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## Full Length Research Article

# ENHANCED OSMOLYTE ACCUMULATION COUNTERACTS SALT STRESS BY CONTROLLED ROS: EVIDENCE FROM BIOCHEMICAL STUDIES

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**ABSTRACT**

Salinity is the most damaging factor towards crop production. Observing the possible role of thiourea in salinity tolerance, experiments were carried out using optimized thiourea level (400 µM) at which two maize hybrids may show improved growth and physiological attributes in autumn and spring seasons. Salt stress caused a substantial suppression of growth and damaging effects were observed as reduction in shoot and root length, fresh and dry weights, leaf area and increased accumulation of hydrogen peroxide ($H_2O_2$), malondialdehyde (MDA) and of some key osmolytes like proline, glycinebetain (GB), soluble sugars, total free amino acids (TFAA), proteins and anthocyanins in both maize hybrids. Medium supplemented thiourea was found to be very effective in improving growth attributes like shoot and root length, fresh and dry weights, improved leaf area, controlled $H_2O_2$, MDA and enhanced accumulation of osmolytes like proline, glycinebetain (GB), soluble sugars, total free amino acids (TFAA), soluble proteins and anthocyanins under control and saline conditions. Salinity tolerance produced by TU was superior in the spring than in autumn grown maize. From the changes in the growth and physiological attributes of maize, it is concluded that thiourea application owes a great potential to alleviate the deteriorating effects of salt stress on maize, and is recommended for improving growth in marginally to moderately saline soils.



## INTRODUCTION

Salinity is the most limiting factor of plant growth (Dasgan *et al*., 2002; Sreenivasulu *et al*., 2007). Salinity is a complex abiotic stress, it mainly effects osmotic components and poses a great threat to agriculture in arid and semi-arid areas of world (Munns *et al*., 2006). Salt stress induces a wide range of trepidations; but it is not always possible to distinguish these negative effects are either associated with ionic toxicity or due to some other abiotic stress. These metabolic alterations results in growth reduction. However in order to withstand these stressful conditions all plants have developed different tolerance mechanisms such as controlled ROS and enhanced osmolyte accumulation (Noreen *et al*., 2010;Perveen*et al*., 2015).This salt tolerance is different among different plant species (Munnes *et al*., 2002) and even among hybrids/cultivars of different species (Al-Karaki, 2000; Ghoulam *et al*., 2002). Salinity reduces growth due to higher accumulation of reactive oxygen species (ROS) (Ashraf, 2009; Miller *et al*., 2010). Under normal conditions ROS are generated at controlled or non-toxic levels (Mittler, 2002; Kaya *et al*., 2015). But under salt stress all these substances are accumulated at higher concentrations that become toxic for normal plant growth and resists in accumulation of other essential osmolytes and nutrients (Raza *et al*., 2007; Hameed *et al*., 2008, 2009, 2013; Golldack *et al*., 2014; Noctor *et al*., 2014). Most degradative effect of salt stress is the production of ROS such as $H_2O_2$ and MDA that results in oxidative damage at cellular levels (Hameed *et al*., 2010, 2011). However to counteract damaging effects of ROS plants enhance accumulation of osmolytes like soluble proteins (Kaya *et al*., 2015; Perveen *et al*., 2015). Higher the accumulation of these solutes, higher will be tolerance (Shafi *et al*., 2011) such as proline and GB considered to actas compatible osmolytes that takes part in salt resistance mechanisms (Ehsanpour and Fatahian, 2003; Sakhanokho and Kelley, 2009). Many stress alleviating chemicals including

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thiols are very important in enhancing crop productivity (Sahu *et al*., 2005; Nathawat *et al*., 2007). Thiourea can completely stop $H_2O_2$ and MDA formation (Zhu *et al*., 2002). Thus thiourea is thought to be direct scavenger of hydrogen peroxide radicals (Kelner *et al*., 1990; Sahu *et al*., 2005). Improved plant growth with thiourea application under stress have been observed in maize (Sahu *et al*., 1993; Perveen *et al*., 2013, 2015), wheat (Sahu *et al*., 2006; Anjum *et al*., 2011), barely (Ikram and Javed, 2015). Thiourea also upgrades accumulation of compatible solutes like proline, GB, free amino acids and proteins to improve plant tolerance in wheat (Asthir *et al*., 2013). In view of above reports, the present study was planned to divulge the effect of thiourea on control of ROS and enhanced osmolyte in leaf and root tissues of two maize hybrids in both autumn and spring growing seasons.

## MATERIALS AND METHODS

### Plant material

Experiments were conducted in the autumn and spring seasons of 2013-14 in the Department of Botany, University of Agriculture Faisalabad. Seeds of two maize hybrids (DK6789 and 33M15) were obtained from Maize and Millet Research Institute, Sahiwal. Seeds were sown in plastic pots containing river sand. An optimized level (400 µM) of TU and 120 mM NaCl salinity were applied to 15 days old plants, mixed in half strength nutrient solution (Hoagland and Arnon, 1950). After fifteen days of treatment, the data were taken for growth attributes, one plant preserved as fresh material for different analysis like determination of osmolytes and reactive oxygen species in freezer at -30$^{o}$C. Number of leaves and roots along with their lengths and fresh weights were measured and plants were placed in dry oven at 70 ºC for 7 days and their dry weights were again measured. Hydrogen peroxide in the leaf samples was determined at an absorbance of 390 nm using a UV-Vis spectrophotometer (IRMECO U-2020) with the method of Velikova*et al*., (2000). The malonyldialdehyde (MDA) was measured at two wavelengths of 532 and 600 nm (Carmak and Horst, 1991). Proline and glycinebetain were estimated using Bates *et al*., (1973) protocol and Grieve and Grattan, (1983) method respectively. Soluble sugars and total free amino acids were estimated using Yemm and Willis, (1954) and Hamilton and Van-Slyke, (1943) methods. Total soluble proteins were estimated using the dye-binding method described by Bradford, (1976). Anthocyanins were estimated by using Stark and Wray (1989) method.

### Statistical analysis

The design of the experiment was completely randomized factorial and each treatment was replicated thrice. The data were subjected to statistical analysis using COSTAT computer software. Analysis of variance were performed separately for each analysis. Treatment means were marked with alphabets when H×S×TU interactions were significant. Bars indicating standard errors.

## RESULTS

**Growth characteristics:** Statistical analysis of data for shoot and root length revealed that 400 µM thiourea produced significant ($P<0.01$) differences on shoot (Fig. 1a) and root length (Fig. 1b). Applied salt stress severely affected the shoot and root length of both hybrids in both the seasons but the medium supplementation of thiourea significantly improved shoot and root length, although the shoot indicated greater improvement than root. Of the two seasons, the effectiveness of thiourea was greater in the spring than autumn season under stress and non-stress conditions. Of the two hybrids, 33M15 performed better than DK-6789 (Fig. 1a, b). Data showed that that both salinity and 400 µM thiourea significantly affected leaf (Fig. 1c) and root number (Fig. 1d) in both the hybrids and in both the seasons. Applied salinity although reduced the production of leaves and roots in both the hybrids over respective controls, the medium supplementation of thiourea increased these numbers under salt stress or non-stress conditions. (Fig. 1c, d). Statistical analysis of data recorded for shoot and root fresh and dry weights showed significant differences ($P<0.01$) in the hybrids, salt levels and thiourea treatments. Applied salt stress severely affected shoot and root fresh and dry weight of both hybrids in both seasons (Fig. 2a, b, c and d). Thiourea significantly improved these attributes. Maximum length, number of leaves and roots were counted and in case of biomass attributes at better results were found at 400 µM thiourea level in spring season for 33M15 hybrid, while DK-6789 performed poorly in both the seasons.

### Biochemical attributes

Data revealed that salt stress and seasons differed significantly ($P<0.01$), while hybrids and thiourea treatments showed non-significant ($P>0.05$) difference for leaf $H_2O_2$ concentration, spring data indicated significant differences among hybrids, thiourea and salt for leaf $H_2O_2$ and MDA (Fig. 3a, b). Applied salinity induced the accumulation of leaf $H_2O_2$ and MDA, but medium supplementation of thiourea significantly reduced $H_2O_2$ and MDA content. The behavior performance of both the hybrids was different in both the seasons. Analysis of data revealed for leaf and root proline showed that salt stress significantly affected ($P<0.01$) while hybrids and thiourea treatments showed non-significant ($P>0.05$) results. Applied salinity considerably enhanced the accumulation of leaf and root proline of both hybrids in both seasons. Thiourea also upregulated proline accumulation in both leaf and root tissues (Fig. 4a, b). Statistical analysis of data revealed non-significant ($P>0.05$) differences in the hybrids while thiourea and salt levels exerted significant ($P<0.01$) differences for glycinebetain of both leaf and root tissues. Salinity affected leaf and root glycinebetain of both hybrids. A marked enhanced accumulation of glycinebetain in salt stressed plants observed. Thiourea also enhanced their accumulation (Fig.4c, d). Data revealed significant differences ($P<0.01$) in salt levels while thiourea treatments and hybrids exerted non-significant ($P>0.05$) effects for leaf and root soluble sugars. Salinity significantly affected leaf and root soluble sugars and marked accumulation of soluble sugars in salt stressed plants was observed. Root application of thiourea significantly improved soluble sugars (Fig. 5a, b). Analysis of recorded data showed significant ($P<0.01$) differences among salt and thiourea levels, but hybrids did not exerted any significant ($P>0.05$) effect for leaf and root total free amino acids. Salinity imposition increased TFAA, but root application of thiourea significantly reduced leaf and root TFAA (Fig. 5c, d).

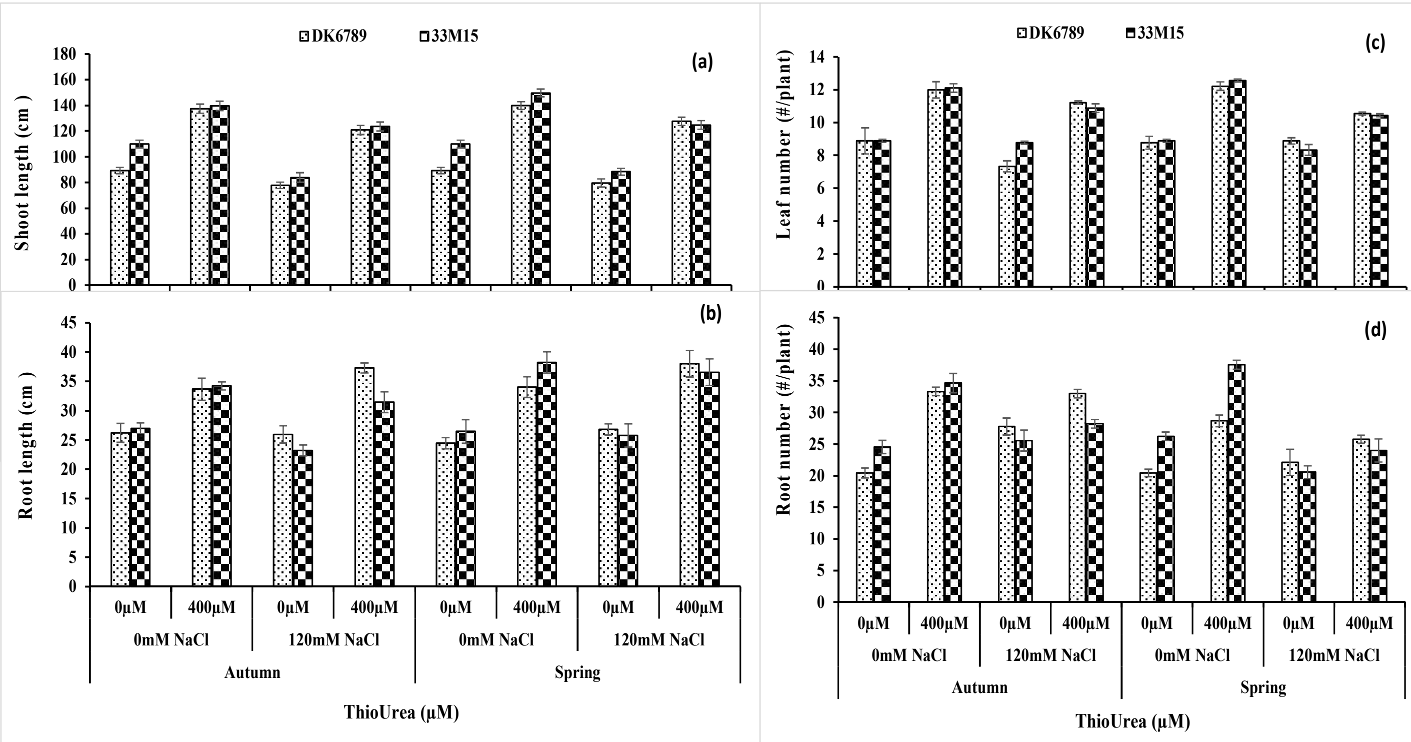


**Fig. 1. Changes in some growth attributes of maize hybrids under salt stress and role of thiourea in alleviating salt stress toxicity in autumn and spring seasons. The bars labeled with alphabets show significant (P<0.01) TU/S/Season (TU=Thiourea level, S=Salt level)**

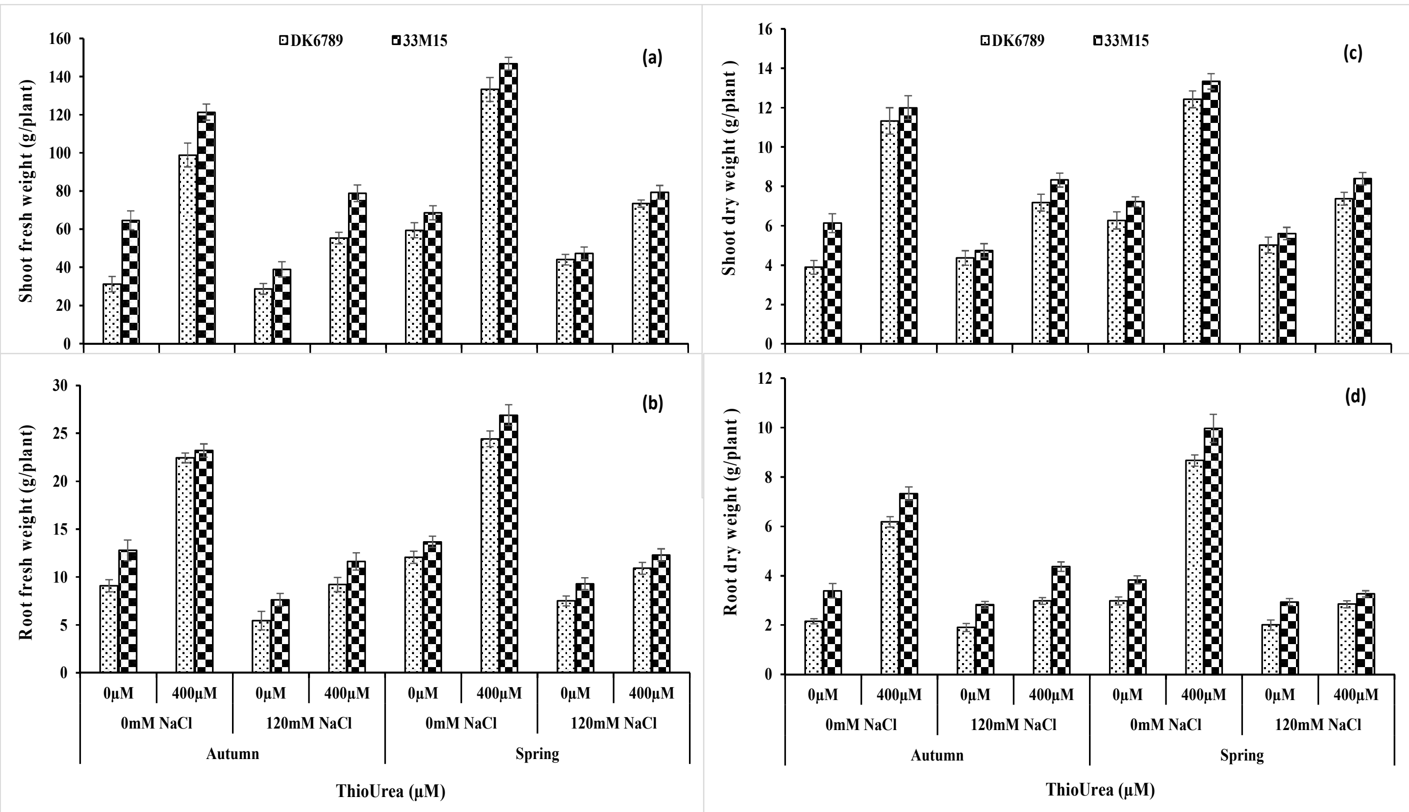


**Fig. 2. Changes in some growth attributes of maize hybrids under salt stress and role of thiourea in alleviating salt stress toxicity in autumn and spring seasons. The bars labeled with alphabets show significant (P<0.01) TU/S/Season (TU=Thiourea level, S=Salt level)**

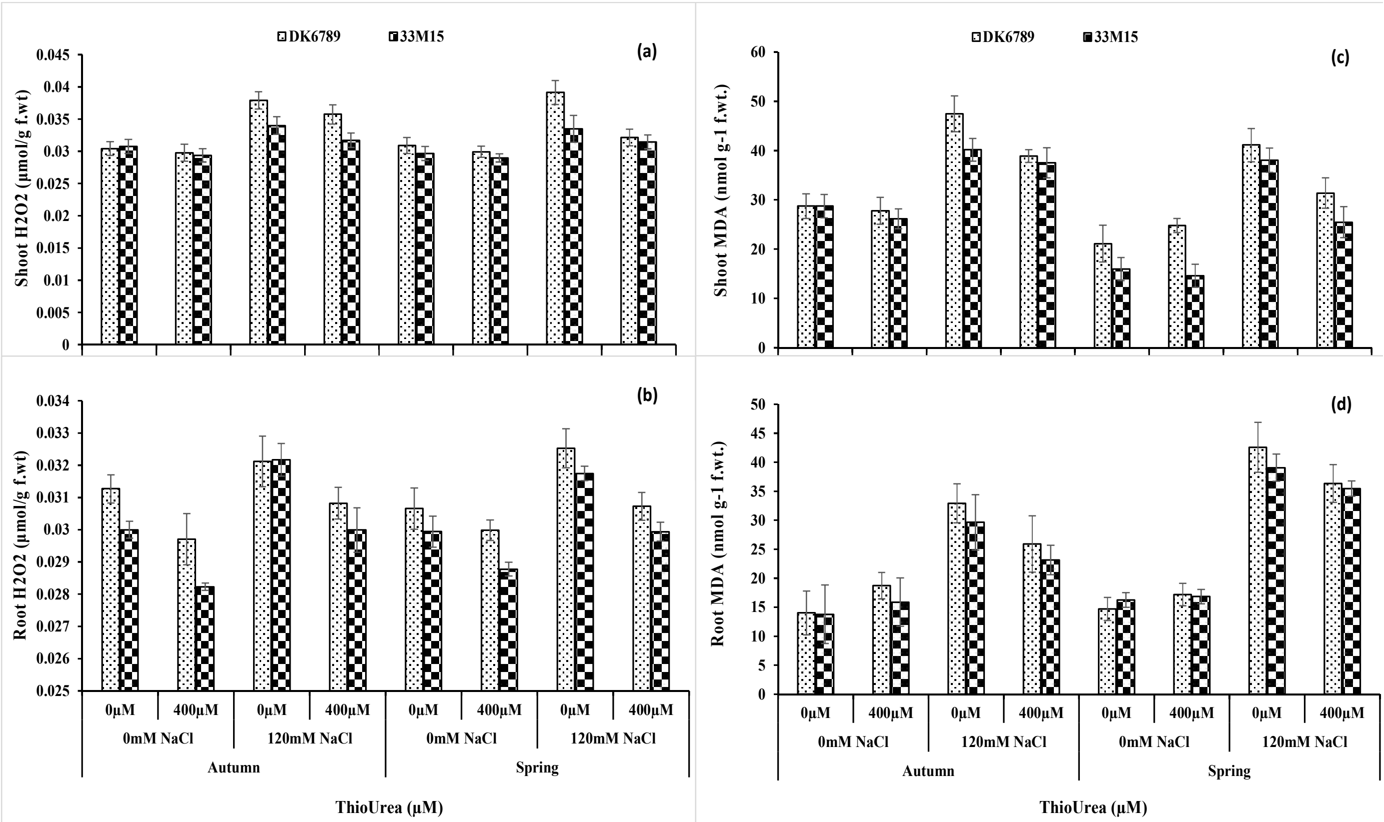


**Fig. 3. Changes in some growth attributes of maize hybrids (Zea mays L.) under salt stress and role of thiourea in alleviating salt stress toxicity in autumn and spring seasons. The bars labeled with alphabets show significant (P<0.01) TU/S/Season (TU=Thiourea level, S=Salt level)**

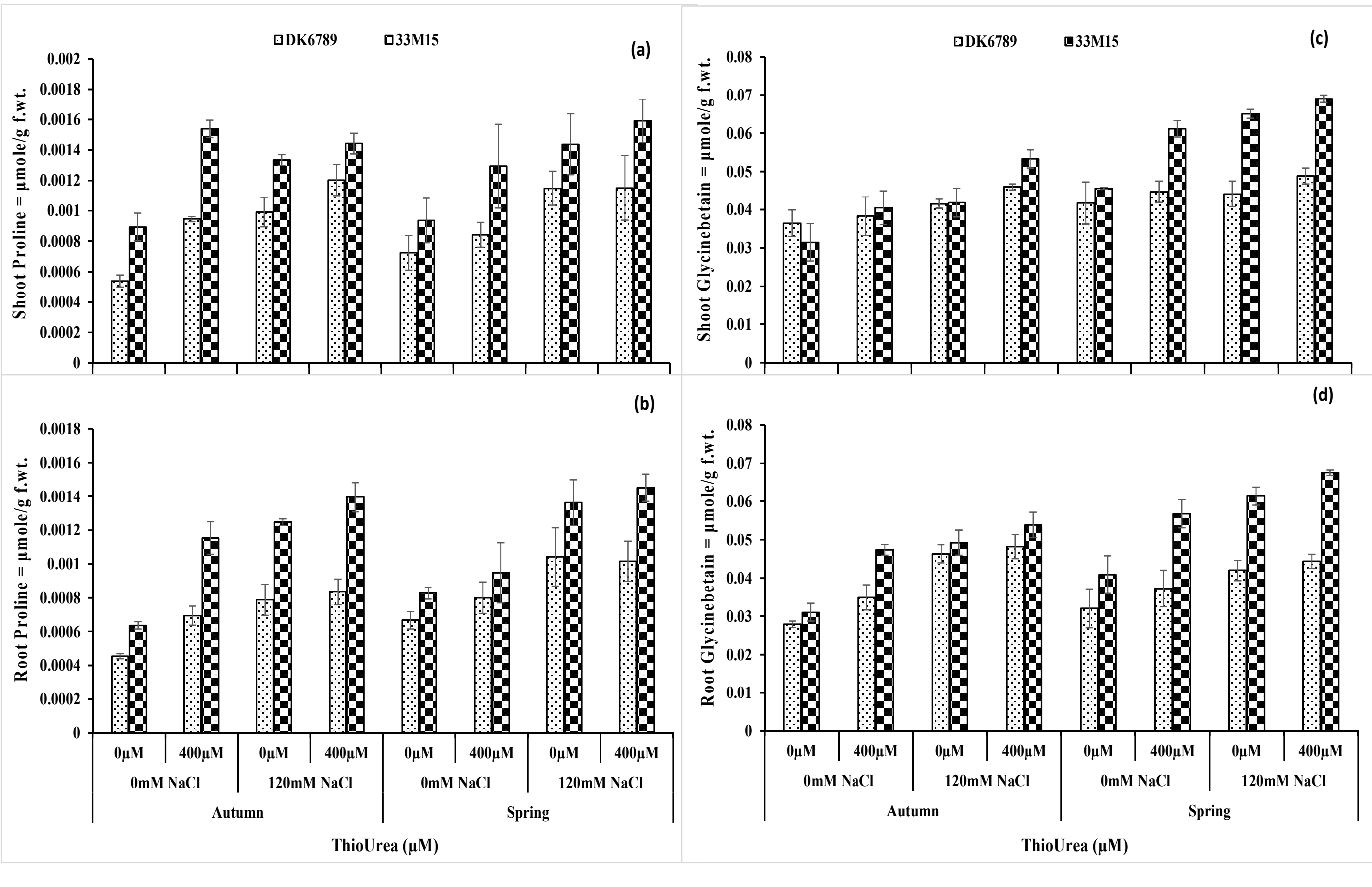


**Fig. 4. Changes in some growth attributes of maize hybrids (Zea mays L.) under salt stress and role of thiourea in alleviating salt stress toxicity in autumn and spring seasons. The bars labeled with alphabets show significant (P<0.01) TU/S/Season (TU=Thiourea level, S=Salt level)**

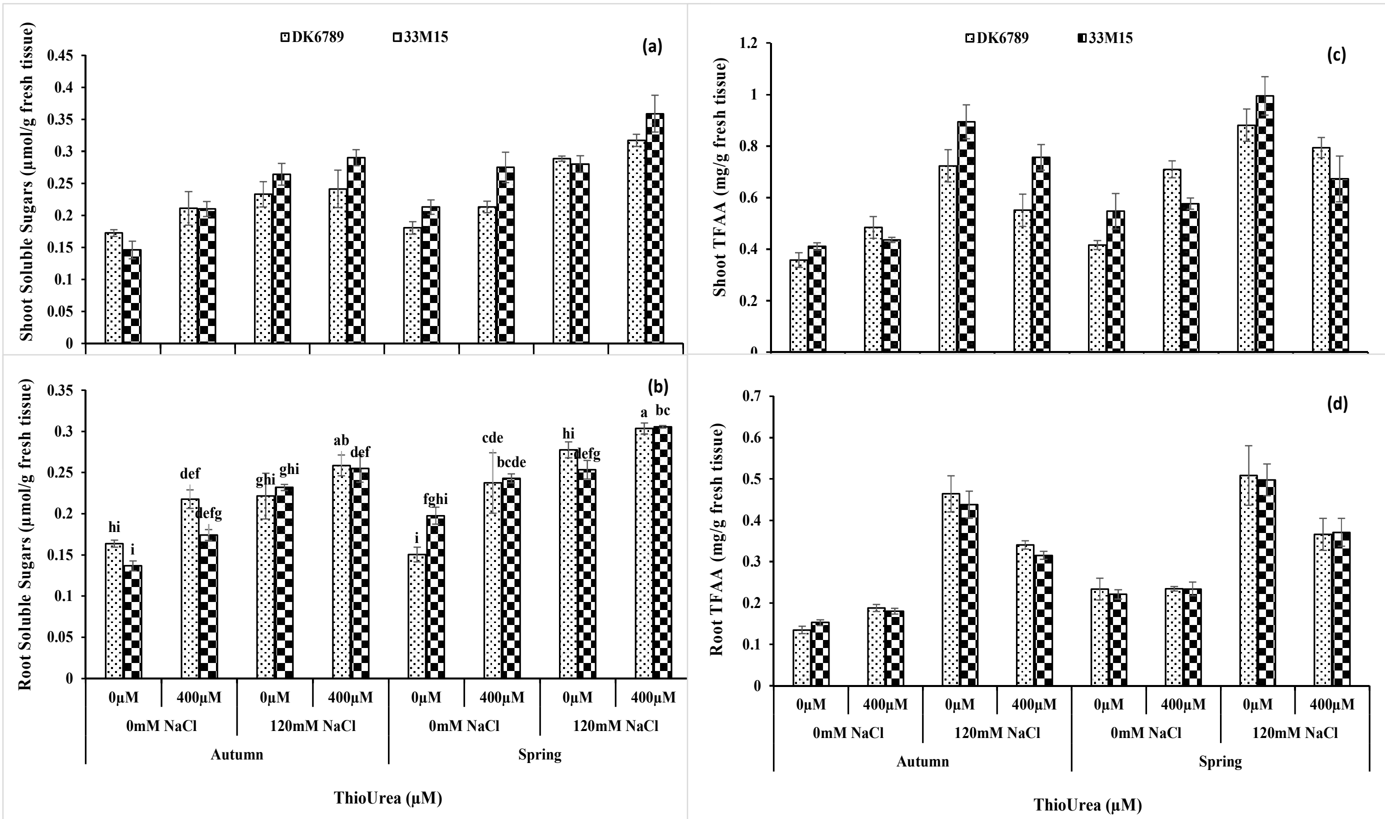


**Fig. 5. Changes in some growth attributes of maize hybrids (Zea mays L.) under salt stress and role of thiourea in alleviating salt stress toxicity in autumn and spring seasons. The bars labeled with alphabets show significant (P<0.01) TU/S/Season (TU=Thiourea level, S=Salt level)**

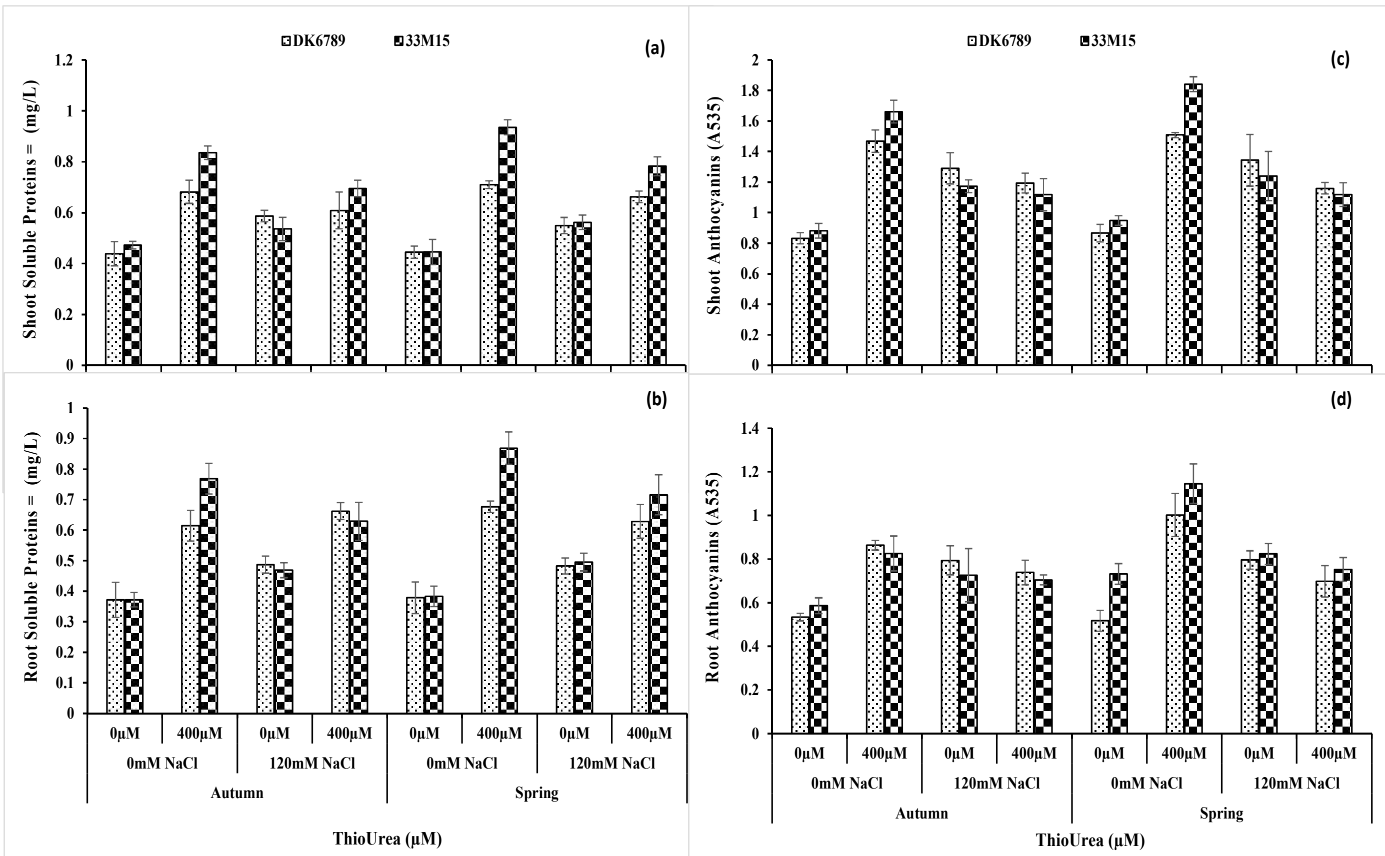


**Fig. 6. Changes in some growth attributes of maize hybrids (Zea mays L.) under salt stress and role of thiourea in alleviating salt stress toxicity in autumn and spring seasons. The bars labeled with alphabets show significant (P<0.01) TU/S/Season (TU=Thiourea level, S=Salt level)**

Leaf and root soluble proteins contents indicated significant ($P<0.01$) differences in the hybrids, seasons, salt and thiourea treatments (Fig. 6a, b). Although leaf and root soluble proteins declined severely under salinity stress, the medium supplemented thiourea improved this attribute in both the hybrids although performance of 33M15 was better than DK6789. With thiourea application soluble proteins contents improved in both the hybrids under salinity stress in both the seasons. Analysis of recorded data showed significant ($P<0.01$) differences among salt and thiourea levels, but hybrids did not exerted any significant ($P>0.05$) effect for leaf and root anthocynins. Salinity imposition enhanced anthocyanins, root application further boosted anthocyanins accumulation (Fig. 6c, d). Hybrid 33M15 performed better for osmolytes estimations and this performance was much better in Season.

## DISCUSSION

Exogenously applied growth regulators induce cell divisions ultimately increasing shoot and root length (Silveira *et al.,* 2006). In this study selected level of thiourea (400 µM) was used for managing salinity toxicity of selected maize hybrids. Salt stress reduced shoot and root length (Fig. 1a, b, c, d) and fresh and dry weights of shoot and root (Fig. 2a, b, c, d) while medium supplemented thiourea proved to be very effective in partially nullifying the damaging effect of salinity on both the hybrids. As a strategy of alleviation the adverse effects of salinity, the exogenous supply of growth bio regulators such as glycinebetaine, proline has been in vogue for quite some time with great success (Francisco *et al*., 2008). There are different facets of salinity damage on plant cells and tissues, but the oxidative damage has been given due consideration (Perveen *et al*., 2015), while the thiourea has been reported to reduce the salinity damage in terms of growth and biomass production (Anjum *et al*., 2011; Perveen *et al*., 2013). The salinity induced disturbances taking place in leaf tissues are due to over-accumulation of some toxic ions particularly $Na^+$ and $Cl^-$, which result in generation of ROS (Mittler *et al*., 2004). In this study salinity stress caused enhanced production of $H_2O_2$ and MDA in the leaves and roots of both hybrids in both seasons, although substantial differences were noticed under salinity stress (Fig. 3a, b, c, d). Increased amounts of $H_2O_2$ and MDA under salinity stress is the result of toxicity of excessive tissue concentration of toxic ions, which alter the biochemical mechanisms in a way that the membranes of the cellular compartments where the ROS metabolism takes place are specifically damaged (Apel and Hirt,2004). In tobacco, salt stress induced higher ROS production as well as increased membrane lipid peroxidation (Katsuhara *et al*., 2005). In this study although there was enhanced production of $H_2O_2$ (Fig. 3a, b) and MDA (Fig. 3c, d), the medium supplementation of thiourea markedly reduced their tissue concentration. To cope with the enhanced accumulation of ROS, the plants have natural tendency to enhance accumulation of osmoprotectants in stressed conditions (Gandonou *et al*., 2011). For normal plant growth accumulation of soluble proteins, proline and glycinebetain are required in minute quantities for proper functioning of cells, however their role becomes much more pivotal under salt stress, since they maintain the cell water balance under abiotic stress conditions (Ashraf and Foolad, 2007; Hayat *et al.,* 2012;Taiz *et al*., 2015).

Proline has multiple functions like protection of membranes, scavenge free radicals, mediate osmotic adjustment, osmotic pressure regulation, stabilize the structure of macromolecule and regulate cellular redox status that participated in tolerance to stress conditions (Jia *et al*., 2002; Zhu, 2002; Larher *et al*., 2003; Kaymakanova and Stoeva, 2008; Misra and Saxena, 2009). It plays a role in osmoregulation causing tolerance to oxidative damage at cellular level (Tripathi *et al*., 2007). In this study enhanced accumulation of proline (Fig. 3a, b) was observed under salt stress. Exogenously applied stress alleviating growth bioregulators proved to be very helpful in improving salinity tolerance by maintenance of cell water balance and shield memberanes (Johnson and Smith, 1992; Banu *et al*., 2010; Hasanuzzaman *et al*., 2012). Salt stress generally trigger accumulation of compatible solutes particularly proline and glycinebetaine (GB) as an adaptive response to salinity in different plants (Ashraf and Foolad, 2007; Park *et al*., 2007). Similarly, in the present investigation, saline growth medium caused a marked increase in GB accumulation in both maize hybrids (Fig. 3c, d). It is well evident that increased accumulation of GB plays a significant role in the protection of various vital enzymes, membranes having a key role in scavenging ROS (Banu *et al*., 2010), osmotic adjustment (Rhodes and Hanson, 1993) and facilitating water uptake (Flowers, 2004; Hassine and Lutt, 2010;Hassine *et al*., 2010). In the present study, exogeneously applied thiourea greatly enhanced the accumulation of GB. These findings cannot be explained in view of the fact that not a single report related to this can be deciphered from the literature, except the enhanced GB accumulation in barely and maize under cadmium stress (Ikram and Javed, 2015; Perveen *et al.,* 2011).

Under stress sugar contents decreased (Riham *et al*., 2001; Perveen *et al*., 2011). Shu *et al*. (2004) studied that sugar metabolisms is negatively affected plants under the stress. Ayari (2014) reported that thiourea increase carbohydrate content under control and salt stress. Maximum sugar was observed at alone thiourea application for 33M15 in spring season (Fig. 5a, b). Amino acids play an essential role in plant metabolism, thus plants respond to environmental stress by increased contents of amino acids (Lesko *et al*., 2002; Leasko and Simno-Sarkadi, 2003). Mahatma *et al*. (2009) also reported increase in amino acid contents with the exogenous application of thiourea. Significant increase in amino acid contents with TU treatment has also been reported by Garg*et al*. (2006). In our findings thiourea significantly improved this attribute under control and salt stress (Fig. 5c, d). Soluble proteins play an important role in plant metabolism by acting as typical osmoprotectant and in sugar signaling and sensing systems (Parida *et al*., 2004, 2005). Root applied thiourea enhanced soluble protein concenteration (Fig. 6 a, b) in both hybrids. When plants subjected to salt stress an increase in soluble protein and anthocyanin contents were showed by maize (Coffeen and Wolpert, 2004) in Albizialebbak (Tripathi *et al*., 2007) and in potato tubers (Germchi *et al*.,2010). Salt stress causes accumulation of pigment contents (Kaymakanova and Stoeva, 2008). In our findings salt stress enhanced anthocyanins accumulation, thiourea further accelerated their accumulation (Fig. 6c, d). Thiourea application improved anthocyanin concentration under stress and thiourea application (Sahu *et al.,* 2005). Thus it can be concluded that the medium supplementation of thiourea can partially alleviate the salinity induced production of ROS in maize, although the response of hybrids was markedly different. Prevailing seasonal conditions has great influence on the plant performance, which is the result of the operation of internal mechanisms (Perveen *et al*., 2011; Taiz *et al*., 2015). This study revealed that spring season was more profound than autumn season for maize growth irrespective of the treatments applied. During spring season, the temperature is within the moderate range, which favors plant growth. The tissue accumulation of toxic ions is relatively lesser than that observed in the autumn season. The use of thiourea was even more effective in the spring than the autumn season. Thus it can be asserted that the use of thiourea for the improvement of salinity induced oxidative damage can be more effective in the spring season. From results it was clear that application of thiourea can effectively promote root growth possibly by enhancing assimilate partitioning in roots. A pronounced improvement in root growth of salt sensitive hybrids indicated that enhanced tendency of roots to acquire the nutrients in greater amounts. Likewise, increased number of roots with root applied thiourea under salinity stress emerged as a major factor in promoting growth and biomass.

**Conclusion**

Salinity stress declined the growth attributes of both maize hybrids, although differentially. Enhanced production of $H_2O_2$ and MDA and some osmoprotectants was the result of salinity induced damage on the leaf tissue. Medium supplementation of thiourea improved the growth of both the maize hybrids by reducing the synthesis of ROS and MDA and increasing osmoprotectants production like proline, GB, soluble sugars, free amino acids, soluble proteins and anthocyanins indicating a possible repair mechanism operated by MDA. Seasonal condition do influence the production of ROS and osmoprotectionefficacy of thiourea application. For achieving better salinity tolerance, the supplementation of thiourea may be made in the spring season.

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