

# Transgenic peanut overexpressing *mtlD* gene confers enhanced salinity stress tolerance via mannitol accumulation and differential antioxidative responses

Kirankumar G. Patel<sup>1</sup> · Viralkumar B. Mandaliya<sup>1</sup> · Gyan P. Mishra<sup>1</sup> · Jentilal R. Dobaría<sup>1</sup> · Radhakrishnan Thankappan<sup>1</sup>

Received: 3 January 2016/Revised: 9 April 2016/Accepted: 13 June 2016/Published online: 20 June 2016  
© Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków 2016

**Abstract** Globally, peanut is an important oilseed crop, which is cultivated under different agro-climatic zones. Soil salinity is one of the major constraints in peanut cultivation. Therefore, to understand the physio-biochemical mechanisms imparting salinity stress, four transgenic peanut lines (cv. GG20) already developed and confirmed by our lab, having bacterial mannitol dehydrogenase gene (*mtlD*), were subjected to different levels of salinity stresses (1, 2 and 3 dS m<sup>-1</sup>) in pots under containment facility. Further, these lines were also characterized for various physio-biochemical parameters at flowering, pegging and pod formation stages. All the transgenic lines recorded significantly higher mannitol dehydrogenase (MTD) activity and mannitol accumulation than the wild type (WT). Under salinity stress, significantly higher levels of superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, glutathione reductase activities, while significantly lower levels of H<sub>2</sub>O<sub>2</sub> and malondialdehyde contents, were recorded in the transgenics compared to WT. Similarly, significantly higher ascorbic acid and relative water content (RWC) were recorded in transgenic lines. The MTD activity showed positive correlation with various antioxidant enzymes, growth parameters and RWC, while negative correlation was recorded with H<sub>2</sub>O<sub>2</sub>

and malondialdehyde content at most of the plant growth stages. The *mtlD* transgenic peanut lines under pot conditions were found maintaining lower oxidative injuries, indicating amelioration of salinity-induced oxidative stress by enhanced protection mechanisms via mannitol accumulation and antioxidative responses. The best lines identified (MTD1 and MTD4) may be used further as pre-breeding source for imparting salinity stress tolerance in peanut. Besides, these lines may also be tested under open-field trials for release as salt-tolerant variety.

**Keywords** Antioxidant enzymes · Groundnut · Mannitol dehydrogenase · Oxidative injury · Soil salinity

## Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
GR	Glutathione reductase
MTD	Mannitol-1-phosphate dehydrogenase
POD	Guaiacol peroxidase
SOD	Superoxide dismutase

## Introduction

Peanut or groundnut (*Arachis hypogaea* L.) is one of the principal oilseed crop, which is cultivated in around 22–24 M ha area in about 120 countries. It is grown under different agro-climatic zones of tropical and subtropical regions of Asia, Africa and North and South America (Mishra et al. 2015). The worldwide peanut production is over 45.6 million tons with an average yield of 1.79 tons per hectare (FAOSTAT 2014). Among oilseeds, peanut is unique since it can be consumed directly as a foodstuff. In

Communicated by MG dos Santos.

**Electronic supplementary material** The online version of this article (doi:10.1007/s11738-016-2200-0) contains supplementary material, which is available to authorized users.

✉ Radhakrishnan Thankappan  
radhakrishnan.nrcg@gmail.com

<sup>1</sup> ICAR-Directorate of Groundnut Research,  
Post Box No. 05, Junagadh 362 001, Gujarat, India

India, peanut is mostly grown under rain-fed conditions, mostly by resource-poor farmers, and, hence, is most likely encountered by abiotic stresses like drought and salinity (Sarkar et al. 2014; Bhauso et al. 2014a, b).

Salinity is one of the major environmental factors responsible for reduction of yield and biomass of crop plants in many parts of the world (Bhauso et al. 2014a). It has been estimated that worldwide, about 830 M ha area is salt-affected (Martinez-Beltran and Manzur 2005) and in India, the area under salinity is around 7.61 M ha (Singh et al. 2007). In major peanut growing states of India, salinity is increasing due to secondary salinization as non-scientific use of poor quality groundwater (Singh et al. 2008). The negative effect of soil salinity stress on plants is a complex phenomenon with major consequences like hyperosmotic stress, ion cytotoxicity (mostly due to  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{SO}_4^-$ ), nutritional imbalance and oxidative stress, which causes a series of changes at physiological, biochemical and molecular level (Conde et al. 2011).

To overcome salt stress, plants have evolved complex mechanisms through the synergistic action of various low-molecular-weight compatible solutes, osmolytes, antioxidants, and sugar alcohols (Rahnama et al. 2011; Miller et al. 2010; Bhauso et al. 2014a). Compatible solutes like betaines, polyols, sugars and amino acids get accumulated under salinity stress and maintain the osmoregulation, and they also function as oxygen free radical scavengers (Khare et al. 2010; Parida and Jha 2013).

Mannitol is a six-carbon, non-cyclic sugar alcohol having different roles like coenzyme regulation, free-radicals scavenging, and osmoregulation (Rahnama et al. 2011; Hema et al. 2014), thereby imparting abiotic stress tolerance. Synthesis of mannitol takes place in mature leaves, which is then translocated via phloem to heterotrophic sink tissues, where it is either stored or oxidized to mannose through mannitol-1-phosphate dehydrogenase (MTD) and used as a carbon and energy source (Conde et al. 2011). It is one of the most commonly occurring polyols in nature, with the exception of Archaea, animals (Bonin et al. 2015) and also not reported in peanut (Abebe et al. 2003; Bhauso et al. 2014b).

Among various ways for imparting the salinity tolerance, accumulation of mannitol using *E. coli mtlD* gene coding for MTD (EC 1.1.1.17), a key mannitol biosynthesis enzyme, has been widely used for the development of transgenic in various crops (Hema et al. 2014; Nguyen et al. 2013) including peanut (Bhauso et al. 2014a, b). Previously, we have reported the successful transfer of *mtlD* gene in peanut (cv. GG20) and also demonstrated the NaCl-induced salt stress tolerance in transgenic events under hydroponic system (Bhauso et al. 2014a, b). The

ultimate aim of this study is to impart salinity stress tolerance in GG20, the most popular variety of Gujarat and neighboring states (India), especially in the coastal belts where salinity stress is a major problem, so that it will receive immediate acceptance. Moreover, GG20 is equally popular for the luxuriant fodder it produces; thus, keeping the Indian agrarian system in consideration, where fodder also fetches handsome price to the farmers, we chose this variety.

In this backdrop, the objective of the present work was to further understand the mechanisms exhibiting salinity stress tolerance in transgenic peanut, overexpressing *mtlD* gene, under different salinity stress levels and at different growth stages. This involves evaluation of key biochemical parameters like MTD enzyme activity, antioxidant responses, accumulation of mannitol and other sugars, and reactive oxygen species (ROS) under progressive life cycle of both T and WT plants grown in pots under transgenic containment facility.

## Materials and methods

### Plant materials and growth conditions

The four peanut transgenic (T) events namely, MTD1 to MTD4 in  $T_4$  generation, having single-copy transgene insertion and confirmed using Southern blot analysis (Bhauso et al. 2014b) were used for salt stress tolerance analysis in pots under containment facility. Wild-type (WT) and T events were sown in pots made up of concrete rings (in triplicate) under salinity stress and controlled conditions (Fig. S1). The size of the concrete ring was 60 cm diameter and 45 cm height, filled with an approximately 200-kg mixture of soil: sand: farm yard manure (1:2:0.1). This soil mixture was measured for its electrical conductivity (EC) which is about  $0.5 \text{ dS m}^{-1}$  and is considered as control condition. Different levels of salinity were created by irrigating with saline water containing 25, 50 and  $75 \text{ mmol L}^{-1}$  NaCl, until EC reaches up to 1, 2 and  $3 \text{ dS m}^{-1}$ , respectively (Chakraborty et al. 2012). Soil samples from various depths (0–5, 5–30 and 30–40 cm) were taken every week from each concrete ring, and EC was measured (Dellavalle 1992). Scheduled saline water irrigation was practised throughout the crop growth period to maintain respective EC levels. Plants were maintained in a containment facility under 16 h light conditions at  $35 \pm 2 \text{ }^\circ\text{C}$  for 120 days. Third and fourth leaf from main stem of WT and T lines were taken at flowering [45 days after sowing (DAS)], pegging (60 DAS) and pod formation (85 DAS) stages for salinity stress tolerance analysis (Boote 1982).

### Mannitol-1-phosphate dehydrogenase assay

For mannitol-1-phosphate dehydrogenase (MTD) assay (Iwamoto et al. 2003), 1 g of fresh leaf tissue was homogenized in liquid nitrogen, and crude extract was obtained using freshly prepared extraction buffer (10 mL) [HEPES–KOH (500 mM; pH 7.0), benzamidine-HCl (1 mM), MgCl<sub>2</sub> (5 mM), Na<sub>2</sub>-EDTA (2 mM), 2-ME (4 mM), phenylmethylsulfonyl fluoride (1 mM), polyvinylpyrrolidone K-90 (100 mg)] and centrifuged (20,000g; 30 min).

For purification, aqueous two-phase partitioning method (Nakamura and Ikawa 1993) was used. PEG 6000 solution (50 %, w/v; prepared in extraction buffer) was added to crude extract, with gentle stirring to get 12.5 % (w/v) final concentration, and then, ammonium sulfate (AS) was slowly added to make 12 % (w/v) fraction. The mixture was then centrifuged, and lower phase is collected as PEG/AS fraction for assay. Fructose-6-phosphate (F6P) reducing activity was measured by monitoring the disappearance of NADH at 340 nm in a 3-mL reaction mixture [HEPES–KOH (50 mM; pH 7.0), Na<sub>2</sub>-EDTA (3 mM), F6P (3 mM), NADH (0.2 mM), NaCl (100 mM), enzyme extract (100 µL)], and reaction was initiated by adding F6P. MTD activity was expressed as F6P reduction µmol mg<sup>-1</sup> - protein min<sup>-1</sup>. The analyses were performed using Spectord 200 (Analytikjena, Germany) spectrophotometer.

### In situ localization of hydrogen peroxide and superoxide radical

In situ localization of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide radicals (O<sub>2</sub><sup>-</sup>) was examined based on histochemical staining by 3,3-diaminobenzidine (DAB; Fryer et al. 2002) and nitroblue tetrazolium (NBT; Ramel et al. 2009) dyes, respectively. To avoid auto-oxidation, DAB (1 mg mL<sup>-1</sup>; pH 3.8) was prepared freshly in distilled water, while NBT (1 mg mL<sup>-1</sup>) was prepared in potassium phosphate buffer (10 mM; pH 7.8) containing NaN<sub>3</sub> (10 mM). Leaflets (60 DAS) were immersed in DAB and NBT solution and kept in dark for 12 h (24 °C), then bleached twice in acetic acid: glycerol: ethanol (1:1:3; v/v/v) solution (80 °C; 5 min), and then stored in glycerol: ethanol (1:4; v/v) solution until photographed. On the bleached leaflets, H<sub>2</sub>O<sub>2</sub> was visualized as brown color spots due to DAB polymerization, while O<sub>2</sub><sup>-</sup> was visualized as blue color spots, due to NBT precipitation.

### Hydrogen peroxide measurement

H<sub>2</sub>O<sub>2</sub> content was measured as titanium–hydro peroxide complex (Rao et al. 1997), for which leaf tissue (1 g) was ground with chilled acetone (10 mL) and filtered using

Whatman No. 1 filter paper. To 5 mL of extract, titanium reagent (2 mL) and concentrated ammonium solution (2.5 mL) were added and centrifuged (5 min; 10,000g). The resulting peroxide–titanium complex was dissolved in H<sub>2</sub>SO<sub>4</sub> (2 M; 5 mL), and absorbance was recorded at 415 nm against blank. The H<sub>2</sub>O<sub>2</sub> content was determined using a standard curve, plotted with known concentrations of H<sub>2</sub>O<sub>2</sub>.

### Relative water content, malondialdehyde and ascorbic acid measurement

For measuring the relative water content (RWC), the fresh weight (FW) of eight fresh leaf discs (1 cm diameter) was measured. These were then hydrated (8 h) in distilled water, and their turgid weight (TW) was measured and subsequently dried in a hot-air oven (80 °C; 72 h) and weighed till a consistent dry weight (DW) was obtained. RWC was calculated as  $RWC = [(FW - DW) / (TW - DW)] \times 100$  (Barrs and Weatherley 1962).

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content determined by thiobarbituric acid (TBA) reaction method (Heath and Packer 1968). Leaf tissue was homogenized in 10 mL of trichloroacetic acid (TCA, 0.1 %, w/v), centrifuged (15 min; 15,000g), and 1 mL supernatant was mixed with 4 mL of TBA reagent (0.5 %, w/v TBA in 20 %, w/v TCA). The mixture was heated (95 °C; 30 min) and then cooled in ice for stopping the reaction. Then, the mixture is centrifuged (10 min; 10,000g), and absorbance of supernatant was measured (532 nm) which was corrected with non-specific absorption (600 nm). The amount of MDA was calculated using the extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup> and expressed as µmol g<sup>-1</sup> FW.

Ascorbic acid (AsA) was extracted with 10 mL of TCA (6 %, w/v) using 250 mg leaf tissue (Mukherjee and Choudhuri 1983). The extract (2 mL) was mixed with 1 mL of dinitrophenyl hydrazine (2 % w/v) and one drop of thiourea solution (10 % w/v prepared in 70 % v/v ethanol). This mixture was then boiled (15 min) and cooled at room temperature, and then, 5 mL of H<sub>2</sub>SO<sub>4</sub> (80 % v/v) was added to obtain hydrazone complex. Absorbance of hydrazone complex was measured (530 nm), and amount of AsA was calculated from standard curve plotted with known concentration of AsA.

### Antioxidant enzyme activity measurement

Enzyme extract for superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) was extracted from 1 g of fresh leaf tissue. Leaf tissues were pulverized in liquid nitrogen and grounded in 10 mL of chilled

extraction buffer [100 mM potassium phosphate buffer (pH 7.5) containing 5 mM EDTA, while 1 mM of ascorbic acid is added in case of APX]. The homogenates were centrifuged (20 min; 15,000g), and supernatants were stored in separate aliquots at  $-20^{\circ}\text{C}$  until analysis.

SOD activity was assayed by measuring its ability to inhibit photochemical reduction of NBT (Dhindsa et al. 1981). Reaction was developed in 3 mL mixture [phosphate buffer (50 mM; pH 7.8), methionine (13 mM), NBT (75  $\mu\text{M}$ ), EDTA (0.1 mM), sodium carbonate (50 mM), riboflavin (2  $\mu\text{M}$ ) and enzyme extract (0.1 mL)]. To start the reaction, 2  $\mu\text{M}$  riboflavin was added to mixture, and tubes were shaken and placed under 15-W fluorescent lamps for 15 min. SOD specific activity ( $\text{U mg}^{-1}$  protein) was measured at 560 nm, and one unit of SOD activity was taken as the amount of enzyme required to inhibit the reduction of NBT by 50 %.

For the measurement of CAT activity (Aebi 1984), the reaction was developed in 3 mL mixture [phosphate buffer (50 mM; pH 7.0),  $\text{H}_2\text{O}_2$  (12.5 mM) and enzyme extract (50  $\mu\text{L}$ )]. Reaction was initiated by the addition of  $\text{H}_2\text{O}_2$  (12.5 mM), and decomposition of  $\text{H}_2\text{O}_2$  was measured as decreasing in absorbance at 240 nm for 2 min at 10 s interval. The initial and final  $\text{H}_2\text{O}_2$  contents were calculated by comparing with a standard curve drawn with known concentrations of  $\text{H}_2\text{O}_2$ , and CAT activity was expressed as  $\mu\text{mol H}_2\text{O}_2$  reduced  $\text{mg}^{-1}$  protein  $\text{min}^{-1}$ .

For POD activity (Castillo et al. 1984), the reaction was developed in 3 mL mixture [potassium phosphate buffer (50 mM; pH 6.1), guaiacol (16 mM),  $\text{H}_2\text{O}_2$  (2 mM) and enzyme extract (25  $\mu\text{L}$ )]. Absorbance due to the oxidation of guaiacol to tetra-guaiacol on addition of 2 mM  $\text{H}_2\text{O}_2$  was monitored at 470 nm for 1 min at 15 s interval. POD activity was calculated using the extinction coefficient of tetra-guaiacol ( $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ) and expressed as mmol tetra-guaiacol formed  $\text{mg}^{-1}$  protein  $\text{min}^{-1}$ .

For APX activity (Nakano and Asada 1981), the reaction was developed in 3 mL mixture [potassium phosphate buffer (50 mM; pH 7.0), EDTA (0.1 mM), ascorbate (0.5 mM),  $\text{H}_2\text{O}_2$  (0.1 mM) and enzyme extract (0.1 mL)]. Reaction was initiated by addition of 0.1 mM  $\text{H}_2\text{O}_2$ , and the rate of ascorbate oxidation was measured by the decrease in absorbance at 290 nm for 2 min at 10 s interval. APX activity was calculated by measuring the decrease in AsA content using standard curve and expressed as  $\mu\text{mol}$  ascorbate oxidized  $\text{mg}^{-1}$  protein  $\text{min}^{-1}$ .

For GR activity (Smith et al. 1988), the reaction was developed in 3 mL mixture [potassium phosphate buffer (100 mM; pH 7.5), 5,5-dithiobis-2-nitrobenzoic acid (DTNB; 0.5 mM), NADPH (66  $\mu\text{M}$ ), oxidized glutathione (GSSG; 0.66 mM) and enzyme extract (0.1 mL)]. Reaction was initiated by adding 0.66 mM GSSG, and increase in

absorbance at 412 nm was monitored for 2 min at 10 s interval. GR activity was expressed as  $\mu\text{mol GSSG}$  reduced  $\text{mg}^{-1}$  protein  $\text{min}^{-1}$ . In all the enzyme preparations, protein was estimated by the Lowry's method using bovine serum albumin (BSA) as the standard (Lowry et al. 1951).

### Soluble sugars measurement

For soluble sugars analysis (Oupadissakoon et al. 1980), fresh leaf samples were extracted in 80 % ethanol, membrane-filtered and aliquot (25  $\mu\text{L}$ ) was injected in the ion chromatograph (Dionex, ICS 3000) equipped with amino trap column, CarboPac PA10 guard column followed by CarboPac PA10 analytical column. Sugars were eluted from column in 150 mM NaOH with a flow rate of 1 mL  $\text{min}^{-1}$ . Myo-inositol, mannitol, glucose, trehalose, fructose, lactose, and sucrose were used as standards. Lactose was used as internal standard. Data integration was done using Chromeleon software supplied with the equipment.

### Growth parameters

Various growth-related parameters, including root and shoot length (cm), were measured from both WT and T lines after harvesting (120 DAS). Thereafter, plants were dried for 72 h in oven at  $70^{\circ}\text{C}$ , and pod weight (g), root weight (g), shoot weight (g) and harvest index (HI, %) were measured.

### Statistical analysis

To find the significance of difference between means of WT and T lines of each treatment group, data were subjected to analysis of variance (ANOVA) and expressed as mean with standard error ( $\pm\text{SE}$ ). Each assay was carried out in three replicates. Significant differences between control and transgenic plants were analyzed using Tukey's multiple range at 5 % probability level ( $\text{LSD}_{P\leq 0.05}$ ). Correlation coefficient was determined using PAST software (PAleontological STatistics, version 1.89).

## Results and discussion

### Mannitol-1-phosphate dehydrogenase (MTD) activity

Accumulation of various osmoprotectants like mannitol is one among the different ways adapted by plants to overcome different types of stresses. As a strategy for imparting salinity tolerance, overexpressing of *mtlD* gene coding for

MTD has been widely used in various crops (Hema et al. 2014; Nguyen et al. 2013), including peanut in hydroponic system in our lab (Bhauso et al. 2014a). In the present investigation, WT plants exhibited almost negligible, while T lines showed notable MTD activities under salinity stress and non-stress conditions, which suggests the expression of transgene in T lines (Fig. 1). However, among T lines, it is significantly higher in MTD1, MDT2 and MDT4 lines compared to MDT3 line. Thus, the MTD enzymatic activity not only ensured the expression of the transgene but also indicated its possible role in imparting the salt tolerance in T peanut as also reported by Bhauso et al. (2014a). It is noted that, with increasing salinity levels up to 2 dS m<sup>-1</sup>, there is a slight increase in the MTD activity, which declined at 3 dS m<sup>-1</sup>. It is hypothesized that, till 2 dS m<sup>-1</sup> of salinity stress, plant might have synthesized enough amount of MTD required for imparting the tolerance, and, thereafter, it declined. The elevated MTD activity in T peanut lines supported the observations made for transgenic tomato, showing elevated MTD activity under abiotic stress conditions (Khare et al. 2010).

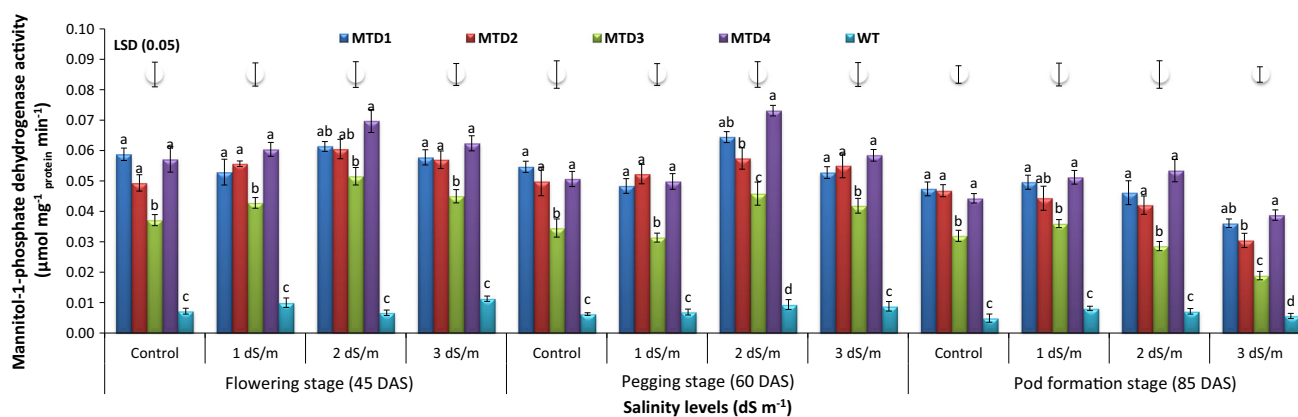
Maximum MTD activity in all the T lines was recorded at pegging stage in 2 dS m<sup>-1</sup> salinity levels, while minimum at pod formation stage in 3 dS m<sup>-1</sup> salinity levels. Thus, pegging stage seems more critical for the peanut plant for combating the salinity stress, while pod formation was least affected with respect to MTD activity. In general, MTD1 and MTD4 lines have shown significantly higher, while MTD3 line has shown significantly lower MTD activity at different salinity treatments (Fig. 1). This variation in the levels of MTD activity in different T events might be caused due to the positional difference in the integration of transgene (Rahnama et al. 2011; Bhauso et al. 2014a, b).

### In situ localization of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> and estimation of H<sub>2</sub>O<sub>2</sub> and malondialdehyde contents

Salinity stress in plants causes metabolic imbalances by impairing the electron transport and increase in the production of ROS, such as singlet oxygen, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide anion (O<sub>2</sub><sup>-</sup>) and hydroxyl radicals (Miller et al. 2010). On the similar note, histochemical in situ localization showed lower accumulation of H<sub>2</sub>O<sub>2</sub> (visualized as deep-brown products in DAB staining; Fig. 2a) and O<sub>2</sub><sup>-</sup> (visualized as dark-blue products in NBT staining; Fig. 2b) under different salt stress conditions in T lines, compared to WT. Also, an increase in the H<sub>2</sub>O<sub>2</sub> content in leaf tissues was reported with increasing salt content in both WT and T lines at different growth stages (Fig. 3a).

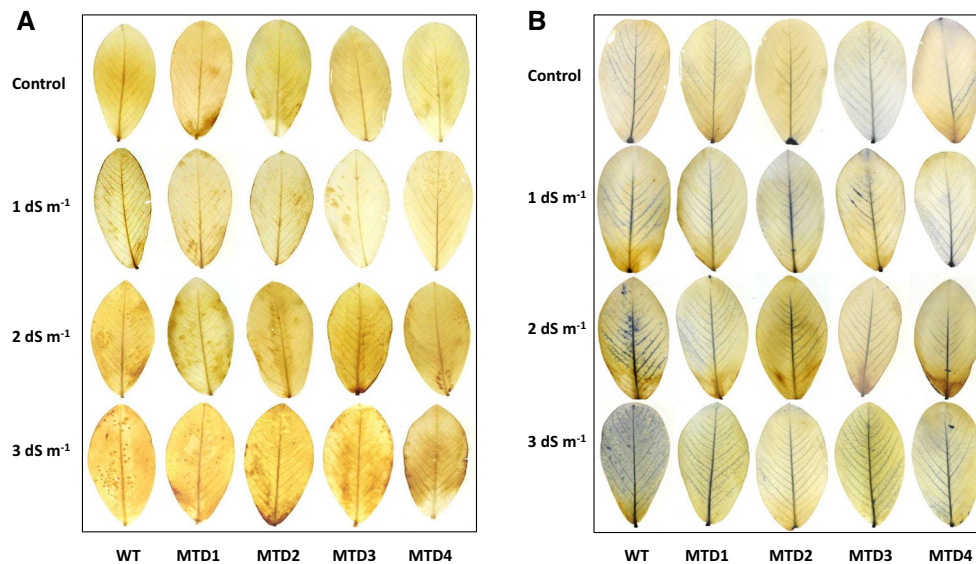
The MDA content in leaves represents the degree of membrane lipid peroxidation resulting from the cell injury by oxidative stress (Tang et al. 2005; Akcay et al. 2010). A gradual increase in MDA content was observed in both WT and T lines with increasing salt stress; however, it was significantly higher for WT than T lines, in higher salt concentrations (Fig. 3b). The relatively lower MDA content in T lines might be due to hydroxyl radicals scavenging activity and osmoprotection function of mannitol which, in turn, lowers the electrolytic leakage (Khare et al. 2010; Adrees et al. 2015; Bhauso et al. 2014a).

During flowering, pegging and pod formation stages, till 2 dS m<sup>-1</sup> of salt treatment, no significant difference ( $P \leq 0.05$ ) in both H<sub>2</sub>O<sub>2</sub> and MDA content was observed between WT and T lines. However, at higher salt treatment (3 dS m<sup>-1</sup>), T line, MTD4 has shown significantly lower H<sub>2</sub>O<sub>2</sub> and MDA accumulation (Fig. 3a, b) compared to other lines at all growth stages, indicating its relatively better ability for salinity stress tolerance. Significantly

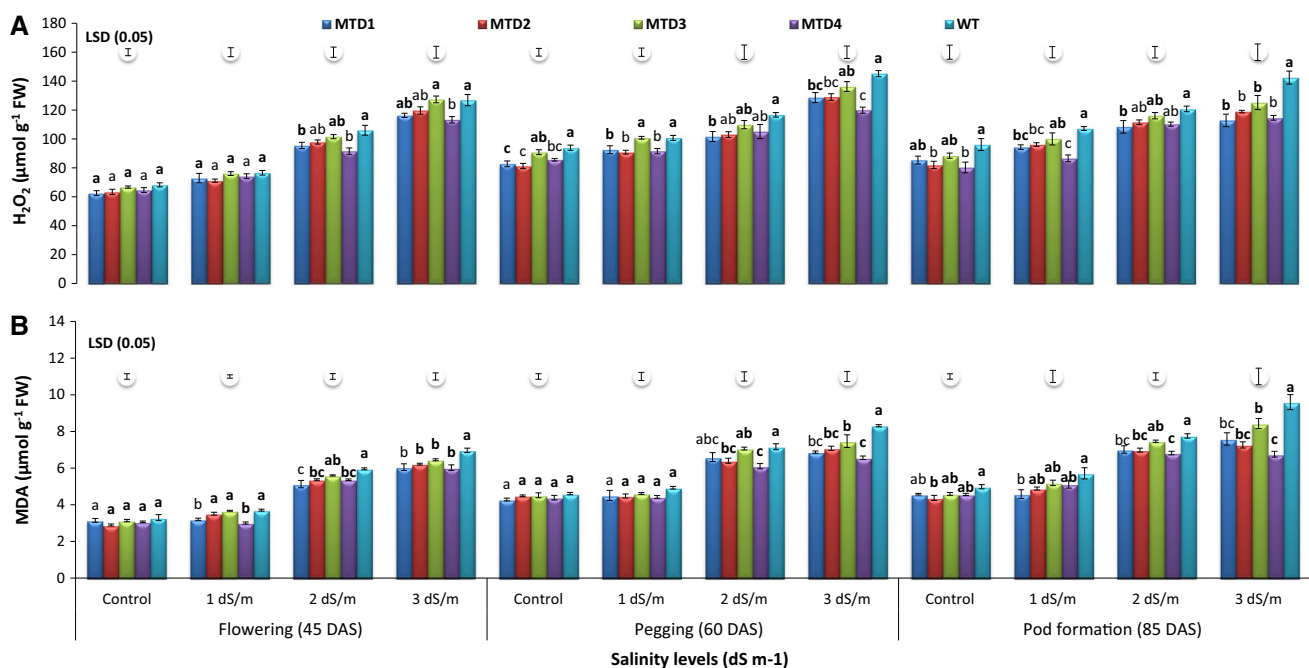


**Fig. 1** Comparison of mannitol dehydrogenase activity in the WT and T lines under different salinity levels. WT is GG20, and MTD1–4 are four independent T lines grown under different salinity levels (control, 1, 2 and 3 dS m<sup>-1</sup>). Sampling was done at flowering,

pegging and pod formation stages. Graph represents the mean  $\pm$  SE ( $n = 3$ ) followed by similar lower case letter are significantly not different according to Tukey's multiple range at  $P \leq 0.05$  (this is the same for Figs. 3, 4 and 5)



**Fig. 2** In situ localization of **a**  $\text{H}_2\text{O}_2$  using DAB staining, and **b**  $\text{O}_2^-$  using NBT staining in WT (GG20) and T lines (MTD1–4) grown under different salinity levels (control, 1, 2 and 3  $\text{dS m}^{-1}$ )



**Fig. 3** **a**  $\text{H}_2\text{O}_2$  and **b** MDA content in WT and T lines under various salinity stress levels

lower  $\text{H}_2\text{O}_2$  and MDA concentration in T lines at higher salt treatment (3  $\text{dS m}^{-1}$ ), compared to WT, suggested that T plants had balanced ROS formation and suffered less oxidative lipid injury than WT lines (Tang et al. 2005; Rai et al. 2013).

Similarly, lower in situ accumulation of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  was reported in *SbMT-2* tobacco (Chaturvedi et al. 2014), *AhAREB1* Arabidopsis (Li et al. 2013), and lower MDA content in *mtID* tomato (Khare et al. 2010) and *MuNAC4*

peanut (Pandurangaiah et al. 2014) during abiotic stress imposition. All these suggest that T peanut lines having *mtID* gene were less affected by oxidative stress than their WT counterpart.

### Relative water content (RWC)

The accumulation of mannitol in the cells is known to prevent intracellular water loss and facilitating osmotic

adjustments (Mahajan and Tuteja 2005). The same has been validated in our results, because a greater decline in RWC was recorded in WT, compared to T peanut lines, with increasing salt treatments across different plant growth stages (Fig S2, Supplementary file).

### Antioxidant metabolite and antioxidant enzyme activities (AsA, SOD, CAT, POD, APX and GR)

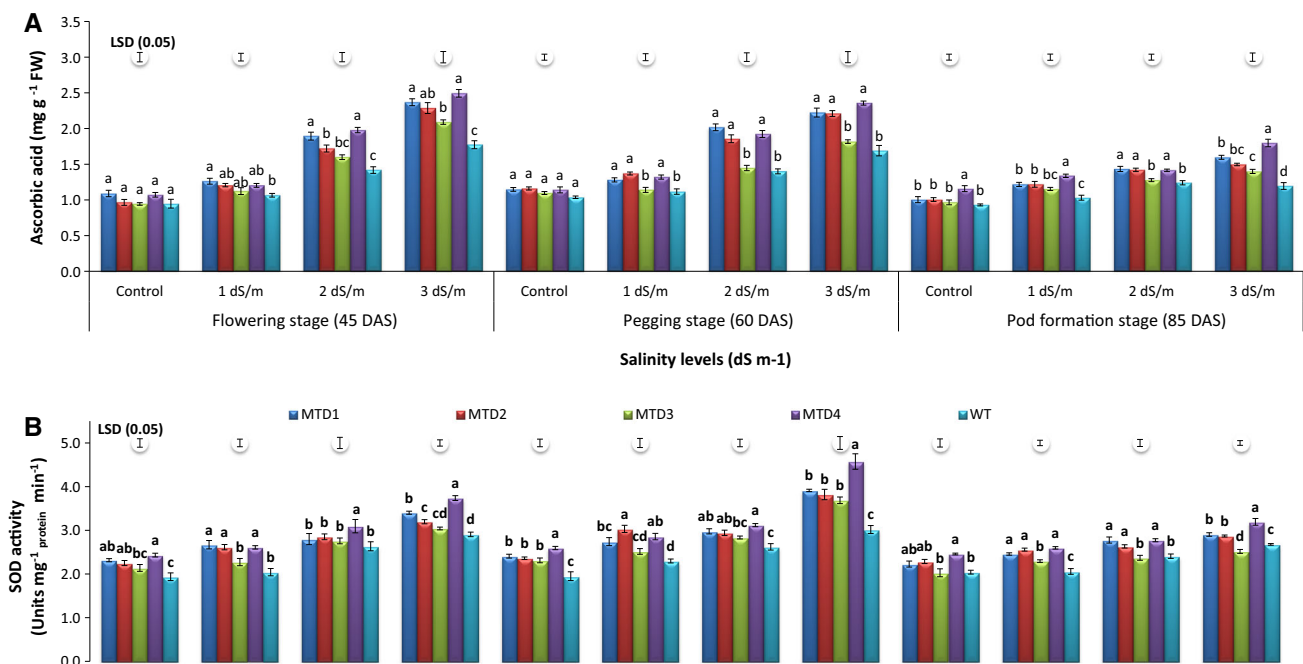
Antioxidants defense system alleviates the oxidative damages caused by ROS produced in response to various stresses and often used for differentiating the salt tolerance response in plants (Chen et al. 2013). This includes low-molecular-mass non-enzyme antioxidant metabolites, such as ascorbic acid and various antioxidant enzymes such as SOD, CAT, APX, GR and POD (Bhatnagar-Mathur et al. 2009; Miller et al. 2010; Chaturvedi et al. 2014).

Ascorbic acid (AsA) is both a secondary metabolite and an antioxidant which interacts enzymatically and non-enzymatically and detoxifies the ROS (Upadhyaya et al. 2011). Therefore, AsA is used as a reference compound in a number of studies associated with stress tolerance in plants (Lopez-Munguia et al. 2011). When compared with WT, an increase in the AsA content was recorded in T lines with increasing salinity stress (Fig. 4a). Moreover, AsA accumulation was found significantly lower when salinity stress was imposed at pod formation stage than other plant growth stages. In all the growth stages, AsA content in the line MTD4 was found higher among other T lines or WT.

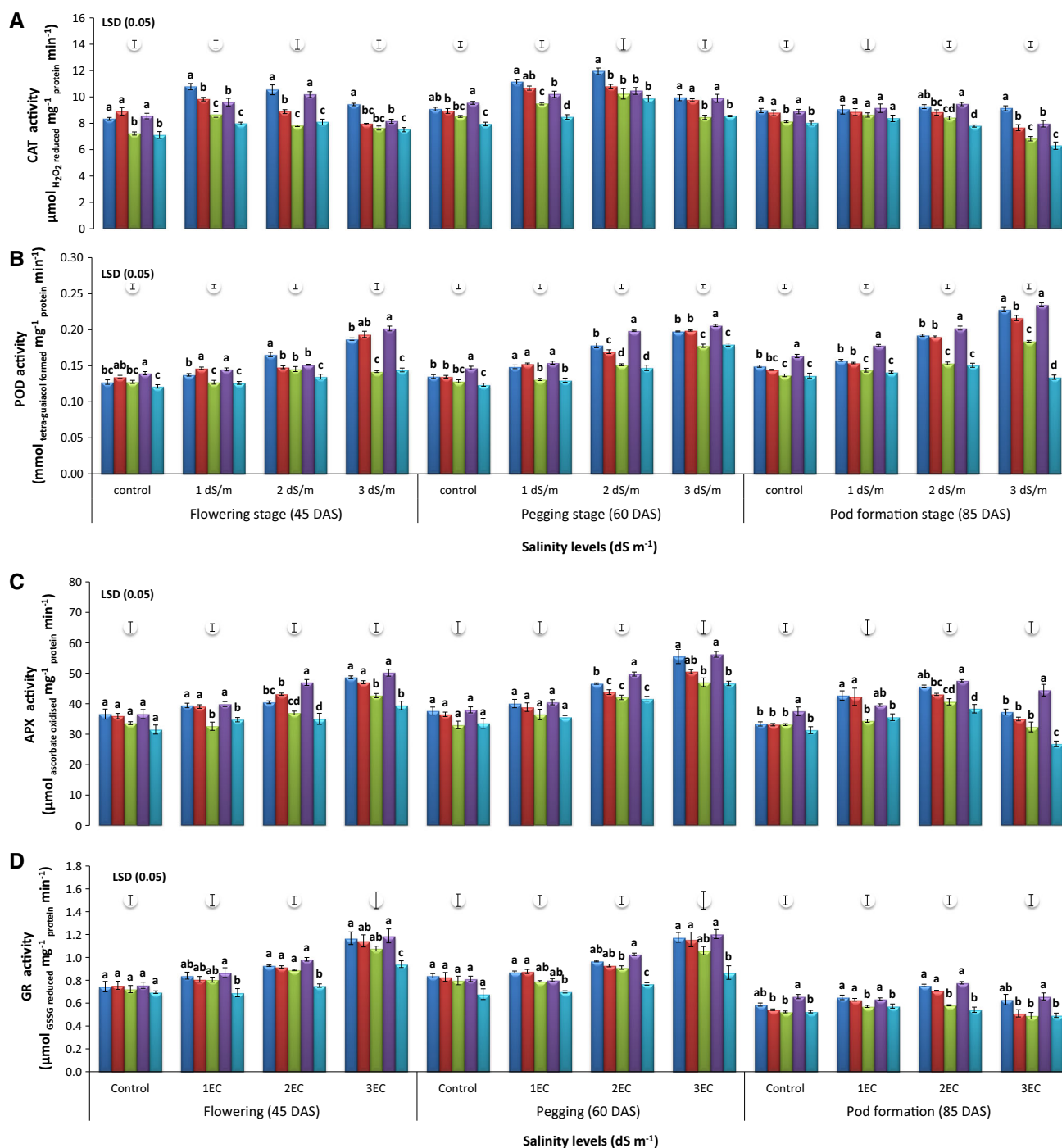
However, MTD3 showed quite fluctuating results, which might be due to the poor production of MTD, which in turn has led to its unstable performance. The phenomenon of increased AsA was also reported in other crops including transgenic-*GalUR* potato (Upadhyaya et al. 2011), and *DHAR* tomato (Li et al. 2012) under salt stress conditions.

Significantly higher activities of SOD, CAT and POD were recorded in T lines compared to WT plants (Figs. 4b, 5a, b). Superoxide radicals generated by oxidative metabolism are dismutated into  $H_2O_2$  and  $O_2$  by SOD. However, CAT dismutates  $H_2O_2$  into  $H_2O$  and  $O_2$ , whereas POD decomposes  $H_2O_2$  by oxidation of co-substrates, such as phenolic compounds and/or antioxidants (Boldt and Scandalios 1997). Although the specific activities of SOD and POD enzymes were increased in both WT and T lines with increasing salinity stress at different growth stages, no clear trend was observed for CAT activity (Figs. 4b, 6a, b). In relation to the plant growth stages, SOD and CAT activities were in the order of pegging > flowering > pod formation stages; while for POD activity, a gradual increase was recorded with progressive growth stages. In general, for SOD and POD activities among different T lines, MTD4 was found better (Figs. 4b, 5b); whereas for CAT, two T lines, MTD1 and MTD4, were found better over others (Fig. 5a) especially at higher salinity levels.

The significant increase in SOD, CAT, and POD activities resulted in efficient detoxification of superoxide radicals so as to minimize the oxidative stress in T peanut lines, compared to WT. This ultimately builds up a better



**Fig. 4** a Relative water content and b superoxide dismutase in WT and T lines under different salinity levels



**Fig. 5** Comparison of antioxidative enzymes viz. **a** Catalase, **b** guaiacol peroxidase, **c** ascorbate peroxidase and **d** glutathione reductase activities in WT and T lines at different salinity levels

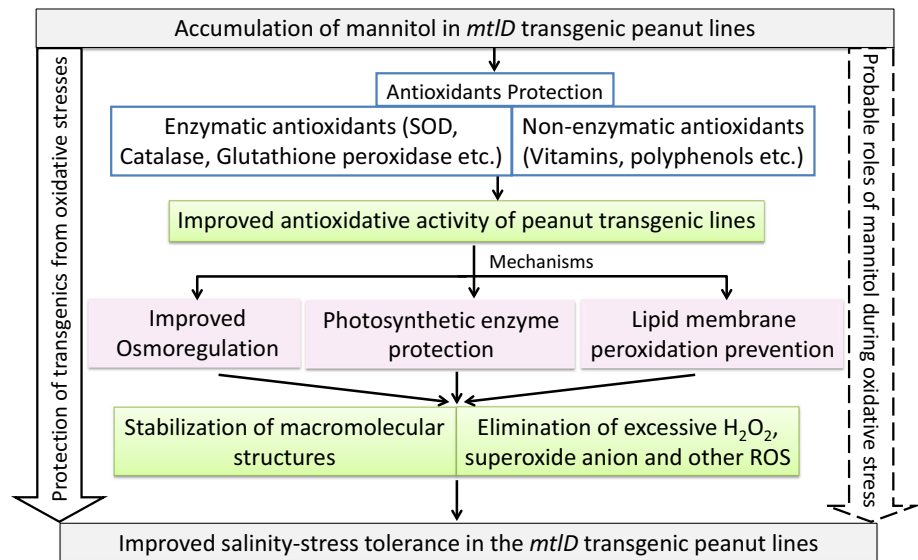
survival capacity in T under higher salinity stress conditions, as also reported by Khare et al. (2010) and Rai et al. (2013). Likewise, in transgenic *MuNAC4* peanut (Pandurangaiah et al. 2014) and *AtDREB1A* peanut (Bhatnagar et al. 2009), *mild* tomato (Khare et al. 2010) and in some peanut cultivars (Chakraborty et al. 2015), enhanced SOD, CAT, and POD activities were reported in response to

various abiotic stresses. Besides, even exogenous application of mannitol is also reported to enhance the antioxidative enzymes activity of metal-induced oxidative stress tolerance in wheat (Adrees et al. 2015).

With increasing salinity stress, a significant increase was recorded for APX and GR activities in T peanut lines than WT, at flowering and pegging stages (Fig. 5c, d). However,



**Fig. 6** Possible roles of mannitol in improving the salinity stress tolerance in the *mtlD* transgenic peanut



a declining trend in their activities was recorded at pod formation stage. APX of the ascorbate–glutathione antioxidant cycle scavenges the  $\text{H}_2\text{O}_2$  (Foyer et al. 1997), whereas GR imparts defense against oxidative stress by sustaining the reduced state of glutathione and maintaining the AsA pool (Miller et al. 2010). Like for many other parameters, T line MTD4 was found exhibiting better APX and GR activities over other lines. Enhanced APX and GR activities were also reported in many other T plants viz., *GalUR* potato (Upadhyaya et al. 2011), *AtDREB1A* peanut (Bhatnagar et al. 2009), *AtDREB1A/CBF3* tomato (Rai et al. 2013), some peanut cultivars (Akçay et al. 2010), giving enhanced tolerance to various oxidative stresses.

In this backdrop, it is hypothesized that the improved antioxidative activity of T peanut lines over WT may be due to the accumulation of mannitol, which might have protected the T lines from the oxidative stresses by eliminating excessive  $\text{H}_2\text{O}_2$ , superoxide anion and other ROS (Cicero et al. 2015). Moreover, mannitol has been shown to play important roles in osmoregulation, photosynthetic enzyme protection and lipid membrane peroxidation prevention, leading to stabilization of macromolecular structures (Rahnama et al. 2011; Adrees et al. 2015). Hence, it is very likely that in the T lines, the mannitol might have protected certain enzymes of various important metabolic and biosynthetic pathways along with the antioxidative machinery, thereby resulting in better salinity tolerance.

#### Accumulation of mannitol and other soluble sugars

Mannitol and other soluble sugars including myo-inositol, trehalose, glucose, fructose and sucrose were determined in both WT and T lines exposed to different levels of salinity

stress (Table 1). Mannitol content in the T peanut lines ranged from 0.09 to 0.27  $\mu\text{mol g}^{-1}$  FW at different levels of salt stress, while it was not detected in WT plants. An increase in the mannitol content was recorded in T lines until 2  $\text{dS m}^{-1}$  salinity levels, which decreased thereafter (3  $\text{dS m}^{-1}$ ). Similarly, a wide range of mannitol accumulation ( $\mu\text{mol g}^{-1}$  FW) was reported for other transgenic plants, such as *Arabidopsis* (0.05–12; Thomas et al. 1995), wheat (0.6–2.0; Abebe et al. 2003), and maize (0.26–0.56; Nguyen et al. 2013). This clearly indicated that even a very low concentration of mannitol can function in maintaining the osmotic adjustments and also improves the osmotic stress tolerance through scavenging of hydroxyl radicals and stabilization of macromolecular structures (Rahnama et al. 2011; Bhauso et al. 2014a).

Besides mannitol, other soluble sugars were also estimated so as to understand their role in salt stress tolerance, which showed different trends (Table 1). Accumulation of hexose sugars, such as glucose and fructose, which also act as osmolytes, got induced as a quick response to mild salt-stress (1  $\text{dS m}^{-1}$ ). However, disaccharides and sugars alcohols like sucrose, trehalose, myo-inositol and mannitol were accumulated at higher salt-stress conditions (3  $\text{dS m}^{-1}$ ), so as to maintain the osmoticum. At higher salt-content (3  $\text{dS m}^{-1}$ ), T lines MTD1 and MTD4 showed higher accumulation of various sugars including mannitol (Table 1). Similarly, *mtlD* transgenic maize has also showed comparatively higher accumulation of glucose, fructose, sucrose and inositol under higher salinity conditions (Nguyen et al. 2013). Thus, the salinity tolerance might be partially due to the induction of antioxidative machinery, and partially because *mtlD* transgenes might have exerted a direct regulatory control on metabolism,

**Table 1** Comparative accumulation of various soluble sugars in T and WT lines at pegging stage under different salinity levels

Salt treatment	Plant ID	Myo-inositol ( $\mu\text{mol g}^{-1}$ FW)	Mannitol ( $\mu\text{mol g}^{-1}$ FW)	Trehalose ( $\mu\text{mol g}^{-1}$ FW)	Glucose ( $\mu\text{mol g}^{-1}$ FW)	Fructose ( $\mu\text{mol g}^{-1}$ FW)	Sucrose ( $\mu\text{mol g}^{-1}$ FW)
Control	WT	12.43 $\pm$ 0.75 <sup>a</sup>	ND	0.48 $\pm$ 0.03 <sup>a</sup>	5.95 $\pm$ 0.24 <sup>a</sup>	3.96 $\pm$ 0.24 <sup>a</sup>	12.83 $\pm$ 0.57 <sup>a</sup>
	MTD1	11.99 $\pm$ 0.57 <sup>a</sup>	0.17 $\pm$ 0.019 <sup>a</sup>	0.47 $\pm$ 0.02 <sup>a</sup>	5.92 $\pm$ 0.46 <sup>a</sup>	3.74 $\pm$ 0.19 <sup>a</sup>	12.96 $\pm$ 0.60 <sup>a</sup>
	MTD2	12.88 $\pm$ 0.80 <sup>a</sup>	0.13 $\pm$ 0.019 <sup>b</sup>	0.52 $\pm$ 0.04 <sup>a</sup>	5.72 $\pm$ 0.20 <sup>a</sup>	3.70 $\pm$ 0.15 <sup>a</sup>	12.77 $\pm$ 0.29 <sup>a</sup>
	MTD3	12.44 $\pm$ 0.40 <sup>a</sup>	0.12 $\pm$ 0.007 <sup>b</sup>	0.47 $\pm$ 0.02 <sup>a</sup>	5.89 $\pm$ 0.42 <sup>a</sup>	3.77 $\pm$ 0.20 <sup>a</sup>	12.53 $\pm$ 0.34 <sup>a</sup>
	MTD4	12.53 $\pm$ 0.81 <sup>a</sup>	0.17 $\pm$ 0.013 <sup>a</sup>	0.48 $\pm$ 0.03 <sup>a</sup>	5.72 $\pm$ 0.30 <sup>a</sup>	3.82 $\pm$ 0.18 <sup>a</sup>	12.61 $\pm$ 0.84 <sup>a</sup>
1 dS m <sup>-1</sup>	WT	15.33 $\pm$ 0.61 <sup>a</sup>	ND	0.54 $\pm$ 0.06 <sup>a</sup>	6.77 $\pm$ 0.55 <sup>a</sup>	4.15 $\pm$ 0.13 <sup>a</sup>	12.67 $\pm$ 0.45 <sup>a</sup>
	MTD1	15.62 $\pm$ 1.10 <sup>a</sup>	0.17 $\pm$ 0.016 <sup>ab</sup>	0.56 $\pm$ 0.04 <sup>a</sup>	7.15 $\pm$ 0.26 <sup>a</sup>	4.24 $\pm$ 0.53 <sup>a</sup>	13.04 $\pm$ 0.65 <sup>a</sup>
	MTD2	15.36 $\pm$ 0.50 <sup>a</sup>	0.15 $\pm$ 0.009 <sup>b</sup>	0.56 $\pm$ 0.02 <sup>a</sup>	6.60 $\pm$ 0.44 <sup>a</sup>	4.13 $\pm$ 0.22 <sup>a</sup>	13.03 $\pm$ 0.21 <sup>a</sup>
	MTD3	14.98 $\pm$ 1.23 <sup>a</sup>	0.11 $\pm$ 0.007 <sup>c</sup>	0.50 $\pm$ 0.05 <sup>a</sup>	6.43 $\pm$ 0.63 <sup>a</sup>	4.07 $\pm$ 0.12 <sup>a</sup>	12.81 $\pm$ 0.41 <sup>a</sup>
	MTD4	15.67 $\pm$ 0.65 <sup>a</sup>	0.19 $\pm$ 0.019 <sup>a</sup>	0.58 $\pm$ 0.03 <sup>a</sup>	7.50 $\pm$ 0.22 <sup>a</sup>	4.18 $\pm$ 0.15 <sup>a</sup>	13.21 $\pm$ 0.31 <sup>a</sup>
2 dS m <sup>-1</sup>	WT	21.23 $\pm$ 0.48 <sup>c</sup>	ND	0.71 $\pm$ 0.04 <sup>b</sup>	4.47 $\pm$ 0.37 <sup>c</sup>	3.45 $\pm$ 0.23 <sup>a</sup>	14.82 $\pm$ 0.41 <sup>b</sup>
	MTD1	25.85 $\pm$ 0.56 <sup>a</sup>	0.25 $\pm$ 0.011 <sup>ab</sup>	0.79 $\pm$ 0.03 <sup>b</sup>	5.47 $\pm$ 0.20 <sup>a</sup>	3.68 $\pm$ 0.43 <sup>a</sup>	15.99 $\pm$ 0.35 <sup>ab</sup>
	MTD2	23.48 $\pm$ 1.25 <sup>b</sup>	0.24 $\pm$ 0.009 <sup>b</sup>	0.75 $\pm$ 0.02 <sup>b</sup>	5.04 $\pm$ 0.30 <sup>abc</sup>	3.43 $\pm$ 0.14 <sup>a</sup>	15.66 $\pm$ 0.35 <sup>ab</sup>
	MTD3	24.44 $\pm$ 0.52 <sup>ab</sup>	0.17 $\pm$ 0.010 <sup>c</sup>	0.73 $\pm$ 0.02 <sup>b</sup>	4.75 $\pm$ 0.25 <sup>bc</sup>	3.21 $\pm$ 0.19 <sup>a</sup>	15.19 $\pm$ 1.03 <sup>ab</sup>
	MTD4	25.28 $\pm$ 0.64 <sup>ab</sup>	0.27 $\pm$ 0.012 <sup>a</sup>	0.88 $\pm$ 0.05 <sup>a</sup>	5.33 $\pm$ 0.17 <sup>ab</sup>	3.70 $\pm$ 0.20 <sup>a</sup>	16.45 $\pm$ 0.18 <sup>a</sup>
3 dS m <sup>-1</sup>	WT	22.79 $\pm$ 1.18 <sup>b</sup>	ND	0.59 $\pm$ 0.02 <sup>c</sup>	3.59 $\pm$ 0.36 <sup>b</sup>	2.48 $\pm$ 0.18 c	12.60 $\pm$ 0.46 <sup>b</sup>
	MTD1	28.65 $\pm$ 0.99 <sup>a</sup>	0.19 $\pm$ 0.013 <sup>a</sup>	0.76 $\pm$ 0.04 <sup>b</sup>	4.86 $\pm$ 0.12 <sup>a</sup>	3.11 $\pm$ 0.11 <sup>a</sup>	14.15 $\pm$ 0.36 <sup>a</sup>
	MTD2	27.16 $\pm$ 0.57 <sup>a</sup>	0.14 $\pm$ 0.010 <sup>b</sup>	0.67 $\pm$ 0.03 <sup>c</sup>	4.08 $\pm$ 0.21 <sup>b</sup>	2.92 $\pm$ 0.14 <sup>ab</sup>	13.25 $\pm$ 0.41 <sup>ab</sup>
	MTD3	24.01 $\pm$ 0.72 <sup>b</sup>	0.09 $\pm$ 0.007 <sup>c</sup>	0.68 $\pm$ 0.04 <sup>c</sup>	3.77 $\pm$ 0.22 <sup>b</sup>	2.68 $\pm$ 0.07 <sup>bc</sup>	12.48 $\pm$ 0.65 <sup>b</sup>
	MTD4	29.09 $\pm$ 1.06 <sup>a</sup>	0.21 $\pm$ 0.012 <sup>a</sup>	0.91 $\pm$ 0.03 <sup>a</sup>	4.68 $\pm$ 0.19 <sup>a</sup>	3.17 $\pm$ 0.18 <sup>a</sup>	14.04 $\pm$ 0.27 <sup>a</sup>

Note The numerical values bearing different lower-case letters as superscripts in a column means that they differ significantly ( $P < 0.01$ ) WT wild type, MTD1–4 independent transgenic events, ND not detected

which could have resulted in protective compounds accumulation either by posttranslational regulation of enzymatic activities or by stabilization of key intermediates, as also reported for herbicide resistance in grass weeds (Cummins et al. 2013).

### Growth parameters

Various plant growth parameters were compared among WT and T lines, which were grown under different salinity levels (control, 1, 2 and 3 dS m<sup>-1</sup>). Rate of seed germination was found higher in T lines at 2 and 3 dS m<sup>-1</sup> of salt stress treatments compared to WT plants (Fig. S3). Moreover, T lines grown under 3 dS m<sup>-1</sup> of salt stress treatment were relatively healthier than WT plants and showed enhanced growth parameters (Fig. S4). The better growth and yield parameters in *mtlD* peanut lines might be due to the better nutrient uptake and various other mechanisms, including osmotic adjustments, detoxification of ROS and stabilization of macromolecules such as membrane proteins and enzymes of different biochemical pathways (Prabhavathi et al. 2002; Rahnama et al. 2011; Adrees et al. 2015) Other details are mentioned in the Supplementary file.

### Correlation studies

Linear correlation analysis was done to find the relationship between key antioxidant enzymes and growth parameters under high salinity stress conditions (3 dS m<sup>-1</sup>) at pod formation stage. MTD activity has recorded strong positive correlation with POD ( $r = 0.94$ ) and RWC ( $r = 0.86$ ), while negative correlation was observed with H<sub>2</sub>O<sub>2</sub> ( $r = -0.72$ ) and MDA ( $r = -0.81$ ) content (Table 2). This suggests the possibility of mannitol helping in scavenging the ROS and stabilizing the macromolecular structures, which in turn improved the plants' ability of abiotic stress tolerance (Prabhavathi et al. 2002; Prabhavathi and Rajam 2007).

Significant and strong correlations were recorded among antioxidant enzymes, like APX and CAT ( $r = 0.83$ ) and APX and POD ( $r = 0.80$ ). In general, strong-to-moderate correlations were observed between antioxidant enzymes with growth and physio-biochemical parameters (Table 2). H<sub>2</sub>O<sub>2</sub> and MDA showed strong negative correlation with antioxidant enzymes, including POD, which showed negative correlation with H<sub>2</sub>O<sub>2</sub> ( $r = -0.68$ ) and MDA ( $r = -0.83$ ). These results indicate that POD is strongly associated in H<sub>2</sub>O<sub>2</sub> scavenging and thereby minimizing the

**Table 2** Correlation analysis among various antioxidant enzymes and growth parameters

	MTD	SOD	CAT	POD	APX	GR	H <sub>2</sub> O <sub>2</sub>	RWC	MDA	AsA	HI	PW	RL	SL	RW	SW
MTD	1															
SOD	0.636*	1														
CAT	0.783**	0.568*	1													
POD	0.937**	0.682**	0.783**	1												
APX	0.858**	0.726**	0.831**	0.803**	1											
GR	0.691**	0.761**	0.677**	0.709**	0.704**	1										
H <sub>2</sub> O <sub>2</sub>	-0.783**	-0.217	-0.624*	-0.677**	-0.622*	-0.236	1									
RWC	0.864**	0.429	0.698**	0.758**	0.799**	0.558*	-0.740**	1								
MDA	-0.813**	-0.623*	-0.750**	-0.830**	-0.755**	-0.514	0.658**	-0.696**	1							
AsA	0.764**	0.577*	0.807**	0.682**	0.783**	0.587*	-0.715**	0.819**	-0.796**	1						
HI	0.816**	0.454	0.862**	0.824**	0.735**	0.634*	-0.735**	0.780**	-0.789**	0.850**	1					
PW	0.781**	0.561*	0.867**	0.797**	0.777**	0.691**	-0.647**	0.742**	-0.799**	0.865**	0.972**	1				
RL	0.527*	0.392	0.675**	0.531*	0.583*	0.509	-0.196	0.600*	-0.598*	0.572*	0.649**	0.704**	1			
SL	0.857**	0.574*	0.564*	0.780**	0.672**	0.647**	-0.490	0.769**	-0.698**	0.586*	0.646**	0.616*	0.646**	1		
RD	0.863**	0.682**	0.845**	0.890**	0.835**	0.632*	-0.687**	0.782**	-0.830**	0.803**	0.878**	0.906**	0.693**	0.726**	1	
SD	0.728**	0.601*	0.704**	0.721**	0.789**	0.620*	-0.531*	0.672**	-0.737**	0.765**	0.797**	0.864**	0.729**	0.662**	0.838**	1

Asterisks represent significant correlation at \*  $P \leq 0.05$  and \*\*  $P \leq 0.01$ . For each parameter, average values of four T lines along with WT were used

MTD mannitol-1-phosphate dehydrogenase, SOD superoxide dismutase, CAT catalase, POD guaiacol peroxidase, APX ascorbate peroxidase, GR glutathione reductase, RWC relative water content, MDA malondialdehyde, AsA ascorbate, HI harvest index, PW pod weight, RL root length, SL shoot length, RW root weight, SW shoot weight

membrane damage. Interestingly, RWC showed strong positive correlations ( $r = 0.70\text{--}0.82$ ) with many antioxidant traits (CAT, POD, APX and AsA) and strong negative correlations with  $\text{H}_2\text{O}_2$  ( $r = -0.74$ ) and MDA ( $r = -0.70$ ) which were significant. Thus, the improvement in RWC has helped in increasing the antioxidants in the T lines and thereby reduced the oxidative stress, which has led to the better salinity stress tolerance. Other growth parameters were found to moderately to strongly correlate with each other (Table 2).

In general, positive correlations were observed among antioxidant enzymes while negative correlation with  $\text{H}_2\text{O}_2$  and MDA (Table 2). This observation strongly implies the possibility that antioxidant enzyme systems are utilized in T lines to alleviate oxidative stress caused by salinity. Here, enhanced oxidative stress tolerance under salt stress was associated with the overexpression of *mtlD* gene, which increased the activities of these enzymes. These findings again support the opinion that mannitol contributed to lower ROS and provides better osmotic adjustments leading to improved abiotic tolerance (Stoop et al. 1996; Prabhavathi et al. 2002). Thus, salt tolerance of transgenic peanut plants overexpressing *mtlD* gene can be associated with transgene-induced activation of cascade of peanut genes involved in providing protection from oxidative injury, as also observed for tobacco expressing *CsGSTUs* gene (Cicero et al. 2015).

## Conclusions

Single gene insertion has been shown to impart salinity stress tolerance in T peanut lines at various growth stages, by mannitol accumulation which, in turn, has affected the overall antioxidative machinery of the T lines. The present study is an extension of our previous work (Bhauso et al. 2014a, b). Here, we report the best lines for their further use in imparting salinity stress tolerance in peanut. Enhanced MTD activity, antioxidant systems, RWC, sugar accumulation, growth parameters and lessened  $\text{H}_2\text{O}_2$  and MDA content are the evidences that the T lines bloomed well even at  $3 \text{ dS m}^{-1}$  salt stress conditions, compared to WT. Further, the role of mannitol and other sugars is also established in imparting the salinity stress tolerance. Moreover, the *mtlD* transgenic lines were also found maintaining lower oxidative injury, indicating amelioration of salinity-induced oxidative stress by enhanced protective mechanisms via mannitol accumulation and antioxidative response. It is, thus, revalidated that not only mannitol is involved in maintaining the cellular homeostasis, but the expression of other antioxidant genes might also be influenced by the expression of *mtlD* gene, thereby modulating ROS scavenging/detoxification during salt stress conditions

and, thus, imparting salinity stress tolerance. A sketch, depicting the most probable roles of mannitol in improving the salinity stress tolerance in *mtlD* transgenic peanut is presented in Fig. 6.

In olive, it is reported that salinity stress tolerance involves both production and transport of mannitol through mannitol transport system (*OeMaT1*) gene expression (Conde et al. 2011). This clearly means that both synthesis and transport of mannitol are equally important in imparting salinity stress tolerance. Therefore, there is a need to further find the mechanism of mannitol transport in transgenic peanut lines so as to better understand the salinity stress tolerance mechanism in different T events.

This is the first report in peanut *mtlD* transgenic giving such a detailed analysis for salinity stress tolerance at various growth stages. Among four T events tested, two lines, MTD1 and MTD4, overexpressing *mtlD* gene showed better tolerance to salinity stress, and these lines will be tested further under open-field trials. Finally, the best event(s) can be utilized directly in the marginal areas having high salinity by the small farmers of India. Besides, these events can also be used in the peanut improvement programs, as a valuable pre-breeding resource for imparting salinity stress tolerance.

**Author contribution statement** Kirankumar G. Patel: Conducted the experiments and collected data, Biochemical analysis. Viralkumar B. Mandaliya: Biochemical analysis. Gyan P. Mishra: Analysis of data. Jentilal R. Dobarra: Planted and maintained of the experiments in glass house. Radhakrishnan Thankappan: Conceived and planned the experiments.

**Acknowledgments** The research work was financially supported by the Indian Council of Agricultural Research, New Delhi, India.

## References

- Abebe T, Guenzi AC, Martin B, Cushman JC (2003) Tolerance of mannitol accumulating transgenic wheat to water stress and salinity. *Plant Physiol* 131:1748–1755. doi:10.1104/pp.102.003616
- Adrees M, Ali S, Iqbal M, Bharwana SA, Siddiqi Z, Farid M, Ali Q, Saeed R, Rizwan M (2015) Mannitol alleviates chromium toxicity in wheat plants in relation to growth, yield, stimulation of anti-oxidative enzymes, oxidative stress and Cr uptake in sand and soil media. *Ecotox Environ Safe* 122:1–8. doi:10.1016/j.ecoenv.2015.07.003
- Aebi H (1984) Catalase in vitro. *Methods Enzymol* 105:121–126
- Akcay UC, Ercan O, Kavas M, Yildiz L, Yilmaz C, Oktem HA, Yucel M (2010) Drought-induced oxidative damage and antioxidant responses in peanut (*Arachis hypogaea* L.) seedlings. *Plant Growth Regul* 61:21–28. doi:10.4161/psb.6.6.15340
- Barrs HD, Weatherley PE (1962) A re-examination of the relative turgidity technique for estimating water deficits in leaves. *Aust J Biol Sci* 24:519–570. doi:10.1071/B19620413

- Bhatnagar-Mathur P, Devi MJ, Vadez V, Sharma KK (2009) Differential antioxidative responses in transgenic peanut bear no relationship to their superior transpiration efficiency under drought stress. *J Plant Physiol* 166:1207–1217. doi:10.1016/j.jplph.2009.01.001
- Bhauso TD, Radhakrishnan T, Kumar A, Mishra GP, Dobaria JR, Rajam MV (2014a) Over-expression of bacterial *mtlD* gene confers enhanced tolerance to salt-stress and water-deficit stress in transgenic peanut (*Arachis hypogaea*) through accumulation of mannitol. *Aust J Crop Sci* 8:413–421
- Bhauso TD, Radhakrishnan T, Kumar A, Mishra GP, Dobaria JR, Patel K, Rajam MV (2014b) Overexpression of bacterial *mtlD* gene in peanut improves drought tolerance through accumulation of mannitol. *Sci World J*. doi:10.1155/2014/125967
- Boldt R, Scandalios JG (1997) Influence of UV-light on the expression of the *CAT 2* and *CAT 3* catalase genes in maize. *Free Radic Biol Med* 23:505–514. doi:10.1016/S0891-5849(97)00111-1
- Bonin P, Groisillier A, Raimbault A, Guibert A, Boyen C, Tonon T (2015) Molecular and biochemical characterization of mannitol-1-phosphate dehydrogenase from the model brown alga *Ectocarpus* sp. *Phytochemistry* 117:509–520. doi:10.1016/j.phytochem.2015.07.015
- Boote KJ (1982) Growth stages of peanut (*Arachis hypogaea* L.). *Peanut Sci* 9:35–40. doi:10.3146/10095-3679-9-1-11
- Castillo FI, Penel I, Greppin H (1984) Peroxidase release induced by ozone in *Sedum album* leaves. *Plant Physiol* 74:846–851. doi:10.1104/pp.74.4.846
- Chakraborty K, Sairam RK, Bhattacharya RC (2012) Differential expression of salt overly sensitive pathway genes determines salinity stress tolerance in *Brassica* genotypes. *Plant Physiol Biochem* 51:90–101. doi:10.1016/j.plaphy.2011.10.001
- Chakraborty K, Singh AL, Kalariya KA, Goswami N, Zala PV (2015) Physiological responses of peanut (*Arachis hypogaea* L.) cultivars to water deficit stress: status of oxidative stress and antioxidant enzyme activities. *Acta Bot Croat* 74. doi:10.1515/botcro-2015-0011
- Chaturvedi AK, Patel MK, Mishra A, Tiwari V, Jha B (2014) The *SbMT-2* Gene from a halophyte confers abiotic stress tolerance and modulates ROS scavenging in transgenic tobacco. *PLoS One* 9:e111379. doi:10.1371/journal.pone.0111379
- Chen P, Yan K, Shao H, Zhao S (2013) Physiological mechanisms for high salt tolerance in wild soybean (*Glycine soja*) from yellow river delta, China: photosynthesis, osmotic regulation, ion flux and antioxidant capacity. *PLoS One* 8:e83227. doi:10.1371/journal.pone.0083227
- Cicero LL, Madesis P, Tsaftaris A, Piero ARL (2015) Tobacco plants over-expressing the sweet orange tau glutathione transferases (*CsGSTUs*) acquire tolerance to the diphenyl ether herbicide fluorodifen and to salt and drought stresses. *Phytochemistry* 116:69–77. doi:10.1016/j.phytochem.2015.03.004
- Conde A, Silva P, Agasse A, Conde C, Gerós H (2011) Mannitol transport and mannitol dehydrogenase activities are coordinated in *Olea europaea* under salt and osmotic stresses. *Plant Cell Physiol* 52:1766–1775. doi:10.1093/pcp/pcr121
- Cummins I, Wortley DJ, Sabbadin F, He Z, Coxon CR, Straker HE, Sellars JD, Knight K, Edwards L, Hughes D, Kaundun SS, Hutchings S, Steel PG, Edwards R (2013) Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds. *Proc Nat Acad Sci USA* 110:5812–5817
- Dellavalle NB (1992) Determination of specific conductance in supernatant 1:2 soil:water solution. Handbook on reference methods for soil analysis. Soil and Plant Analysis Council, Athens, pp 44–50
- Dhindsa RA, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. *J Exp Bot* 126:93–101. doi:10.1093/jxb/32.1.93
- FAOSTAT (2014) FAO statistics division 2015. <http://faostat.fao.org/> Accessed 03 September 2015
- Foyer CH, Lopez-Delgado H, Dat JF, Scott IM (1997) Hydrogen peroxide and glutathione associated mechanisms of acclimatory stress tolerance and signaling. *Physiol Plant* 100:241–254. doi:10.1111/j.1399-3054.1997.tb04780.x
- Fryer MJ, Oxborough K, Mullineaux PM, Baker NR (2002) Imaging of photo-oxidative stress responses in leaves. *J Exp Bot* 53:1249–1254. doi:10.1093/jexbot/53.372.1249
- Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* 125:189–198. doi:10.1016/0003-9861(68)90654-1
- Hema R, Vemanna RS, Sreeramulu S, Reddy CP, Senthil-Kumar M, Udayakumar M (2014) Stable expression of *mtlD* gene imparts multiple stress tolerance in finger millet. *PLoS One* 9:e99110. doi:10.1371/journal.pone.0099110
- Iwamoto K, Kawanobe H, Ikawa T, Shiraiwa Y (2003) Characterization of salt regulated mannitol-1-phosphate dehydrogenase in the red alga *Caloglossa continua*. *Plant Physiol* 133:893–900. doi:10.1104/pp.103.026906
- Khare N, Goyary D, Singh NK, Shah P, Rathore M, Anandhan S, Sharma D, Arif M, Ahmed Z (2010) Transgenic tomato cv. Pusa Uphar expressing a bacterial *mannitol-1-phosphate dehydrogenase* gene confers abiotic stress tolerance. *Plant Cell Tissue Organ Cult* 103:267–277. doi:10.1007/s11240-010-9776-7
- Li Q, Li Y, Li C, Yu X (2012) Enhanced ascorbic acid accumulation through overexpression of dehydroascorbate reductase confers tolerance to methyl viologen and salt stresses in tomato. *Czech J Genet Plant Breed* 48:74–86
- Li XY, Liu X, Yao Y, Li Y, Liu S, He C, Li J, Lin Y, Li L (2013) Overexpression of *Arachis hypogaea AREB1* gene enhances drought tolerance by modulating ROS scavenging and maintaining endogenous ABA content. *Int J Mol Sci* 14:12827–12842. doi:10.3390/ijms140612827
- Lopez-Munguia A, Hernandez-Romero Y, Pedraza-Chaverri J, Miranda-Molina A, Regla I, Martinez A, Castillo E (2011) Phenylpropanoid glycoside analogues: enzymatic synthesis, antioxidant activity and theoretical study of their free radical scavenger mechanism. *PLoS One* 6:e20115. doi:10.1371/journal.pone.0020115
- Lowry OH, Rosenbrough JJ, Farr AL, Randall RJ (1951) Estimation of protein with the folin phenol reagent. *J Biol Chem* 193:265–275
- Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: an overview. *Arch Biochem Biophys* 444:139–158. doi:10.1016/j.abb.2005.10.018
- Martinez-Beltran J, Manzur CL (2005) Overview of salinity problems in the world and FAO strategies to address the problem. In: Proceedings of the international salinity forum, Riverside, pp 311–313
- Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ* 33:453–467. doi:10.1111/j.1365-3040.2009.02041.x
- Mishra GP, Radhakrishnan T, Kumar A, Thirumalaisamy PP, Kumar N, Bosamia TC, Nawade B, Dobaria JR (2015) Advancements in molecular marker development and their applications in the management of biotic stresses in peanuts. *Crop Prot*. doi:10.1016/j.cropro.2015.07.019
- Mukherjee SP, Choudhuri MA (1983) Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. *Physiol Plant* 58:166–170. doi:10.1111/j.1399-3054.1983.tb04162.x

- Nakamura Y, Ikawa T (1993) Purification and properties of NADH: nitrate reductase from the red alga *Porphyra yezoensis*. Plant Cell Physiol 34:1239–1249
- Nakano Y, Asada K (1981) Spinach chloroplasts scavenge hydrogen peroxide on illumination. Plant Cell Physiol 21:1295–1307
- Nguyen TX, Nguyen T, Alameldin H, Goheen B, Loescher W, Sticklen M (2013) Transgene pyramiding of the *HVA1* and *mtlD* in T3 Maize (*Zea mays* L.) plants confers drought and salt tolerance, along with an increase in crop biomass. Int J Agron (article ID 598163). doi:10.1155/2013/598163
- Oupadissakoon C, Young CT, Mazingo RW (1980) Evaluation of free amino acid and free sugars contents in five lines of Virginia-type peanuts at five locations. Peanut Sci 5:31–34. doi:10.3146/10095-3679-7-1-13
- Pandurangaiah M, Rao GL, Sudhakarbabu O, Nareshkumar A, Kiranmai K, Lokesh U, Thapa G, Sudhakar C (2014) Overexpression of horsegram (*Macrotyloma uniflorum* Lam. Verdc.) NAC transcriptional factor (*MuNAC4*) in groundnut confers enhanced drought tolerance. Mol Biotechnol. doi:10.1007/s12033-014-9754-0
- Parida AK, Jha B (2013) Inductive responses of some organic metabolites for osmotic homeostasis in peanut (*Arachis hypogaea* L.) seedlings during salt stress. Acta Physiol Plant 35:2821–2832. doi:10.1007/s11738-013-1315-9
- Prabhavathi V, Rajam MV (2007) Mannitol accumulating transgenic eggplants exhibit enhanced resistance to fungal wilts. Plant Sci 173:50–54. doi:10.1016/j.plantsci.2007.04.004
- Prabhavathi V, Yadav JS, Kumar PA, Rajam MV (2002) Abiotic stress tolerance in transgenic eggplant (*Solanum melongena* L.) by introduction of bacterial mannitol phosphate dehydrogenase gene. Mol Breed 9:137–147. doi:10.1023/A:1026765026493
- Rahnama H, Haghghat V, Hossain F, Behzad G (2011) Enhanced salt stress tolerance in transgenic potato plants (*Solanum tuberosum* L.) expressing a bacterial *mtlD* gene. Acta Physiol Plant 33:1521–1532. doi:10.1007/s11738-010-0690-8
- Rai GK, Rai NP, Rathaur S, Kumar S, Singh M (2013) Expression of *rd29A: AtDREB1A/CBF3* in tomato alleviates drought-induced oxidative stress by regulating key enzymatic and non-enzymatic antioxidants. Plant Physiol Biochem 69:90–100. doi:10.1016/j.plaphy.2013.05.002
- Ramel F, Sulmon C, Bogard M, Couee I, Gouesbet G (2009) Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose induced tolerance in *Arabidopsis thaliana* plantlets. BMC Plant Biol 9:28. doi:10.1186/1471-2229-9-28
- Rao MV, Paliyath G, Ormrod DP, Murr DP, Watkins CB (1997) Influence of salicylic acid on H<sub>2</sub>O<sub>2</sub> production, oxidative stress and H<sub>2</sub>O<sub>2</sub> metabolizing enzymes. Plant Physiol 115:137–149. doi:10.1104/pp.115.1.137
- Sarkar T, Thankappan R, Kumar A, Mishra GP, Dobarra JR (2014) Heterologous expression of the *AtDREB1A* gene in transgenic peanut-conferred tolerance to drought and salinity stresses. PLoS One 9:e110507. doi:10.1371/journal.pone.0110507
- Singh R, Issar D, Zala PV, Nautiyal PC (2007) Variation in sensitivity to salinity in groundnut cultivars during seed germination and early seedling growth. JSAT Agric Res 5(1):1–7
- Singh AL, Hariprassana K, Solanki RM (2008) Screening and selection of groundnut genotypes for tolerance of soil salinity. Aust J Crop Sci 1:69–77
- Smith IK, Vierheller TLI, Thorne CA (1988) Assay of glutathione reductase in crude tissue homogenates using 5, 5'-dithio bis (2-nitrobenzoic acid). Anal Biochem 175:408–413. doi:10.1016/0003-2697(88)90564-7
- Stoop JMH, Williamson JD, Pharr DM (1996) Mannitol metabolism in plants: a method for coping with stress. Trends Plant Sci 1:139–144. doi:10.1016/S1360-1385(96)80048-3
- Tang W, Peng X, Newton RJ (2005) Enhanced tolerance to salt stress in transgenic loblolly pine simultaneously expressing two genes encoding mannitol-1-phosphate dehydrogenase and glucitol-6-phosphate dehydrogenase. Plant Physiol Biochem 43:139–146. doi:10.1016/j.plaphy.2005.01.009
- Thomas JC, Sepahi M, Arendall B, Bohnert HJ (1995) Enhancement of seed germination in high salinity by engineering mannitol expression in *Arabidopsis thaliana*. Plant Cell Environ 18:801–806. doi:10.1111/j.1365-3040.1995.tb00584.x
- Upadhyaya CP, Venkatesh J, Gururani MA, Asnin L, Sharma K, Ajappala K, Park SW (2011) Transgenic potato overproducing L-ascorbic acid resisted an increase in methylglyoxal under salinity stress via maintaining higher reduced glutathione level and glyoxalase enzyme activity. Biotechnol Lett 33:2297–2307. doi:10.1007/s10529-011-0684-7