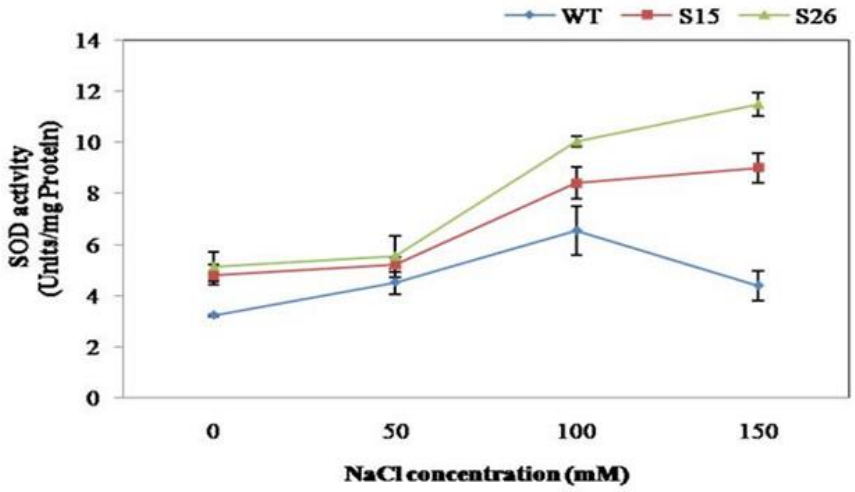

Figure 5. Superoxide dismutase activity in *Cu/Zn-SOD* transgenics and WT *Arabidopsis* plants after three weeks on different concentrations of NaCl. Total SOD levels were analyzed as a function of NBT reduction

Salt stress induces increase in aminoacid, soluble sugars, RWC and Electrolyte leakage in transgenics

An increase in proline and simple sugars against stress is commonly known phenomena in Arabidopsis and other plants [34]. Under normal conditions, more or less similar amounts of proline content was recorded in all the plants, but higher levels of proline were found under stress in the transgenic lines when compared to corresponding WT plants (Table 2). Similar trend was observed in the case of soluble sugars under salt stress (Table 2). Overall, these findings suggested that the content of proline and soluble sugars, which are known osmo-protectants, accumulated more in transgenic plants under cold stress**,** thus providing better stress tolerance and better growth. Electrolyte leakage was also observed to be significantly less in transgenic lines as compared to WT (Table 2), followed by relative water content which was again less in WT under stress conditions but transgenic retained RWC even in high salt stress (Table 2). This indicates that transgenic was more to salinity stress and were able to cope up the stress conditions with respect to WT.

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Table 2: Biochemical analysis of WT and transgenic lines (S15 and S26) under control and stress conditions salt stress.

Attributes	0 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl
Relative water content(%)	83.66 ± 2.27^{bc}	78.53 ± 2.96^{de}	76.81 ± 2.87^{de}	68.33 ± 0.61^{ef}
Electrolyte Leakage (%age)	19.94 ± 2.36^{fg}	17.85 ± 2.36^{g}	21.64 ± 1.84^{ef}	30.78 ± 1.01^{c}
TSS (mg/ml FW)	14.60 ± 0.04^{f}	20.23 ± 0.15^{d}	22.31 ± 0.06^{cd}	23.29 ± 0.06^{c}
Proline content (mg/gFW)	3.68 ± 0.20^{i}	7.86 ± 0.04^{g}	27.70 ± 0.03^{a}	24.33 ± 0.60^{b}

Data represent themean ± SE of three independent experiments (n = 3). Different letters on top of the bars indicate significant difference at a level of P < 0.05, as determined by Duncan's multiple range test (DMRT)

Overexpression of Cu/Zn-SOD enzyme protects cellular damage and ROS production:

Accumulation of ROS may cause damage to many bio molecules of the cells. As the transgenic lines were more tolerant to cold stress than WT, an attempt was made to compare accumulation of ROS in their leaf samples at the end of the salt stress by staining with NBT. Histo-chemical staining of leaves from WT and transgenic plant with NBT revealed that it could also be stained ROS without cold stress also (Fig. 6.). The figure showed that the transgenic lines had slightly lower ROS accumulation relative to WT without stress. Salinity resulted in significantly higher levels of ROS accumulation in WT leaves whereas the transgenic lines S26 and S15 low levels accumulation as evidenced by the lower intensity of the blue colour (Fig. 6.).

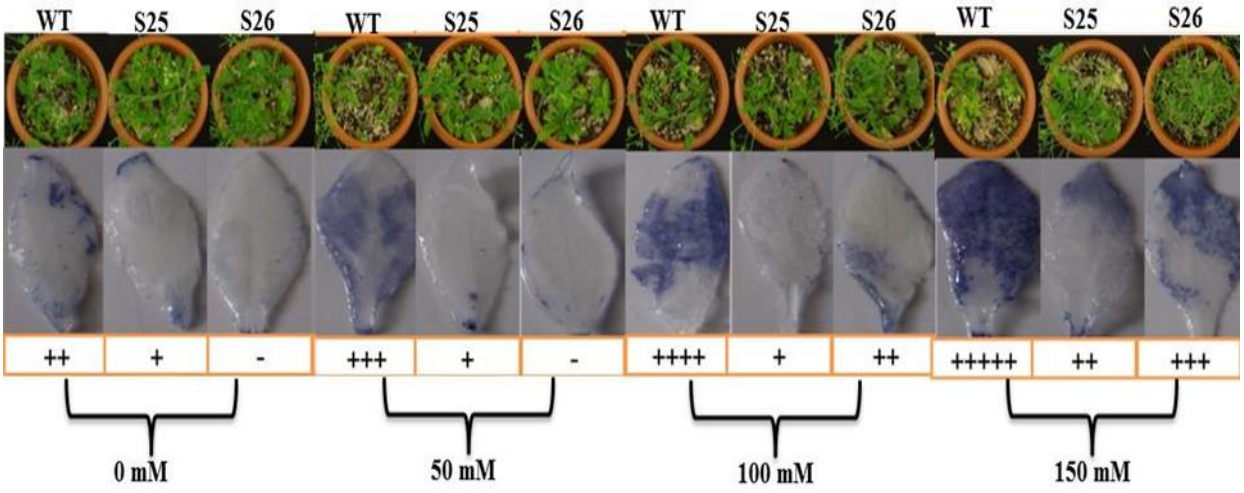

Figure 6. *In situ* ROS visual analysis of WT and transgenic lines after 10 d of different concentrations of NaCl (0, 50, 100 and 150 mM). (*Cu/Zn-SOD*,- S15 , S26 and WT. (-) absence of stain; (+) presence of stain.

Growth and Development of transgenic and WT under salinity stress

After three weeks of plant growth on salt stress, root length, number of leaves and rosette areas were measured as general indicators of plant growth and development under stress (Fig. 7.). Root length, particularly at 150 mM NaCl stress was higher in the transgenic lines S15 and S26 as compared to the WT (Fig. 7A.). On the same stress level, the number of leaves was significantly higher in S15 as well as in S26 transgenic lines when compared to the WT. At lower levels of NaCl stress (50 and 100 mM) the difference between the number of leaves between and WT was not significant (Fig. 7 C.). The rosette area of transgenic plants was significantly larger in S26 at all concentrations of NaCl stress as compared to S15 as well as WT (Fig. 7 B.). Improved growth in terms of increase in biomass during cold stress in transgenic plants might be because of presence of the additional copy of antioxidant gene(s), implies increase in the activity of corresponding enzymes.

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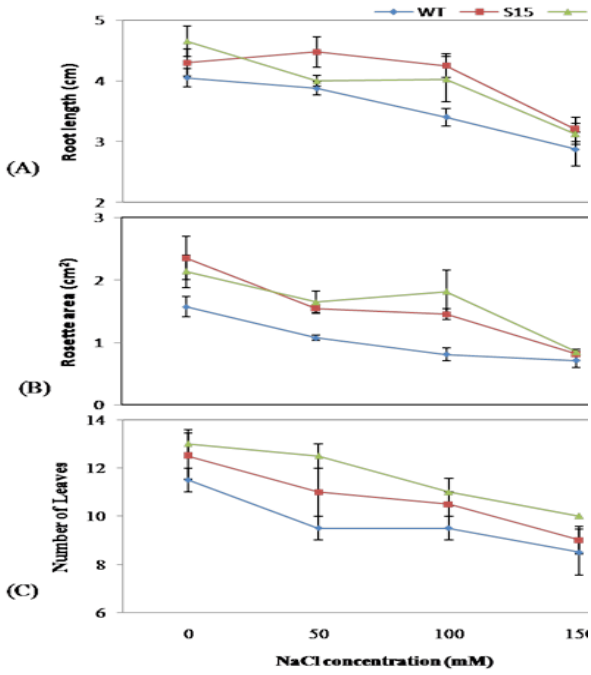

Morphological and Developmental Phenotypes of Transgenic Lines under stress:

The internal structure of the stems was characterized in WT and transgenic lines under control and stress conditions (Fig. 8.), which revealed that vascular bundles were well connected with interfascicular fibers forming a continuous cylinder of lignified tissue around the pith. Under control conditions (0 mM), WT and transgenic lines vascular system show normal morphology with intact cells and xylem system (Fig. 8.). Under stress conditions especially at 100 and 150 mM, vascular system in WT sections were collapsed and normal morphology of cells were not seen, While in transgenic lines (S15 and S26), stress (100 mM and 150 mM) did not affect the vascular system and xylem cells were clearly intact. This is the indication that transgenic were much more tolerant to salinity stress and retained their normal anatomical architecture, while same trend could not be observed in WT. This can be due to tolerance provided by the extra set of antioxidant gene present in transgenic lines.

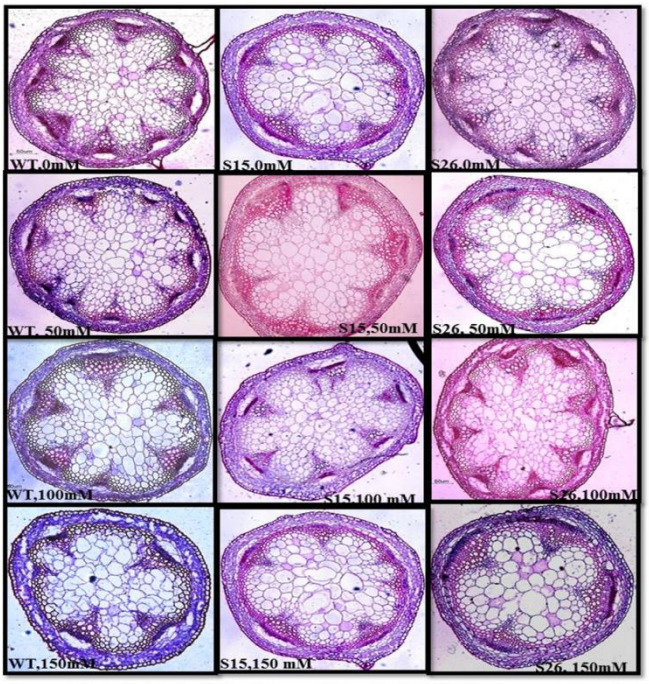

Figure. 8 Anatomical study of WT and transgenic lines after 10 d of different concentrations of NaCl (0, 50, 100 and 150 mM). (*Cu/Zn-SOD*,S15, S26) and WT.

4. CONCLUSION

This work clearly demonstrates that the modulation of endogenous ROS scavenging capacity against abiotic stresses can be successfully engineered by the over expression of Cu/Zn-SOD in Arabidopsis. Overall, Cu/Zn-SOD transgenic lines were able to express greater salinity tolerance and thus the present work would pave way for the judicious use of these genes effectively into the relevant crop plants leading to optimum growth and enhanced yield under environmentally stressed conditions. In addition, the results outlined the importance of the cytosolic antioxidant machinery in the cross-protection from multiple stresses in agriculturally important plants.

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