Phytology

Biochemical parameters affected by sorbitol induced osmotic stress in *Zea mays* Ganga Safed-2 leaves

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Abstract

In the present investigation, effect of osmotic stress imposed by the supply of sorbitol was analyzed on biochemical parameters, such as, protein, RNA, DNA and proline content in leaves of *Zea mays*, Ganga Safed-2 genotype. For the study, maize leaf segments obtained from light grown seedlings were treated with varying concentrations of sorbitol (0.0-1.0 M) under illumination. Total protein, total RNA and DNA were found to decrease markedly and significantly by the supplementation of 0.2 to 1.0 M sorbitol in excised leaf segments. The relative water content decreased marginally with the sorbitol treatment, but a several-fold increase in proline content was noted. SDS- PAGE analysis of sorbitol treated maize leaf tissue revealed the appearance of a protein (approx. 70 kDa) in 0.6M sorbitol treatment. Further, extensive homology (Protein score: 270) was found for that protein with chloroplast heat shock protein 70 from *Cenchrus americanus.* Concentration-dependent decrease in total chlorophylls and carotenoids was observed with an increasing supply of the osmotic inducer. It is suggested that sorbitol interferes with the growth and metabolic parameters of the *Zea mays* Ganga Safed-2 leaf tissue and stimulates the synthesis of stress induced proteins.

Keywords: osmotic stress, maize leaf, protein, sorbitol

1. Introduction

One of the major environmental factors limiting the worldwide productivity and distribution of cereal crops is osmotic stress resulting from drought (Zhu, 2002) [32]. The consequences of osmotic stress manifest in the inhibition of cell elongation, stomata closure, disturbances in water and ion uptake, translocation of assimilates and changes of various metabolic processes. Prolonged water deficit can cause wilting of plants, inhibits protein synthesis, increases the protein folding and processing, protein-protein interaction which leads to their aggregation and denaturation (Mahajan and Tuteja, 2005) [20]. Osmotic adjustment is one of the drought avoidance mechanisms. Osmotic stress triggers the accumulation of amino acids, such as proline and their derivatives, like glycine betaine, soluble sugars, such as, fructose, galactose, sucrose etc, sugar alcohols, (eg. Mannitol, sorbitol etc.) and polyamines, putrescine, spermidine, spermine etc. However, the production and accumulation of these osmoprotectants depend on environmental conditions, type, duration and sensitivity of plants and genotype (Bowne *et al*., 2012; Marcek *et al*., 2019) [6, 21].

Photosynthesis is a key phenomenon which contributes substantially to the plant growth and hence crop yield. However, osmotic stress hampers the process of photosynthesis in most plants by altering the ultra-structure of various pigments and metabolites including enzymes (Rapacz *et al*., 2010) [28]. Stress response on one hand leads to protein damage and down regulation of certain genes (Diller, 2006) [9], on the other hand it activates the expression of certain genes commonly known as stress genes. As a consequence of activation of the stress genes; stress proteins, known as heat shock proteins or heat stress proteins (HSPs) are synthesized to overcome stress factors. These proteins are known to play a critical role in cell protection against various environmental stresses as well as in maintaining cellular homeostasis (Schroder *et al*., 2005) [29]. Stress proteins normally exist in low concentrations within the cell; however, their concentration enhanced many folds under stress conditions. Sorbitol, a six carbon sugar alcohol, has been used in osmotically induced water stress studies in plants (Hsu and Kao, 2003) [14]. Alleviation of salt stress by improving some biochemical parameters with exogenously applied sorbitol has been reported in *Spinacea oleracia L.* (Gul *et al*., 2017) [13]. Research on osmotically mediated water deficit has been focused mainly on the evaluation of its effects on germination, plant morphology, biomass production and biochemical processes (Bhargava and Paranjpe, 2004, Grzesiak et. al., 2006, Ahmad et. al., 2007) [5, 12, 1], and little attention has been devoted to its action at the cytological level. The objective of the present study was to analyze the effect of osmotic stress induced by sorbitol on biochemical parameters in light grown maize leaf segments.

2. Materials and Methods

Zea mays L.cv. Ganga safed-2 seeds were surface sterilized with 0.1% $HgCl_2$ and were grown in small plastic pots in continuous light for 7-8 d at 25 ± 3 ºC. Hoagland solution (½ strength) containing 5 mM ammonium nitrate was supplied to the seedlings for providing nutrients for analysis of biochemical parameters, excised segments of primary leaves were treated with different concentrations of sorbitol (0.0 - 1.0M) in continuous light supplied with fluorescent tubes for 24 h at 25 ± 3ºC. Before analysis, treated leaf segments were washed carefully with distilled water.

Measurement of relative water content: Relative water content was measured by the method of Barr and Weatherly (1962) [2] and calculated by using the following equation-

RWC (%) = (FW – DW)/ (TW - DW) *100

FW is the fresh weight of the leaf segments; DW is the dry weight and TW is the turgid weight of the leaf segments.

Estimation of protein, RNA content and DNA content: Protein content was estimated with Folin Ciocalteau reagent according to the method of Lowry *et. al.,* 1951 [19]. Total RNA was extracted and estimated by the method of Webb and Levy (1958) [31] using Orcinol reagent. DNA was extracted from leaves by CTAB method described by Doyle and Doyle (1990) [10].

Protein analysis by gel electrophoresis: Treated leaf segments (1.0 g) were homogenized in liquid nitrogen; frozen powder was mixed with 4.0 ml of extraction buffer, and incubated at 4 °C till the homogenate got thawed completely with occasional mixing. The supernatant obtained after centrifugation at 4000 rpm for 30 min in a cooling centrifuge at 4°C using 'Sorvall RC 5B plus' was used to perform one – dimensional polyacrylamide gel electrophoresis (SDS-PAGE) using 10% separating gel and 5% stacking gel according to the procedure of Laemmli (1970) [16]. The molecular mass (14.3 kDa- 205 kDa) standards were run alongside the extracted proteins.

Masss spectrometry: Mass spectrometric analyses of the selected protein band from light grown maize leaf segments treated with 0.6M, 0.8M and 1.0M sorbitol was carried out by peptide mass fingerprinting (PMF) and MS/MS analysis on 4800 plus MALDI TOF/TOF platform (Applied Bios stems®, USA). The protein band was chopped into small pieces of approximately 1 mm3 size and destained completely. The digestion of selected protein band and extraction of peptides was carried out as described in Upadhyay *et. al.,* 2010 [30]. Monoisotopic peptides obtained from MALDI TOF/TOF were analyzed by the 4000 Series Explorer software (version 3.5, ABI, USA). On the basis of mass signals, protein identification was performed with the Mascot search engine (http://www.matrixscience.com) in NCBI nr database. Following parameters were used for database search:

a) mono isotopic mass accuracy, <100 ppm;
b) missed cleavage, 1;
c) carbamidomethylation of cysteincysteine and oxidation of methionine as fixed and variable modification respectively.

Estimation of proline content: Proline content was assayed spectrophotometrically by Bates *et. al*. (1973) [3].

Estimation of pigment content: For the estimation of pigment content, leaf tissue was extracted with 80% acetone in cold. The extract was centrifuged and the absorbance of clear supernatant was measured at 646, 663 and 470 nm. The chl a, chl b and carotenoid contents were calculated using equation of Lichtenthaler and Welburn (1983) [18].

Chlorophyll a (µg ml-1) = 12.21(A_{663}) - 2.81(A_{646}) Chlorophyll b (µg ml-1) = 20.13(A_{646}) – 5.03(A_{663})

Carotenoids (µg ml-1) = [1000 (A_{470}) – 3.27 (Chl a) - 104 (Chl b)] / 229

Data presented are average with ± standard error (SE) of quadruplets (n=4). In each replicate, 20 seeds were used for seedling growth characteristics such as fresh/dry weight of seedling, RWC and metabolic characteristics. Statistical differences were analysed by the student's t-test using Microsoft Excel software. Level of significance used were 'p' values < 0.05 *, < 0.01 **, < 0.001 ***compared with control. The correlation was analyzed for sorbitol treatment and various parameters using Microsoft Excel chart type X-Y scatter.

3. Results

Treatment of leaf segments obtained from light grown maize seedlings decreased the fresh weight and turgid weight prominently at 0.8 and 1.0M sorbitol, but, for dry weight at 1.0M sorbitol only (Table 1). Marginal reduction in RWC was observed at all the concentrations of sorbitol (Table 1). Correlation analysis between sorbitol concentrations and these parameters resulted significant R squared values, being 0.841 with fresh weight, 0.795 with turgid weight and 0.762 with dry weight (Fig.1a, 1b and 1c respectively).

Total protein as well as total RNA content of the leaf segments treated with 0.2-1.0M concentration of sorbitol decreased substantially with increasing concentration (Table 2). However, decline in protein content was more prominent at higher concentrations of sorbitol (Table 2). Correlation analysis generated highly significant R squared values for these parameters, 0.988 for protein and 0.981 for RNA (Figure 2a and 2b). DNA content of the leaf tissue treated with varying concentrations of sorbitol lessened with increasing concentration, substantial reduction in the DNA content was noted from 0.6 - 1.0M sorbitol (Table 2). R squared value of 0.968 was resulted from correlation analysis between sorbitol concentration and DNA content (Figure 2c).

Protein profile obtained with 12% SDS-PAGE for leaf segments from light grown maize seedlings treated without or with sorbitol is shown in Figure 3. The excised protein band was subjected to MS-MS analysis and data were analyzed by mascot search against reported protein database of NCBI. Upon search, extensive homology (Protein score: 270) was found with chloroplast heat shock protein 70 from *Cenchrus americanus* (Nominal Mass: 73137; Calculated pI: 5.23; Number of mass values matched: 5; sequence coverage: 8%) as indicated in Figure 4. Sequences of peptides matched with different mass values are presented in Table 3.

On treatment of leaf segments with sorbitol, total chlorophylls and carotenoids declined with increase in concentration of sorbitol (Table 4). Further, the decrease at each concentration of sorbitol was to almost the same extent for both the parameters. Highly significant R squared values were obtained; 0.863 with total chlorophylls and 0.956 with carotenoids (Figure 5a and 5b). Further, substantial increased proline content was noticed with increasing level of sorbitol (Table 4). There was a 1.7 fold increase in proline level at 1.0M sorbitol treatment. Correlation analysis yielded a highly significant R squared value of 0.902 (Figure 5c).

4. Discussion

The results of the present study demonstrate decrease in fresh weight and turgid weight prominently by the supply of 0.8 and 1.0 M sorbitol in leaf segments obtained from light grown maize seedlings, however, the dry weight was reduced at 1.0 M sorbitol only (Table 1). Relative water content maintains cell

turgor under water stress environment to give relative high yield (Bayoumi *et al*., 2008) [4]. It has been found to decrease marginally with the sorbitol treatment (Table 1). Hence, water status seems to be less affected by the sorbitol. Significant decrease in RWC in leaf disc of *Ganga* 2 *maize* genotype under different levels of water stress caused by PEG solutions has been shown by Moussa and Abdel- Aziz (2008) [24]. Water deficit is one of the common abiotic stresses that affects the growth and development of plants through alteration in metabolism and gene expression (Leopold, 1990; Bray, 1997) [17, 7]. In the present study, concentration dependent decrease in total protein, total RNA as well as DNA content is observed with increasing supply of sorbitol (Table 2), while several fold increase in proline content is observed (Table 4). High accumulation of proline has been reported in both leaves and roots of wheat by sorbitol treatment (Darko *et al*., 2019) [8].

Molecular control mechanisms for abiotic stress tolerance are based on the activation and regulation of specific stress related genes. Abiotic stresses usually cause protein dysfunction. Maintaining the proteins in their functional conformation and preventing the aggregation of non-native proteins are particularly important for cell survival under stress (Park and Seo, 2015) [25]. Experimental stress could evoke compensatory metabolic changes through modification and modulation of the quantity and quality of proteins (Ramagopal, 1987) [27]. Several types of proteins accumulate as a result of drought stress in plants and many of them offer protection. In *Withania somnifera* 34 to 32 kDa proteins have been found to accumulate in stressed leaves, which are considered as an adaptation of drought stress (Kannan and Kulandaivelu, 2011) [15]. Pruvor *et. al.* (1996) [26] also reported similar increase in the contents of these proteins under drought. Further, these proteins were found to be located in chloroplast and their synthesis was induced by high – osmolarity related signals and abscisic acid related signaling systems. Their involvement in the reorganization of thylakoid structure to tolerate the stress was suggested. In my study, SDS- PAGE analysis of sorbitol treated maize leaf tissue revealed the appearance of a protein (approx. 70 kDa) in 0.6M sorbitol treatment. (Figure 3). Further, extensive homology (Protein score: 270) was found for that protein with chloroplast heat shock protein 70 from *Cenchrus americanus* (Table 3 and Figure 3).

Chlorophyll (Chl) has unique and essential roles in harvesting and transducing light energy in antenna systems, and charge separation and electron transport in reaction centers (Eckhardt, and Grimm, 2004; Masuda, 2008) [11, 22]. Chlorophyll level is an important index used to evaluate photosynthetic capacity. In the present study, a concentration dependent decrease in total chlorophylls and carotenoids with increasing supply of osmotic inducer is observed (Table 4). Similarly, a concentration dependent decline in chlorophyll content with increasing concentration of polyethylene glycol- 6000 has been reported in peanut leaves (Meher *et al*., 2018) [23].

Table 1: Sorbitol induced osmotic stress effect on fresh weight, turgid weight, dry weight and RWC in excised maize leaf segments from light grown plants

Sorbitol Conc. M	Fresh weight mg	Turgid weight mg	Dry weight mg	RWC %	
0.0	233 ± 4 (100)	209 ± 3 (100)	21 ± 1.0 (100)	113 ± 2	(100)
0.2	226 ± 5 (97)	207 ± 5 (99)	22 ± 0.8 (104)	110 ± 1	(97)
0.4	201 ± 2.***(86)	187 ± 1***(89)	21 ± 0.2 (100)	108 ± 1	(96)
0.6	210 ± 2*** (90)	194 ± 2** (93)	19 ± 0.6 (90)	108 ± 4	(96)
0.8	170 ± 2*** (73)	159 ± 3***(77)	19 ± 0.7 (90)	108 ± 1	(96)
1.0	178 ± 4***(76)	169 ± 4***(81)	15 ± 0.7***(71)	106 ± 1*	(94)

Leaf segments from light grown maize seedlings were treated with varying concentrations of sorbitol in continuous light for 24 h at 25 ± 2° C.

Values relative to control are given in parentheses.

Level of significance: *'p'* values < 0.05 *, < 0.01 **, < 0.001 ***compared with control.

Table 2: Sorbitol induced osmotic stress effect on total protein, total RNA and DNA content in excised maize leaf segments from light grown plants

Sorbitol Conc. M	Total protein mg g-1 fr.wt.	Total RNA mg g-1 fr.wt.	DNA content mg ml-1
0.0	10.4 ± 0.08 (100)	9.8 ± 0.04 (100)	3.95 ± 0.98 (100)
0.2	8.5 ± 0.06*** (82)	8.1 ± 0.02*** (83)	3.5 ± 0.19 (89)
0.4	7.5 ± 0.04*** (72)	7.3 ± 0.04*** (74)	2.78 ± 0.01 (70)
0.6	6.2 ± 0.06*** (60)	6.1 ± 0.02*** (62)	2.03 ± 0.16 (51)
0.8	4.0 ± 0.04*** (38)	5.4 ± 0.02*** (55)	1.42 ± 0.33 (36)
1.0	3.1 ± 0.06*** (30)	4.5 ± 0.03*** (46)	1.36 ± 0.18 (34)

Leaf segments from light grown maize seedlings were treated with varying concentration of sorbitol in continuous light for 24 h at 25 ± 2 °C.

Values relative to control are given in parentheses.

Level of significance: *'p'* values < 0.05 *, < 0.01 **, < 0.001 ***compared with control.

Table 3: The MALDI TOF mass spectrum sequence of peptides matched with chloroplast heat shock protein 70 from *Cenchrus americanus*

S. No.	Peptides Identified by MS / MS	Mr (expt)	Mr (calc)
1	QAVVNPENTFFSVKR	1735.2343	1734.9053
2	QFAAEEISAQVLR	1461.0489	1460.7623
3	AVITVPAYFNDSQR	1580.1024	1579.7995
4	LQFKDIDEVILVGGGSTR	1889.376	1889.0258
5	DIDEVILVGGGSTR	1372.9911	1372.7198

Mr: Nominal Mass

Table 4: Sorbitol induced osmotic stress effect on total chlorophylls, carotenoids and proline content in excised maize leaf segments from light grown plants

Sorbitol conc. M	Total Chlorophylls µg g-1 fr.wt.	Carotenoids µg g-1 fr.wt.	Proline content mg g-1 fr.wt.
0.0	465 ± 20 (100)	138 ± 14 (100)	77 ± 0.4 (100)
0.2	385 ± 30* (83)	119 ± 11 (86)	100 ± 0.2*** (130)
0.4	350 ± 23** (75)	108 ± 7 (78)	114 ± 0.4*** (148)
0.6	345 ± 21** (74)	101 ± 7 (73)	116 ± 0.8 *** (151)
0.8	326 ± 19***(70)	95 ± 5* (69)	125 ± 0.7*** (162)
1.0	303 ± 6*** (65)	85 ± 5** (62)	131 ± 0.5*** (170)

Leaf segments from light grown maize seedlings were treated with varying concentration of sorbitol in continuous light for 24 h at 25 ± 2 °C.

Values relative to control are given in parentheses.

Level of significance: '*p*' values < 0.05 *, < 0.01 **, < 0.001 ***compared with control.

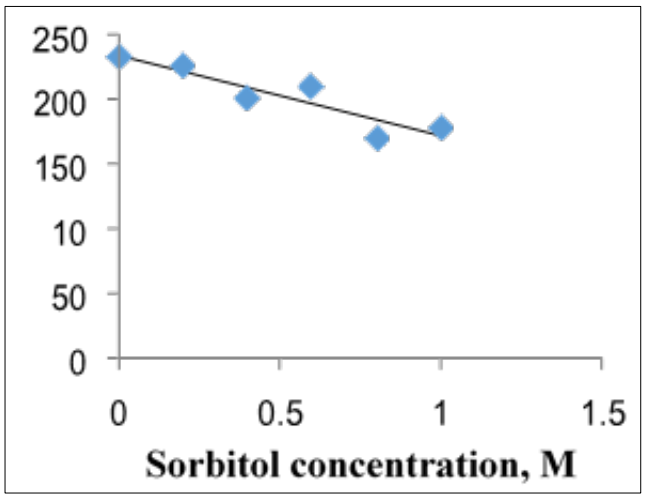

Fig 1a: Correlation analysis of sorbitol concentration and fresh weight

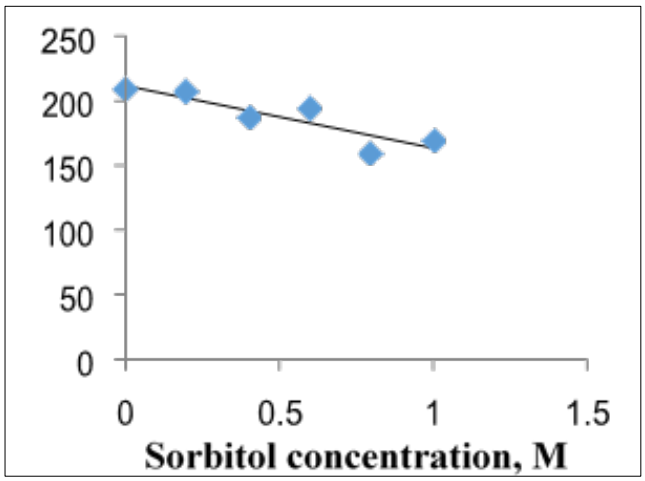

Fig 1b: Correlation analysis of sorbitol concentration and turgid weight

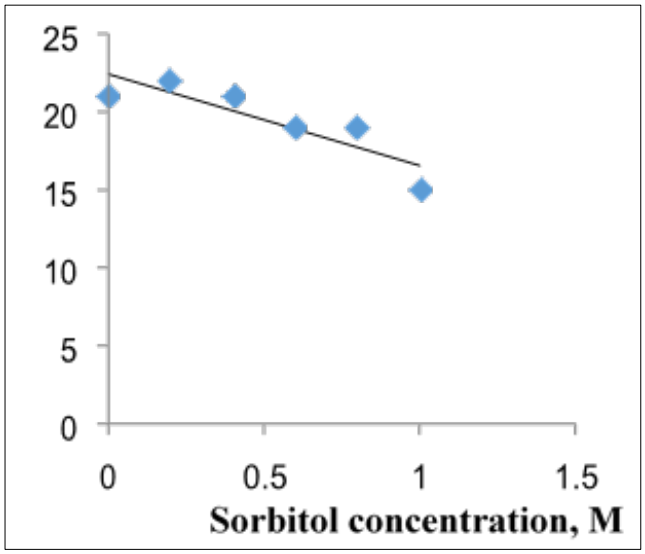

Fig 1c: Correlation analysis of sorbitol concentration and dry weight

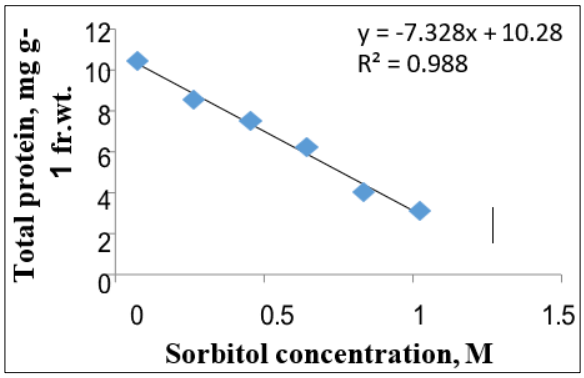

Fig 2a: Correlation analysis of sorbitol concentration and total prot

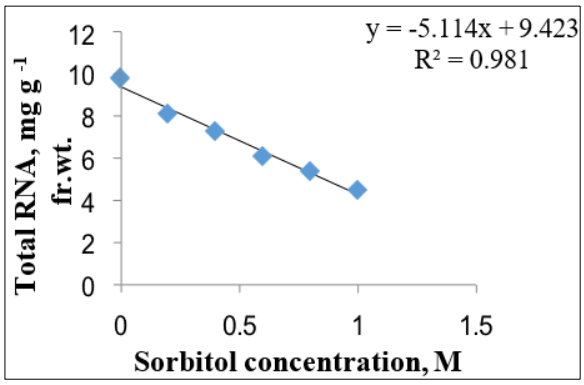

Fig 2b: Correlation analysis of sorbitol concentration and total RNA

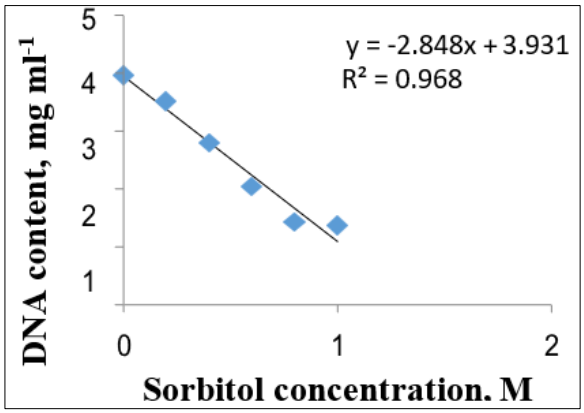

Fig 2c: Correlation analysis of sorbitol concentration and DNA content

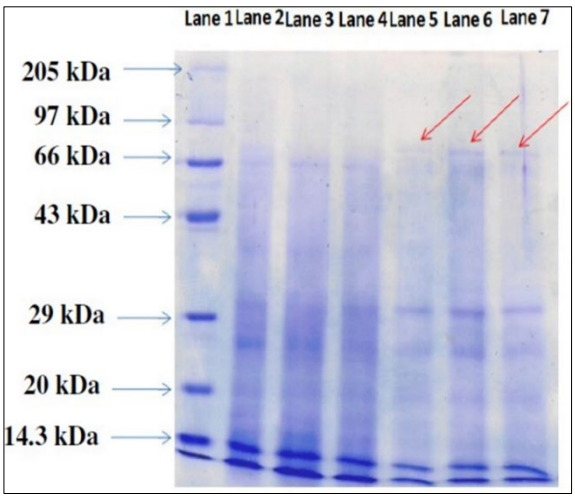

Fig 3: Sorbitol induced osmotic stress effect on protein profile from excised maize leaf segments from light grown plants

Leaf segments from light grown maize seedlings were treated with varying concentration of sorbitol in continuous light for 24 h at 25 ± 2 °C.
Lane 1: Markers
Lane 2: 0.0
Lane 3: 0.2M sorbitol
Lane 4: 0.4M sorbitol
Lane 5: 0.6M sorbitol
Lane 6: 0.8M sorbitol
Lane 7: 1.0M sorbitol

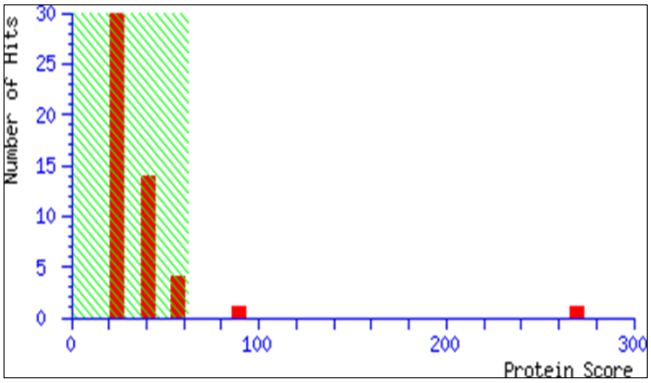

Fig 4: The top score (270) matches the correct *Cenchrus americanus* accession

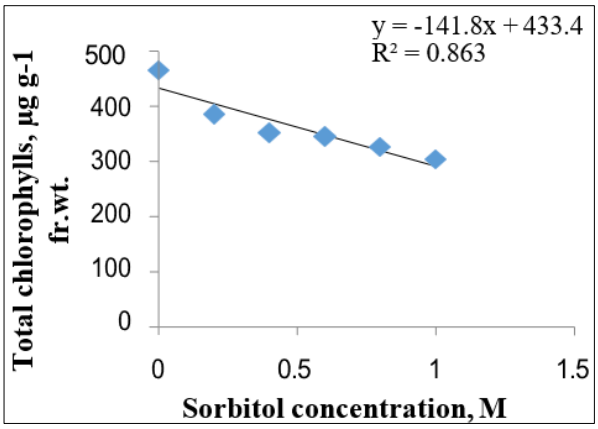

Fig 5a: Correlation analysis of sorbitol concentration and total chlorophylls

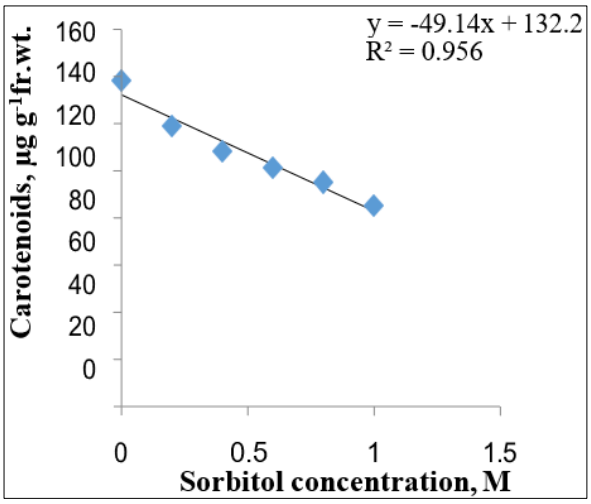

Fig 5b: Correlation analysis of sorbitol concentration and carotenoids

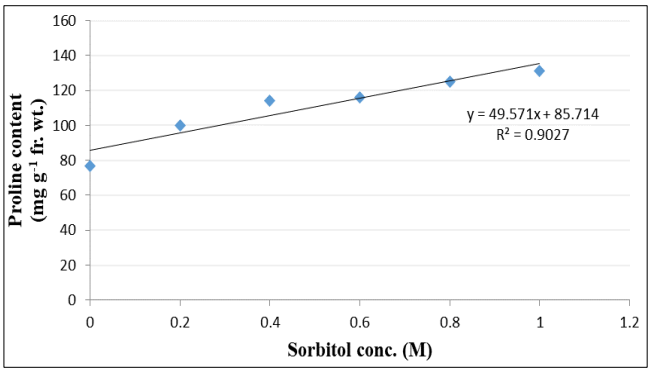

Fig 5c: Correlation analysis of sorbitol concentration and proline content

5. Conclusion

The effect of sorbitol induced osmotic stress was analyzed on biochemical parameters, viz., protein, RNA, DNA and proline content in maize leaves. The outcome of the investigation suggested a significant decrease in these parameters, except proline that enhanced many times with sorbitol treatment. There was concentration-dependent decline in total chlorophylls and carotenoids with increasing supply of the osmotic inducer. Appearance of new protein bands in leaf tissues treated with sorbitol on SDS-PAGE indicates the synthesis of stress induced proteins.

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