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Determination of Proline Accumulation, an Abiotic Stress Marker in Pearl Millet Inbred Lines under Salt Stress using Spectrophotometry

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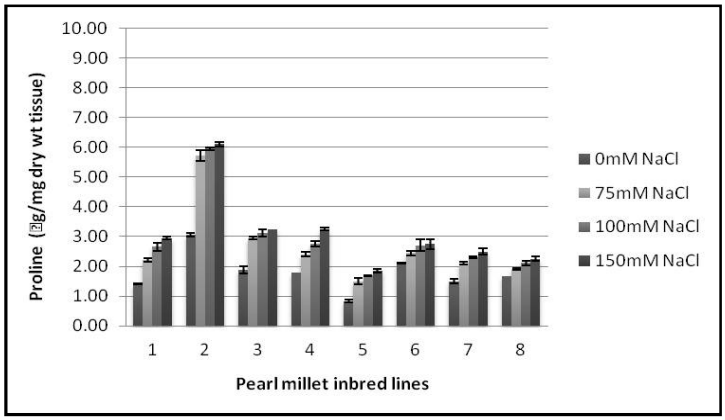
ABSTRACT

Proline accumulation is a widespread response observed in plants experiencing abiotic stresses like drought and salinity. Apart from acting as an osmolyte for osmotic adjustment, proline contributes to stabilizing sub-cellular structures, scavenging free radicals and buffering cellular redox potential under stress conditions. The determination of this amino acid is therefore very useful to assess the physiological status and to understand the stress tolerance in plants. State of the art spectroscopy and spectrophotometry are being successfully used to decipher the underlying mechanisms of stress tolerance. For a clearer understanding of proline accumulation on plant response to salinity stress, 10-day old pearl millet seedlings were subjected to various levels of salt stress, followed by spectrophotometric determination of the accumulated proline content. Whole plant free proline levels were seen to increase with increasing salinity levels in all the pearl millet lines, specially the tolerant varieties. Accumulation of proline to a higher degree under salinity stress is indicative of the fact that proline acts as cytoplasmic osmoticum and perhaps protects proteins against denaturation. Further use of mass spectrometry (MS)-based analytical platforms to profile stress-responsive metabolites will allow these crop plants to adapt to adverse environmental conditions.

Graphical Abstract



Proline content (µg mg^{-1} dry wt. tissue) under salt stress in eight salt-sensitive lines of pearl millet

Keywords: Spectrophotometry, Salinity stress, Proline accumulation, Osmolyte, pearl millet.

INTRODUCTION

Salinity stress one of the major abiotic stresses which drastically affects crop production [1]. Soil contaminated with salts (ECe > 4 dS m-1 or 40 mM NaCl or osmotic potential < 0.117 MPa) are defined as saline lands, which directly affects plant growth and development in vegetative growth prior to reproductive stage, especially cereal crop species [2-4]. Osmotic regulation is an important mechanism for plant cellular homeostasis in saline conditions. Under salt stress, plants accumulate several compatible solutes in the cytosol, such as polyols, glycinebetaine, trehalose, proline and others [5]. As a most common osmolyte for osmoprotection, proline has been extensively researched [6]. At the molecular level, the differential accumulation of proline in reproductive tissues is thought to be primarily determined by upregulation of proline synthesis and transport genes. Besides acting as an excellent osmolyte, proline plays three major roles during stress, i.e., as a metal chelator, an antioxidative defense molecule and a signaling molecule [7]. Proline accumulation normally occurs in cytoplasm where it functions as molecular chaperons stabilizing the structure of proteins and its accumulation buffer cytosolic pH and maintains cell redox status. Hence, proline is an important abiotic stress marker and its determination is therefore very useful to assess the physiological status and to understand the stress tolerance in plants. State of the art spectroscopy and spectrophotometry are being successfully used to decipher the underlying mechanisms of stress tolerance. In current study, spectrophotometry was used to determine the accumulated proline content in salt-stressed pearl millet seedlings to understand the underlying mechanism of osmolyte accumulation as a tolerance mechanism for salinity stress.

MATERIALS AND METHODS

Collection of plant material: Twenty-eight inbred pearl millet genotypes (LGD 1-B-10. ICMP 85410-P7, Tift 23D2B1-P1-P5, WSIL-P8, 81B-P6, ICMP 451-P8, ICMP 451-P6, H 77/833-2-P5(NT), H77/833-2, PRLT 2/89-33, W504-1-P1, P310-17-Bk, PT732B-P2, P1449-2-P1, ICMB 841 (=841B)-P3, 863BP2; IP 18293-P152, Tift 238D1 -P158, Tift 186, Tift 383,ICMB 89111. ICMB 90111, ICMB 92666. ICMB 95333.843B, ICMB 98004, ICMB 99022 and ICML 22) were obtained from International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India.

Salinity stress treatment: Seeds of pearl millet inbred lines were soaked in 0.1% Bavistin solution for 30 sec, washed with sterile distilled water and surface-sterilized with 70% ethanol for 1-2 min. Surface sterilized seeds were washed three times with sterile distilled water and germinated on filter-paper boats in balanced nutrient solutions [8] of pH 6.7 at 20°C for 10 days and later transferred to Hoagland medium containing four different concentrations of NaCl (0 mM, 75 mM, 100 mM, and 150 mM) in triplicates for each experiment. The experiments were repeated 4 times for each line and the genotype × salinity treatment means of each experiment were taken for the statistical analysis. Seedlings from germinating seeds were allowed to grow for 10 days at 25°C under 16 h photoperiodism.

Estimation of proline: Proline was determined by modification of the method outlined by Bates et al. (1973) [9]. Approximately 0.1 g of dry weight of tissue was homogenized in l ml of 3% aqueous sulfosalicylic acid in a chilled mortar and pestle. Neutral glass powder was used for homogeneous grinding. Two ml of 3% sulphosalycylic acid was added followed by centrifugation at 2000 rpm, 4°C for 10 min. One ml of the supernatant was reacted with 1 ml of glacial acetic acid and 1 ml of acid-ninhydrin (2.5 g ninhydrin was dissolved in 50 ml of solvent prepared by mixing glacial acetic acid and 6M phosphoric acid) for 30 min at 100°C in a boiling water bath. The reaction mixture was terminated in an ice bath after 30 min. Four ml of toluene was added to the reaction mixture and then vigorously mixed using a cyclomixer for 15-20 seconds. The two phases were then allowed to separate and brought back to room temperature. The reactant chromophore containing toluene (upper phase) was aspirated and absorbance was read at 520 nm using toluene as a blank using spectrophotometry. Proline concentrations in the samples were determined from the standard curve

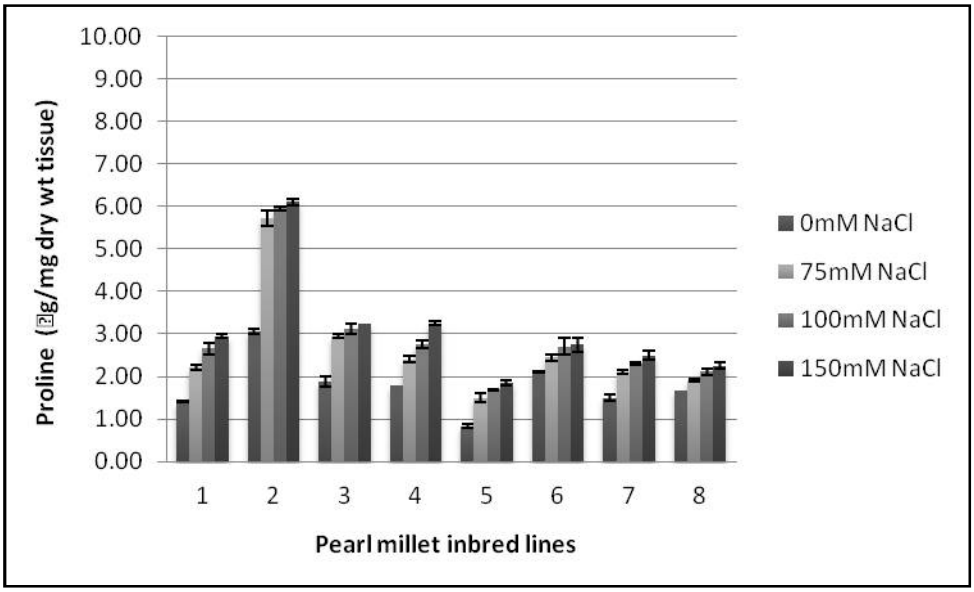
calibrated with different concentrations of the standard proline. Proline content was expressed in terms of μg mg^{-1} dry weight of tissue.

Statistical analysis: Experimental data were analyzed statistically using the GenStat software package [**10**] to ascertain the levels of significance for each source of variation (replications, genotypes, salinity levels, genotype x salinity level interact ions, and error) in the experiment.

RESULTS AND DISCUSSION

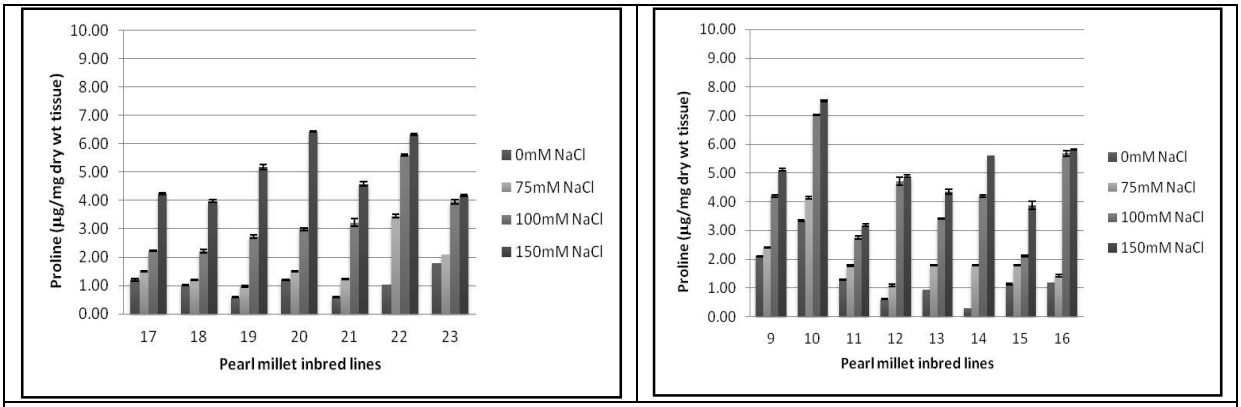
The pearl millet in breeds were categorized as sensitive, moderately tolerant and highly tolerant to salinity based on their relative abilities to maintain high germination levels and good early seedling growth across NaCl levels of 75 mM , 100 mM and 150 mM, respectively. Seven of the pearl millet inbred lines were categorized as sensitive (ICMB 90111, PRLT 2/89-33. P1449-2-P1, Tift 238D1 - P152, 81B-P6, WSIL-P8 and ICMP 85410-P7), fifteen as moderately tolerant, and five as highly tolerant (Tift 23D2B1 -P1-P5, ICMB 841-P3, P310-17-Bk, ICML 22 and ICMB 95333) [**11**].

Proline was estimated for all the 28 varieties and expressed in terms of μg/mg dry weight tissue and compared to the levels in control plants. In general, there was an increase in the proline content in the salt treated plant tissues with an increase in salt concentration. Its accumulation to a higher degree under salinity stress is indicative of the fact that, proline acts as a cytoplasmic osmoticum and also protects the proteins against denaturation. Proline is seen to accumulate according to the categories made above (based on growth of seedlings during salt stress) as shown in Figures 1 to 3. Whole free proline levels were observed to increase with increasing salinity levels in all of the pearl millet lines included in this study, irrespective of their tolerance to salt. Significantly greater proline accumulation was observed in the tolerant lines with increasing salinity levels as compared to the sensitive lines (Figures 1-3). Perhaps this enables the tolerant genotypes to cope with the salt stress conditions more efficiently as was reported in a similar study [**12**]. Similar increases in proline content was also reported in rice, pigeon pea, niger [**13**] and many other plants in response to increasing salinity levels. Proline accumulation was also significant under salinity stress in salt tolerant cultivars of green gram [**14**], and is correlated with salt tolerance of many higher plants.



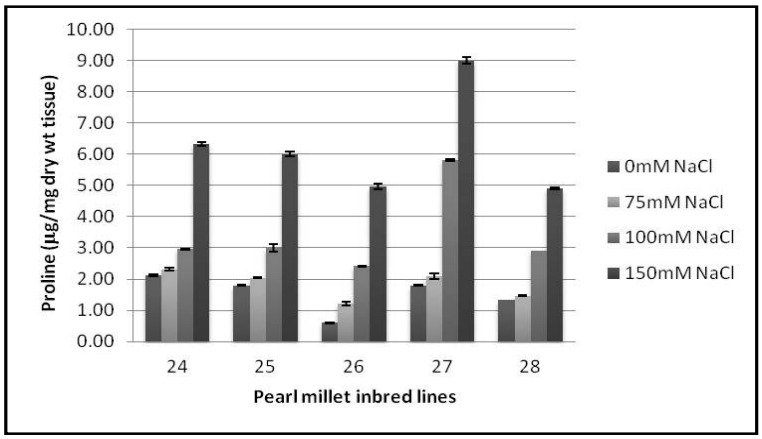
[1. ICMP85410-P7, 2. WSIL-P8, 3. 81B-P6, 4. PRLT2/89-33, 5. P1449-2,
6. ICMB90111, 7. Tift238D1, 8. IP18293].

Figure 1. Proline content (μg/mg dry wt. tissue) under salt stress in eight salt-sensitive lines of pearl millet.



[9. ICMP451-P6, 10. ICMP451-P8, 11. H77/833-2, 12. H77/833-2-P5, 13. W504-1-P1, 14. PT732B-P2, 15. 863B-P2, 16. LGD1-B-10, 17. Tift186, 18. Tift383, 19. ICMB89111, 20. ICMB92666,21. 843B, 22. ICMB98004, 23. ICMB99022].

Figure 2. Proline content (µg mg^{-1}dry wt. tissue) under salt stress in fifteen moderately salt-tolerant lines of pearl millet.



[24. Tift23DB-P5, 25. P310-17Bk, 26. 841B-P3, 27. ICMB95333, 28. ICML22].

Figure 3. Proline content (µg mg^{-1} dry wt. tissue) under salt stress in five highly salt-tolerant lines of pearl millet

Higher proline accumulation is related to salt tolerance in the salt-tolerant genotypes, does not occur as a consequence of tissue dehydration or tissue reaction to stress damage [**15**]. Accumulation of free proline is correlated with tissue Na^+ concentration for numerous plant species. This strongly suggests a possible role of proline in osmoregulation during salt stress. Among various compatible solutes, proline is the only molecule that was to plants against singlet oxygen and free radical induced damages. Since proline can act as a single oxygen quencher [**16, 17**] and as a scavenger of OH radicals, it is able to stabilize proteins, DNA and membranes. Hydroxy-radical scavenging activity has been measured for sorbitol, mannitol, myo-inositol and proline and it was found that proline is an effective hydroxy radical scavenger [**18**] Thus, proline is not only an important molecule in redox signaling, but also an effective quencher of reactive oxygen species formed under salt, metal and dehydration stress conditions in all plants, including algae [**19, 20**].

Further, proline estimation can also be performed by Gas Chromatography (GC) coupled to a mass spectrometer (MS) for sensitive identification of analytes. Reports suggest that GC-MS has been employed for the analysis of proline after derivatization with a range of derivatising agents. For instance, GC analysis of proline along with 150 other metabolites was achieved with a mixture of MSTFA and TMCS; mass spectrometry (MS) was employed for detection [**21**]. GC-MS using MTBSTFA to derivatize proline was employed to study the performance of alfalfa plants exposed to water stress [**22**].

APPLICATION

Pearl millet is an important crop of arid and semi-arid areas which is gaining a lot of importance currently due to its high nutritional quality. Pearl millet can grow in drought, low soil fertility and low pH, but salinity greatly affects growth and yield of this crop. The accumulation of the osmoprotectant proline in salt-tolerant lines is suggestive of the fact that this mechanism can be exploited at the genetic level to increase the adaptability of pearl millet for salt stress conditions and hence be applied to develop more number of salt-tolerant varieties of pearl millet by genetic engineering in future.

CONCLUSION

Pearl millet is one of the most important crops produced prominently from western Rajasthan of India and occupies significant place in Indian agriculture. There were very less literature available on volatile profiling of salt-stress induced metabolites in pearl millet. Current study conducted to evaluate the accumulation of proline in pearl millet genotypes under salt stress using spectrometry suggested that proline is an important osmoprotectant in plants and protects the photosynthetic machinery against salt induced damage. In pearl millet seedlings exposed to salt stress, more accumulation of proline in salt-tolerant lines as compared to salt-sensitive was correlated with increasing concentrations of NaCl. Further use of mass spectrometry (MS)-based analytical platforms to profile stress-responsive metabolites will allow these crop plants to adapt to adverse environmental conditions.

ACKNOWLEDGEMENTS

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