

Oxidative Stress Response Induced by PEG in Arabidopsis Engineered with the Cu/Zn-SOD and Mn-SOD Antioxidant System Genes

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ABSTRACT

Among abiotic stresses, water stress is one of the major stress causing growth retardation and yield loss of plants along with an increased accumulation of reactive oxygen species (ROS). To cope with oxidative stress, plants employ a host of antioxidants and enzymatic systems. This consists of enzymes like superoxide dismutases, catalases, peroxidases. In the present study, the genetically engineered Arabidopsis with copper-zinc superoxide dismutase (Cu/Zn-SOD) and manganese superoxide dismutase (Mn-SOD) genes isolated from *Potentilla atrosanguinea* and *Camellia sinensis* respectively, both of which are high altitude cold niche area plants of Western Himalaya, were evaluated against drought stress induced by polyethylene glycol (PEG). The genes, Cu/Zn-SOD and Mn-SOD were. It was found that the transgenic plants over-expressing both the genes were more tolerant to PEG-induced stress during growth and development. In both transgenic plants higher levels of total antioxidant enzyme activities, chlorophyll content, total soluble sugars, proline content and lower levels of ROS, ion leakage were recorded when compared to the WT during PEG stress. Transgenic plants showed 2-3 fold increase in SOD activity resulting in the enhanced stress tolerance to PEG stress than WT. In terms of growth and development in number of leaves, rosette area and root length, which was observed to be significantly higher in transgenic lines compared to WT. Also, the transgenic lines showed higher germination percentage at various levels of PEG. Overall, Cu/Zn-SOD and Mn-SOD transgenic lines were able to express greater drought 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.

Keywords: Cu/Zn-SOD, Mn-SOD, Water stress, PEG, ROS, Germination

I. INTRODUCTION

Plants are subjected to various abiotic stresses from low temperature and drought to salinity. To survive, they have evolved several different mechanisms to enhance their overall tolerance, including stress signal perception and transduction, and associated molecular regulatory networks. During development, however, plants encounter a variety of environmental stresses. Among them, water stress is the major abiotic stress affecting plant ecological distribution, crop growth, and productivity (Hsiao, 1973; Reddy et al., 2004). Recent studies have provided evidence to show that when plants are subjected to water stress, root growth is strongly inhibited, although root development is less

sensitive to water stress than that of shoots (Westgate and Boyer, 1985; Sharp and Davies, 1989; Spollen et al., 1993). Importantly, maintenance of root growth under water stress has been considered as an important adaptive trait for plants to increase deep water uptake and ensure their survival (Rodrigues et al., 1995).

The frequency and intensity of environmental extremes are expected to increase with climate change (Khaled et al., 2015). How plants cope with drought stress is a topic of an intense debate. In addressing this problem, geneticists and breeders have focused mainly on exploiting high yield potential and genotype selection for morphological, physiological and agronomic traits indicative of drought tolerance under field

conditions (Dhanda et al., 2004). Developing an understanding of plant responses to drought is a fundamental part of developing stress-tolerant varieties (Oneto et al., 2016; Reddy et al., 2004). Screening for drought tolerance under field conditions involves considerable resources (land, people and power) and requires suitable environmental conditions for the effective and repeatable phenotypic expression of drought tolerance attributable to the genotype. It is therefore necessary to use simple but effective early screening methods that relate to the field phenotypes (El Siddig et al., 2016). Overexpression of antioxidant genes is one of the latest and most reliable methods used worldwide with the objective of improving stress tolerance and productivity (Mickelbart et al., 2015), and often enabling applications across plant species. Overexpression of *Potentilla atrosanguinea* copper zinc superoxide dismutase (PaSOD) has improved cold and salt stress tolerance in *Arabidopsis thaliana* (Shafi et al., 2014; 2015a, b). Apparently, overexpression of the same SOD in potato, enhanced photosynthetic performance under salinity stress (Shafi et al., 2017). In another study, over accumulation of lignin in vascular bundles was found to be the molecular mechanism which underlies the improved stress tolerance induced by the over-expression of SOD in *Arabidopsis thaliana* (Shafi et al., 2015a, b). Genetic manipulation of antioxidant enzymes is one of the effective measures to impart stress tolerance in plants. Activities of superoxide dismutase and catalases were increased in plants subjected to stress (Scebba et al., 1999).

High altitude ecosystems are often inhabited by a very few plant species due to prevailing harsh environmental conditions. The genes/proteins isolated from high altitude plants are being used as molecular tools for engineering crop and other plants for better stress tolerance and adaptability against the present scenarios of climate change. In this pursuit, previously we identified and characterized a thermo-tolerant copper-zinc superoxide dismutase from a high altitude plant *Potentilla atrosanguinea* (Cu/Zn-SOD), which retains its activity even after autoclaving and Mn-SOD from *Camellia sinensis*. In the present study, we have overexpressed these genes in *Arabidopsis* and evaluated for tolerance against PEG stress. The detailed analysis revealed that these transgenics exhibit improved PEG tolerance, higher germination rates and yield as compared to wild type (WT). This study will elaborate and validate that higher altitude plants can serve as

ample source of potential molecular tools which can be successfully utilized for engineering abiotic stress tolerance in plants and yet not disturbing native physiology of the plants.

II. METHODS AND MATERIAL

2.1 Plasmid construction and transgenic plant development

Full length cDNAs of Copper-Zinc Superoxide Dismutase (Cu/Zn-SOD) and Manganese-SOD (Mn-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: altitude 4517 m; 32°24' 20" N; 077° 38' 40" E) and *Camellia sinensis* (Himachal Pradesh: altitude 4000 m; 32° 22' 19" N; 077° 14' 46" E), respectively, from Western Himalaya, were cloned in *Arabidopsis thaliana* as described earlier by Gill et al. (2010). Briefly, coding nucleotide sequences of these genes were amplified using the gene specific primers with incorporated NcoI and BglIII restriction sites at 39 end. PCR products were cloned into a cloning vector pGEMT easy (Promega) and then sub-cloned into binary plant vector pCAMBIA1302 under the Cauliflower mosaic 35 S promoter. The prepared plasmid construct was mobilized into *Arabidopsis* plants via *Agrobacterium* mediated vacuum infiltration method (Bechtold et al. 1993). Seeds were collected and screened in Murashige and Skoog (Murashige and Skoog, 1962) medium supplemented with 20 mg/ml hygromycin.

2.2 SOD enzyme activity assay

Total enzyme activity of SOD was estimated at different time points during PEG stress. Total SOD activity was estimated as described earlier (Gill et al. 2010b). Briefly, leaf samples (100 mg) were homogenized in a pre-cooled 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 Eppendorf and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was used to estimate total SOD activity. The total SOD activity was measured by adding 5 ml enzyme extract to a reaction mixture (200 ml) containing 1.5 mM Riboflavin, 50 mM 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 at 25°C. Total protein content was estimated according to the dye binding method of Bradford (1976) using BSA as standard.

2.3 Gene-specific semi-quantitative

Total RNA was isolated from control and PEG treated 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 ml reaction using of Superscript III Reverse transcriptase (Invitrogen). Transcripts of Cu/Zn-SOD and Mn-SOD were quantified with PCR using gene specific primers. Constitutively expressed 26s RNA was amplified simultaneously in 27 cycles to ensure equal amounts of cDNA used.

2.4 Evaluation of PEG stress tolerance

Arabidopsis (ecotype coloumbia) plants were grown on soil 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, 21d old seedlings of wild type, and hygromycin selected transgenic seedlings were given PEG stress (0, 2.5, 5, and 7.5 %). Samples were collected PEG treatment for the analyses of transcript levels, enzyme activity, proline and soluble sugars accumulation.

2.5 In-situ ROS staining

In situ ROS staining was done in accordance with Beyer and Fridovich (1987), 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.

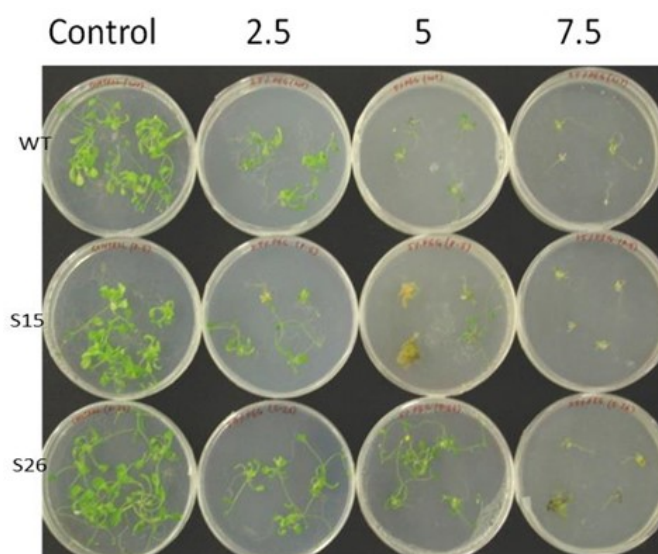
2.6 Statistical analysis

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.

III. RESULTS AND DISCUSSION

3.1 Overexpression of SOD increased tolerance to drought stress in transgenic lines

The phenotype of plants that overexpress SOD was examined under PEG treatment. As shown in Fig.1, no significant difference in phenotype was observed for the transgenic plants when compared with the wild type (WT) plants under normal conditions. The one-week-old soybean seedlings were treated with (2.5, 5, and 7.5 %) PEG 6000 for 4 weeks. During this time, the shoot of transgenic plants grew stronger and higher, whereas the leaves of WT plants curled and exhibited signs of dwarfism (Figure 1), indicating stressed plants. These results suggested that SOD overexpression could improve the response to drought stress in transgenic Arabidopsis.



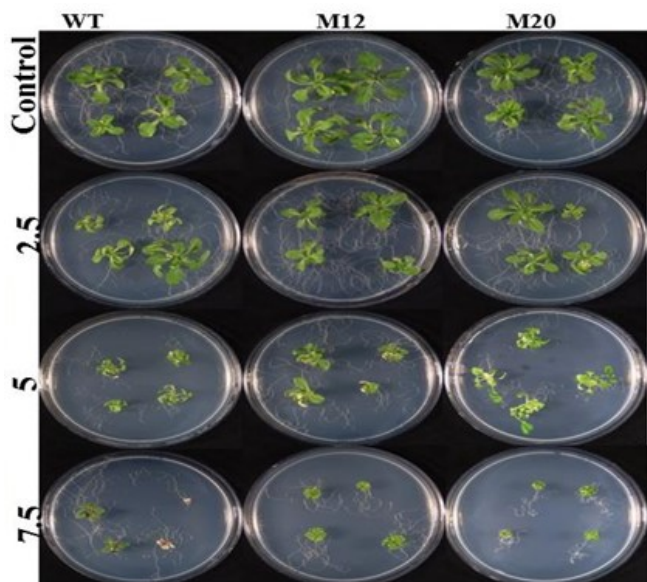


Figure 1. Growth assessment of WT, Cu/Zn-SOD (S15, S26) transgenic line and Mn-SOD (M12 and M20) at three week stage on MS medium having various concentration of PEG stress (2.5, 5 and 7.5%).

3.2 Increased SOD activity and expression under PEG stress

Biochemical analysis of transgenic and WT plants under control and stress conditions was evaluated. It was observed that SOD activity was significantly higher in transgenic lines than WT and activity increased with the increased stress conditions. The transgenic in the present study showed 2-3 fold increase in SOD activity (Figure 2) resulting in the enhanced stress tolerance to PEG stress than WT. These results indicated that SOD expression was also induced by PEG stress, imparting plants with higher tolerance towards PEG stress.

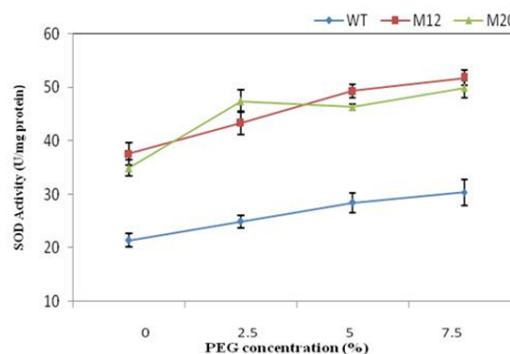
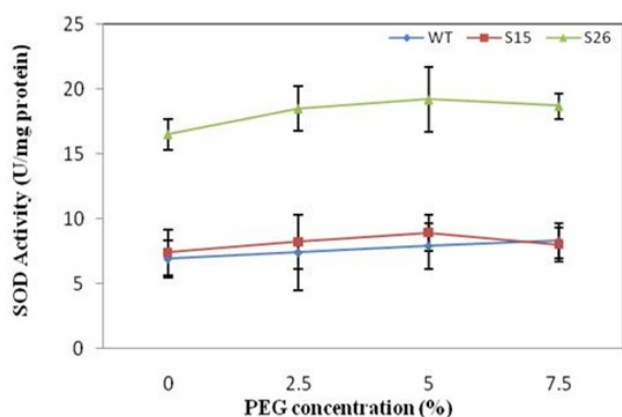


Figure (2): Superoxide dismutase activity in WT, Cu/Zn-SOD transgenic lines (S15 and S26), and Mn-SOD (M12, M20) *Arabidopsis* after PEG treatment (0, 2.5, 5, 7.5 %).

3.3 Expression analysis of stress responsive genes under different PEG stress treatments

Our previous study showed that the expression of SOD increases during stress conditions (Shafi et al., 2014; 2015a; 2015b). We did the expression analysis of SOD under different PEG stress (0, 2.5, 5, and 7.5%), it was observed that the expression of both Cu/Zn-SOD and Mn-SOD increased under stress conditions in transgenic lines (Figure 3). This expression was not observed in WT plants under control or stress conditions which is an indication of weak antioxidant system in WT and strong in transgenic lines, which imparts an extraordinary ability to transgenic for better survival under stress conditions. Higher SOD activity was also observed by Mittler et al., 2010, under drought and salt stress conditions.

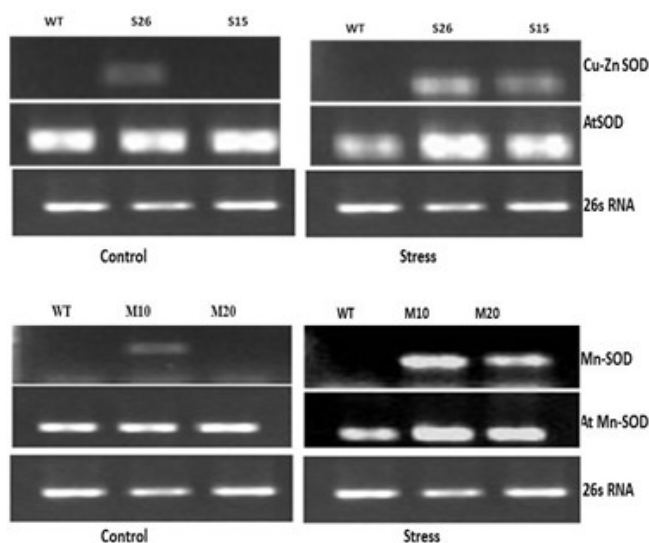


Figure (3): Expression analysis of *Cu/Zn-SOD*, *At Mn-SOD* and *Mn-SOD* at control and under PEG stress 26S rRNA was used as the loading control.

3.4 Reduced ROS accumulation in transgenic lines under PEG stress

In-situ NBT analysis was carried out in transgenic and WT plants to measure reactive oxygen species (ROS). Accumulation of blue coloration is an indication of increased ROS. Under control conditions, it was observed that ROS accumulation was less in both WT and transgenic lines, but under PEG stress conditions ROS accumulation starts increasing (Figure 4). At highest PEG concentration (7.5 %) blue coloration was intense in WT plants, while this condition was not observed in transgenic lines (Cu/Zn-SOD and Mn-SOD); they showed less ROS content even under higher PEG stress.

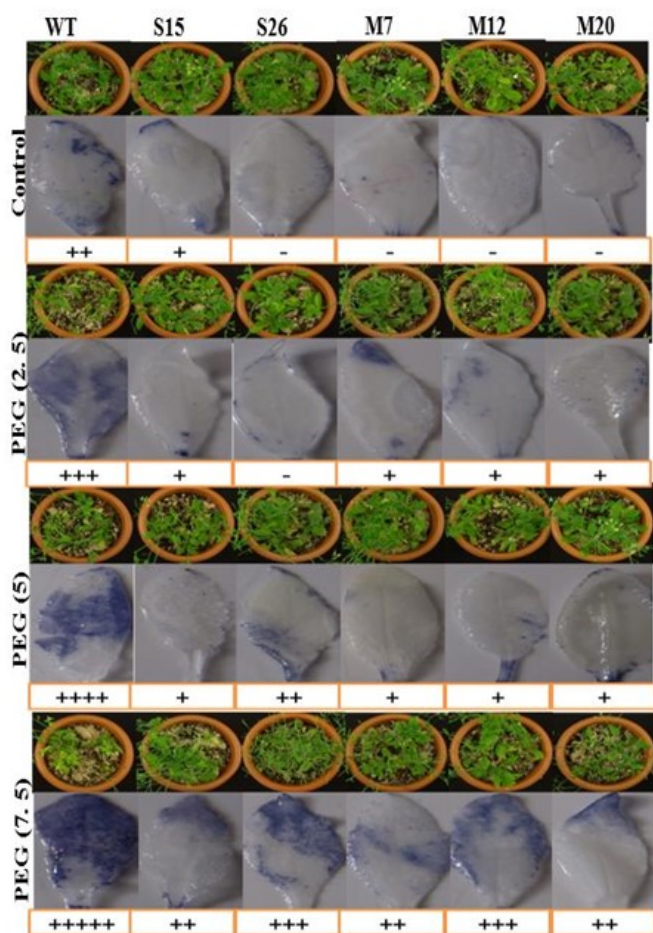


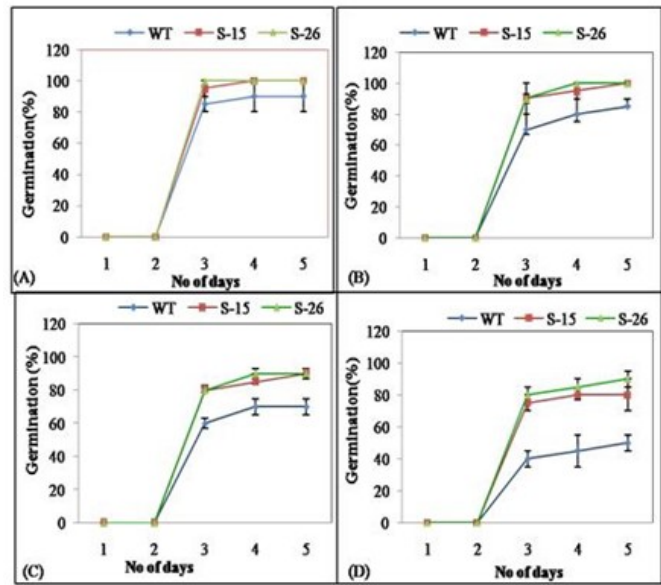
Figure (4): *In situ* ROS visual analysis of WT and transgenic lines after PEG treatment (0, 2.5, 5, and 7.5 %). WT and transgenic lines (S15, S26, M7, M12 and M20). (-) absence of stain; (+) presence of stain.

In all transgenic plants higher levels of total antioxidant enzyme activities (Figure 2) and lower levels of ROS (Figure 4), when compared to the WT plants during PEG stress, which indicates higher antioxidant power of transgenic under PEG stress. Reactive oxygen species

(ROS) produced in plants under drought, salt, and temperature stress conditions cause oxidative stress (Mittler et al., 2004). The results of NBT indicate the efficient scavenging of ROS in SOD-expressing transgenic plants.

3.5 Improved germination rates under Stress

Under normal conditions, there was no obvious difference in phenotypes of the transgenic and WT seedlings (Figure 5). Under different stress conditions, transgenic lines exhibited a higher survival rate as compared with WT. After a 2.5 % and 5 % PEG treatment, the WT seeds had a lower germination rate (86.7%) than the transgenic seeds (100%), as depicted in (Figure 5). After a 5% PEG treatment, the germination rate of the transgenic seeds began to decrease but was still significantly higher (86.6%) than that of WT (40%). These results suggested that *SOD* overexpression could increase PEG tolerance in transgenic plants germination.



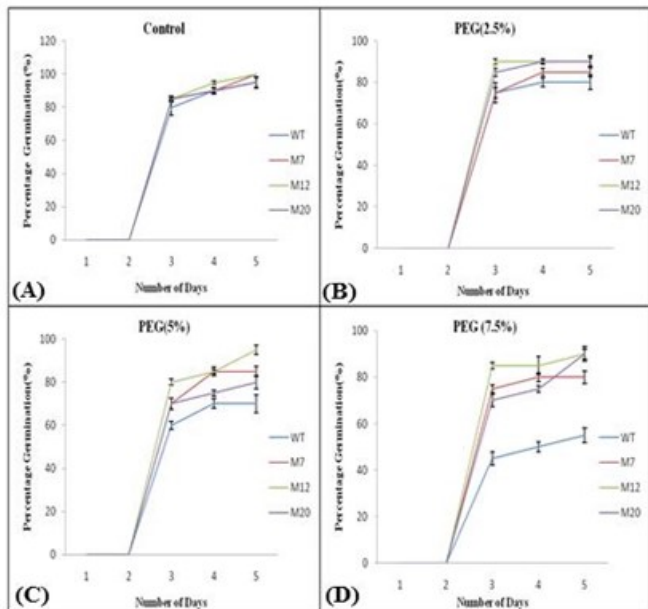


Figure (5): Percentage germination of *Arabidopsis* (WT), *Cu/Zn-SOD* transgenic lines (S15 and S26) and *Mn-SOD* transgenic lines (M7, M12, M20) under controlled conditions and PEG stress (A) control; (B) 2.5% PEG; (C) 5.0% PEG and (D) 7.5% PEG.

3.6 Growth and development of transgenic plants under PEG stress

Transgenic lines subjected to drought stress showed higher biomass accumulation with improved growth than WT. It was found that the transgenic plants over-expressing both the genes were more tolerant to PEG-induced stress during growth and development (Figure 6). In terms of growth and development in number of leaves, rosette area and root length, which was observed to be significantly higher in transgenic lines (Figure 6) compared to WT. Moreover, the transgenic lines showed increased root length when compared with WT, which indicative of stress tolerance.

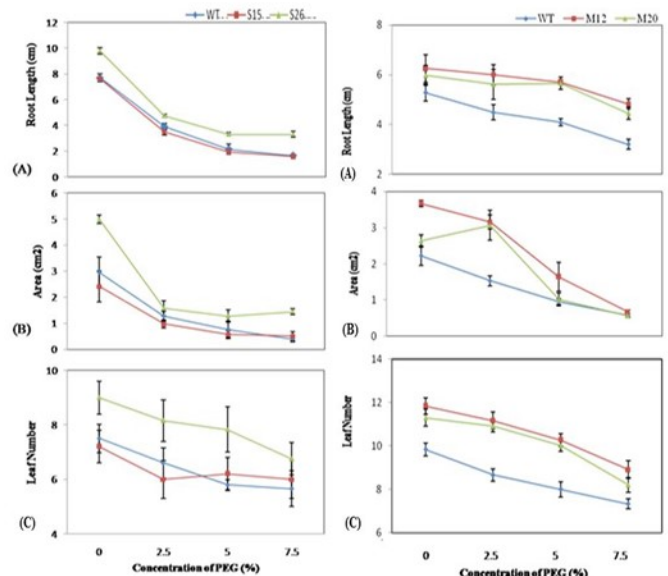


Figure (6): Overexpression of *Cu/Zn-SOD* and *Mn-SOD*, gene in *Arabidopsis* improves (A) root length; (B) rosette area and (C) leaf number. WT and transgenic plant after three weeks growth on MS medium supplemented with different concentrations of PEG.

IV. CONCLUSION

This work clearly demonstrates that the modulation of endogenous ROS scavenging capacity against abiotic stresses can be successfully engineered by the simultaneous over expression of *Cu/Zn-SOD* and *Mn-SOD* in *Arabidopsis*. Overall, *Cu/Zn-SOD* and *Mn-SOD* transgenic lines were able to express greater drought 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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