INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–1095/2020/23–3–630–636
DOI: 10.17957/IJAB/15.1333
http://www.fspublishers.org

Full Length Article

Physiological and Biochemical Characterization of Linseed Genotypes under Salinity Stress

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Received 05 July 2019; Accepted 25 November 2019; Published 04 February 2020

Abstract

Linseed (*Linum uistatissimum* L.) is among the most valuable dual purpose oilseed crops and is used for the extraction of oil from seeds and fibers from plant's stems. Linseed has great adaptability, product diversity and researchers around the globe are conducting experiments for producing its bio-products. However, very scarce information is available regarding salinity tolerance in linseed. Therefore, diverse linseed germplasm was subjected to various rates of NaCl salt stress to assess their comparative performance and correlation between defense system and osmotic adjustment. Three salt treatments (control, 100 and 200 m*M* NaCl) were applied in hydroponic conditions and four linseed genotypes (two salt tolerant 637-72 and NO-303; two salt sensitive S-907 and C-99-3-115) were evaluated. Salinity reduced induction of carbonic anhydrase and nitrate reductase (activities which may cause hampered growth in linseed genotypes. Linseed gradually improved the accumulation of glycine betaine and proline in cytosol with increasing salt stress, however, this accumulation was more for glycine betaine when compared with proline. An increased activity of antioxidant enzymes especially superoxide dismutase, peroxidase and ascorbate peroxidase played an important role in reducing lipid peroxidation and malondialdehyde contents in salt tolerant genotypes than salt sensitive genotypes with poor antioxidant system. A positive correlation of glycine betaine with the activity of superoxide dismutase indicated activation of antioxidative enzyme system to improve salt tolerance. Reduced malondialdehyde contents can be used as an important biochemical indicator for salt tolerance in linseed genotypes.

Keywords: Biochemical; Antioxidants; Organic osmolytes; NR; MDA

Introduction

Salinity poses several undesirable changes like modifications in plasma membrane architecture and production of active oxygen species (AOS) due to toxicity of certain ionic species (Hasegawa *et al.* 2000). High salt concentration in root medium causes disruption of photosynthetic rate and production of nitrogenous compounds by altering the activity of certain enzymes particularly of CO_2 and nitrate assimilation. The C_3 and C_4 plants possess carbonic anhydrase (CA) enzyme in photosynthetic tissues which regulates the CO_2 assimilation (Badger and Price 1994). Similarly, nitrate assimilation is carried out by regulating the production of substrate specific nitrate reductase (NR) enzyme and it is considered an important process in nitrogen acquirement by plants (Flores *et al.* 2002). Under salt stress, CO_2 uptake is reduced which ultimately decreases carbon reduction by Calvin cycle and hence photosynthetic rate. This phenomenon causes the non-availability of oxidized $NADP^+$ as electron acceptor which is a major stimulating factor of AOS production in salt stressed plants (Peltzer *et al.* 2002). These AOS lead to the generation of other destructive

To cite this paper: Qayyum MA, J Akhtar, F Bashir, T Naz, MM Iqbal, O Farooq, Atique-ur-Rehman, MI Zafar, M Ali, M Imtiaz, MA Sarwar, ZA Saqib, SMA Basra, Z Guoping, Yinglan (2020). Physiological and biochemical characterization of linseed genotypes in response to NaCl stress. *Intl J Agric Biol* 23:630–636

species like lipid peroxides (Vaidyanathan *et al.* 2003).

To cope with the osmotic and oxidative stress under saline conditions, plants adopt certain mechanisms of complex nature. These salt stressed plants increase active uptake of certain inorganic ions like K^+ from growth medium or synthesize organic solutes like soluble sugars, amino and imino acids in the cytoplasm which act as osmotica and also protect different macro molecules and proteins in plant cells from salt injury (Ghoulam *et al.* 2002; Farooq *et al.* 2015). These mechanisms help to restore cell turgor, prevent water loss and regulate osmotic potential of plants (Naidoo and Naidoo 2001).

To alleviate the damage of AOS, plants are well equipped with an effective antioxidant system, which comprised of non-enzymatic compounds and AOS scavenging enzymes. The non-enzymatic compounds include glutathione, ascorbate, β-carotene and α-tocopherol while superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD) as scavengers of AOS (Apel and Hirt 2004). Oxidative damage occurs when production of AOS is more than the scavenging capacity of antioxidant system and hence it results in protein denaturation, DNA disruption and peroxidation of lipid molecules (Shalata and Neumann 2001). The SOD dismutases superoxide radical to H_2O_2 while CAT and POD enzymes convert H_2O_2 into water and oxygen (Hussain *et al.* 2007). Similarly, ascorbate peroxidase (APX) catalyzes the reduction of H_2O_2 to water by using ascorbate as reducing agent. Malondialdehyde (MDA) contents are considered as the general indicator of lipid peroxidation (Meloni and Martinez 2009). Thus, production of organic osmolytes for cell turgor maintenance and regulation of antioxidant system for coping oxidative damage are among the major mechanisms of conferring salt tolerance in plants under salinity (Farooq *et al.* 2017.

Production of antioxidant enzymes in response to oxidative stress (ROS) under salt stress is considered the most obvious effect (Naz *et al.* 2019). The activity of these antioxidative enzymes is supported by the presence of organic osmolytes concentration in plant cells (Naz *et al.* 2018). Therefore, the current work explored and compared the activities of different antioxidant enzymes, contribution of different organic osmolytes in restoration of osmotic shock in diverse linseed genotypes under saline conditions and observed the correlation between production of organic osmolytes and antioxidant enzyme activity.

Materials and Methods

Seedling growth and treatments

The present experiment was conducted in College of Agriculture and Biotechnology, Zhejiang University, China. Seeds of two salt tolerant (*i.e.*, 637-72 and NO-303) and two salt sensitive (*i.e.*, S-907 and C-99-3-115) genotypes of linseed were germinated in moist quartz sand in green house. Fifteen days old seedlings with uniform growth were selected and transplanted into 20 L containers covered with a foamed plastic plate containing evenly spaced holes. The containers contained half strength Hoagland's nutrient solution (Hoagland and Arnon 1950). The design of the experiment was CRD with factorial arrangement using three replicates. After one week of transplanting, salinity levels (*i.e.*, control, 100 and 200 m*M*) were developed with NaCl salt in three increments, whereas in control no NaCl salt was added. The pH of solution was maintained to 6.5 ± 0.5 throughout the experiment with 1 *M* NaOH and/or HCl. The nutrient media in the pots was continuously aerated using aquatic pumps and media was changed after every five days. Measurements were made after 30 days of salt stress.

Biochemical parameters

The carbonic anhydrase (CA) activity was measured by following the method of Dwivedi and Randhawa (1974). The activity of nitrate reductase (NR) was determined by following the method of Jaworski (1971). Proline contents were determined spectrophotmetrically following Bates *et al.* (1973). Glycine betaine (GB) contents were estimated following the method of Grieve and Grattan (1983).

Total soluble sugars (TSS) were determined by following the method of Homme *et al.* (1992). Residues having no sugar were extracted using 1.5 N H_2SO_4 as done in the method of Naguib (1963). After the hydrolysis of polysaccharides, total soluble sugars were calculated using anthrone following Fairbairn (1953). Extraction of soluble proteins was carried out by using the method of Hassanein (1977). After protein extraction, water insoluble residues were extracted with 1 N NaOH. Then total soluble proteins were estimated using BIO-RAD dye following the method by Bradford (1976).

Assay of antioxidant enzymes

For enzyme analysis, 0.5 g fully expanded leaves were homogenized with 5.0 mL 50 m*M* Na-phosphate buffer (pH 7.8) in an ice bath in pre-chilled mortar. The mixture was centrifuged at 13,000×g for 30 min and supernatant used for determining antioxidative enzymes. Activity of SOD was recorded by measuring the photoreduction of nitro blue tetrazolium (NBT) at 560 nm as described by Giannopolitis and Ries (1977).

Activity of CAT was recorded following the method of Aebi (1974). Activity of POD was noted by using the method of Amako *et al.* (1994) with some modification. The reaction mixture (3 mL) consisted of 100 μL enzyme extract, 100 μL guaiacol, 300 m*M* H_2O_2 (100 μL) and 25 m*M* K-phosphate buffer (2.7 mL) with 2 m*M* EDTA (pH 7.0). The magnitude of absorbance was determined through spectrophotometer using 470 nm wave lengths. Activity of APX *(Ascorbate Peroxidase)* was estimated by the method of Nakano and Asada (1981). Lipid peroxidation in terms of MDA (Malondialdehyde) contents in plant leaves were determined by method of Hodges *et al.* (1999).

Statistical analysis

The data gathered were analyzed statistically following analysis of variance technique (ANOVA) and least significant difference (LSD) test was applied to differentiate the treatment effectiveness (Steel *et al.* 1997) using "Statistix 8.1" statistical computer software package(s).

Results

Carbonic anhydrase (CA) and nitrate reductase (NR) activities

The CA and NR activity of all the genotypes significantly decreased by the increased salinity. Linseed genotypes showed similar activity of CA at all salinity levels but a marked variation was observed among genotypes in their NR activity (Fig. 1). Salt tolerant genotypes showed the maximum NR activity (0.48–0.37 μmol NO_2 h^{-1} g^{-1} LFW s^{-1}) when compared with salt sensitive genotypes (0.35–0.26 μmol NO_2 h^{-1} g^{-1} LFW s^{-1}) at 100 and 200 m*M* NaCl. Except genotype 637-72 (81%), all genotypes showed similar increase (78%) for CA activity at 100 m*M* NaCl while this increase was 56% in CA activity in all genotypes at 200 m*M* NaCl.

Proline and glycinebetaine (GB) accumulation

Proline and glycinebetaine (GB) contents of all the genotypes were highly affected by the increased salinity and genotypes showed progressive increase in accumulation of both osmolytes with increasing salinity (Fig. 2). Maximum increase in proline contents (112–130% of control) was recorded in salt tolerant genotypes while in salt sensitive genotypes, proline contents remained relatively low (104–128% of control) with gradual increase in salt concentration. Similarly, GB concentration increased by 123% of respective control in salt tolerant genotype 637-72, however, salt sensitive genotype S-907 showed 114% increase in GB contents with respect to control. It was also observed that linseed genotypes accumulated relatively high GB than proline contents under same set of treatments (*i.e*., control, 100 and 200 m*M* NaCl).

Total soluble sugars (TSS) and total proteins (TP)

Total soluble sugar and total protein contents in all genotypes were affected remarkably by addition of salts in growth medium (Fig. 1) and a sharp increase was observed in TSS and total proteins with increasing NaCl salinity. At 100 m*M* NaCl, TSS concentration increased to 110% of respective control in salt tolerant genotype while at same stress level, sensitive genotypes showed 105% increase in TTS. Salt tolerant genotypes accumulated similar concentration of total proteins that was 116% compared to their respective controls. Similarly, salt sensitive genotypes showed a decline (Fig. 1) in the accumulation of total proteins (111% of control) in their cytoplasm at low level of salt stress (100 m*M* NaCl). The accumulation of total proteins was much higher as compared to TSS in all the genotypes of linseed with increasing level of salinity. In addition salt tolerant genotypes showed a much higher accumulation rate of total proteins (248–249%) when compared with salt sensitive genotypes (217%) at same salinity level.

Superoxide dismutase (SOD) and catalase (CAT) enzymes activities

The activity of SOD and CAT enzymes in linseed genotypes are depicted in (Fig. 2). The results revealed that the activity of SOD enzyme increased progressively with increasing levels of salt stress while the activity of CAT enzyme first increased with increasing growth medium salinity but declined at higher level of salt stress. The maximum increase in SOD (182%) and the maximum decline in CAT activity (80%) were recorded at 200 m*M* NaCl. Salt tolerant genotypes possessed efficient antioxidant system and SOD activity increased to 173% and 182% of respective control with increasing salinity. However, in sensitive genotypes antioxidant enzyme system was comparatively less efficient in scavenging AOS with 124% and 132% increase in SOD activity. CAT activity showed a different trend in stressed genotypes and a sharp decrease in the activity of CAT ranged from 94% to 80% of respective control in tolerant genotypes with increasing salinity. On the other hand, salt sensitive genotypes (S-907 and C-99-3-115) expressed a comparatively less decline in CAT activity which ranged from 97% to 90% of respective control with increasing NaCl stress. High activity of SOD in salt tolerant genotypes and CAT in salt sensitive genotypes indicated a contrasting mechanism of scavenging AOS in linseed genotypes.

Peroxidase (POD) and ascorbate peroxidase (APX) enzyme activity

The activity of POD enzyme was significantly decreased in salt sensitive genotypes but increased in salt tolerant genotypes under salinity (Fig. 3) while salt concentration significantly increased the APX enzyme activity in all four genotypes of linseed. The POD activity increased to 115% of respective control in salt tolerant genotypes at 100 m*M* NaCl but in salt sensitive genotypes a decline in POD activity (93%) was observed at same stress level. APX activity expressed a different trend and increased with increasing salinity in all genotypes of linseed. The increase in APX activity was more (155%–202%) in salt tolerant genotypes as compared to sensitive genotypes (127%–144%) by increasing the NaCl concentration from 100 m*M* to 200 m*M* respectively.

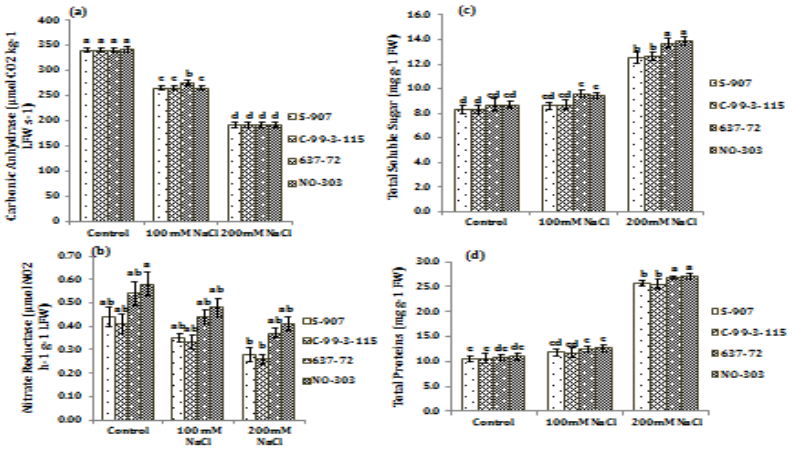

Fig. 1: Carbonic Anhydrase (CA), Nitrate reductase (NR), Total Soluble Sugars (TSS) and Total Proteins (TP) in the leaves of linseed genotypes exposed to salinity

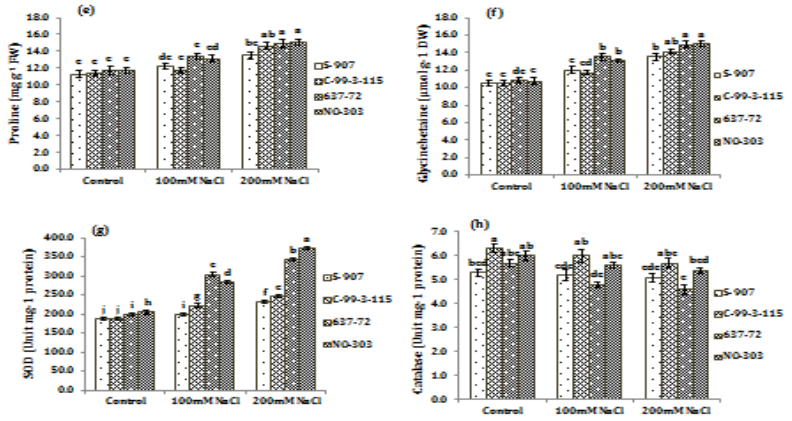

Fig. 2: Proline (Pro), Glycine Betaine (GB), Superoxide Dismutase (SOD) and Catalase (CAT) in the leaves of linseed genotypes exposed to salinity

Lipid peroxidation (MDA) contents

A wide range of difference was noticed among different genotypes regarding MDA (melondialdehyde) contents showing lipid peroxidation levels (Fig. 3). In salt sensitive genotypes, MDA contents greatly increased (206–229% of respective control) with increasing salt concentration when compared with salt tolerant genotypes (174–181% of respective control). The lower contents of MDA in salt tolerant genotypes indicated that these genotypes had an efficient system to control lipid peroxidation as compared to salt sensitive linseed genotypes.

Discussion

The genetic variations between plants provide a precious tool in selecting phenotypes with required characteristics. The present study clearly demonstrated some biochemical traits of salt tolerance in linseed genotypes subjected to different levels of salinity.

Proline accumulation in salt stressed plants is a key defense response to sustain the osmotic pressure in many crops (Koca *et al.* 2007). The GB accumulates in chloroplasts and hence plays major role in thylakoid protection and maintains plant photosynthetic efficiency (Genard *et al.* 1991). Higher accumulation of GB as compared to proline under salinity in present study indicate significant role of GB in osmotic adjustment of linseed (Cha-um *et al.* 2006) investigated that high level of GB in salt-tolerant lines of rice (*Oryza sativa* L. spp. *indica*) played a significant role in salt tolerance by stabilizing chlorophyll pigments and water oxidation in PS-II which significantly improved photosynthetic rate and ultimately growth. A positive correlation was also observed between proline, GB and SOD activity in linseed genotypes (Table 1) which might indicate its role in up-regulation of SOD activity. The antioxidant enzymes activity depends on osmolyte concentration in cell, as these enzymes need availability of *in vivo* melieu for maximal catalytic activity (Burg and Ferraris 2008).

Many reports indicate that sucrose is produced or accumulated in plants tolerating drought or salt stress (Nabati *et al.* 2001). *De novo* synthesis of proteins may also occur under saline conditions and their concentration increases when plants are salt stressed (Pareek *et al.* 1997).

Table 1: Correlation showing significance level for each antioxidant enzymes and with MDA contents in linseed genotypes grown under NaCl stress

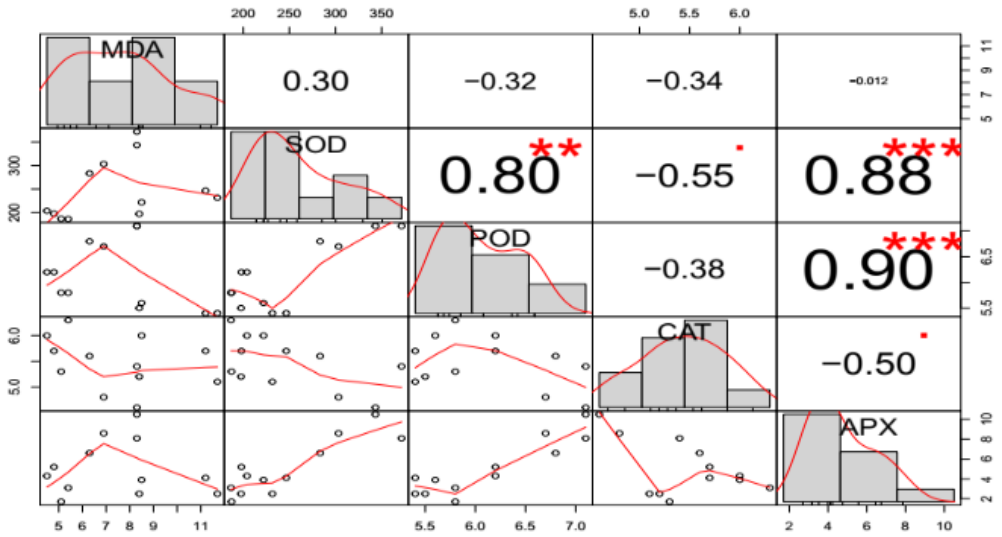

Table 2: Mean Squares of various traits in linseed under NaCl salinity stress

Source	DF	CA	NR	Pro	GB	TSS	TP	SOD	CAT	POD	APX	MDA
Genotypes (G)	3	27.4**	0.049 ^{NS}	2.33**	2.899**	2.093 **	1.975 **	15801.6**	1.661**	4.049**	62.375**	10.589**
Treatments (T)	2	67463.3**	0.078 ^{NS}	26.695**	42.02**	78.285**	887.264**	32730.9**	1.239**	0.177 ^{NS}	23.641**	73.246**
G×T	6	24.3 **	0.0001 ^{NS}	0.521 ^{NS}	0.528 ^{NS}	0.191 ^{NS}	0.293 ^{NS}	3196.3**	0.130 ^{NS}	0.444 *	3.582 **	1.829 **
Error	24	0.4	0.029	0.436	0.343	0.381	0.391	0.5	0.161	0.144	0.208	0.092
Mean		266.81	0.406	12.897	12.528	10.244	16.389	247.95	5.469	6.136	5.091	7.447
CV%		0.23	42.13	5.12	4.67	6.03	3.82	0.27	7.35	6.19	8.95	4.07

** Significant at $P \leq 0.01$, * Significant at $P \leq 0.05$, NS= Non-significant at $P > 0.05$. CA; Carbonic Anhydrase, NR; Nitrate reductase, Pro; Proline, GB; Glycinebetaine, TSS; Total Soluble Sugars, TP; Total Proteins, SOD; Superoxide dismutase, CAT; Catalase, POD; Peroxidase, APX; Ascorbate peroxidase, MDA; Melondialdehyde

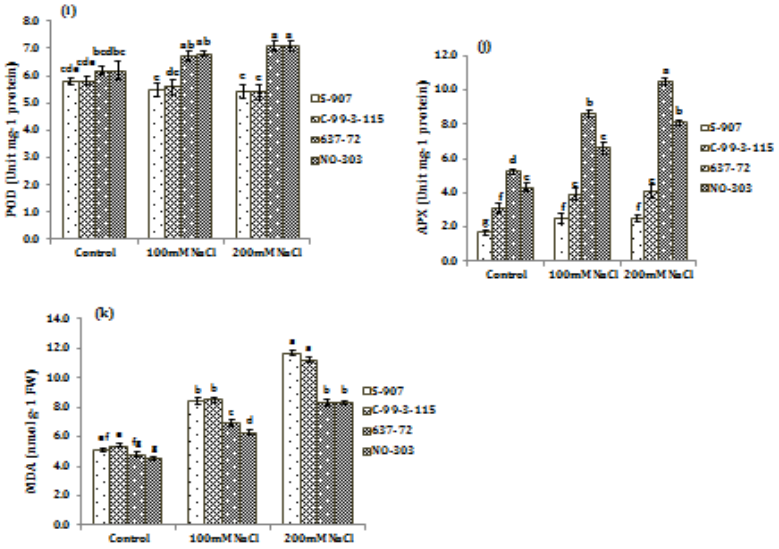

Fig. 3: Peroxidase (POD), Ascorbate Peroxidase (APX) and melondialdehyde (MDA) in the leaves of linseed genotypes exposed to salinity

In linseed genotypes, concentration of total soluble sugars and total proteins increased with increasing salinity which may play its role in the adjustment of osmotic stress in linseed genotypes under saline conditions (Guo *et al.* 2012; Naz *et al.* 2016) (Table 2).

To eliminate or reduce the oxidative damage by AOS, SOD is a key scavenger and is thought to be the first defense line (Hamilton and Heckathorn, 2001) which dismutates superoxide anion to H_2O_2 (Costa *et al.* 2005). Thus antioxidants protect cell organelles from AOS damage

during stress (Reddy *et al.* 2004). In this study, relatively high activity of antioxidative enzymes were noted in tolerant compared to salt sensitive genotypes, indicating the important role of this defensive system under salinity stress. The data gave a clue that APX and POD activity coordinated with SOD activity played a vital role in superoxide and H_2O_2 scavenging under salt stress (Amako *et al*. 1994). An enhanced level of mRNA in plant tissues is a cause of increase in the SOD, APX and POD levels (Sairam *et al.* 2002) and using enzymatic antioxidants as biochemical indicators of salt tolerance were reported by (Wang and Huang, 2004; Farhoudi *et al*. 2012). Salinity caused peroxidation of membrane lipids, which is often used as an indicator of oxidative damage (Du and Huang 2008). A reduction in lipid peroxidation in salt tolerant genotypes and an increase in lipid peroxidation in salt sensitive genotypes of linseed suggests that salt tolerant genotypes have relatively better protective mechanism against oxidative stress. The MDA contents decreased because of enhanced activity of antioxidant enzymes eliminating H_2O_2 and hence membrane damage (Hernandez and Almansa 2002). A similar correlation between lipid peroxidation and antioxidant defense activity was also observed (El-Beltagi *et al.* 2008).

Conclusion

The results revealed that correlation of proline, GB with the activity of SOD was more pronounced as compared to other antioxidant enzymes which give a clue that organic osmolytes help in the induction of antioxidative enzyme system and cause salt tolerance. An increased antioxidant enzymes activity especially SOD, POD and APX significant played important role in reducing lipid peroxidation and MDA contents in salt tolerant genotypes of linseed.

Acknowledgements

The current hydroponic study was conducted at College of Agriculture and Biotechnology, Zhejiang University, China, financially supported by Higher education Commission of Pakistan.

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