In silico and in vitro analysis of salt stress tolerance by Selenium application in proso millet

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Research Article

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Abstract

Background and aim

In order to withstand abiotic stress conditions, plants accumulate a wide range of metabolic products such as proline and there exists a co-relation between proline accumulation and salt stress alleviation in plants, suggesting its role in stress mitigation and osmotic adjustments.

Methods

In this study we assessed the effect of Selenium (Se) under salt (NaCl) stress on Pyrroline-5-carboxylate synthase (*P5CS*), Proline dehydrogenase (*ProDH*) and Ornithine aminotransferase (*OAT*) proteins using *in silico* and *in vitro* studies.

Results

Based on in silico analysis it was found that these enzymes demonstrated a better interaction with Se in comparison to NaCl. The predicted results implied the merit of using Se as a NaCl mitigant. The morphological results revealed a decline in the growth parameters of plants under NaCl stress in a dose dependent manner. However, the application of Se relieved the NaCl induced symptoms. It was found that 1μM Se mitigated the NaCl stress effectively and there was an increase in proline content. The transcriptomic studies also showed that Se up-regulated the expression of *P5CS, ProDH* and *OAT*. Based on above results Se fulfills the criteria as an excellent mitigant for exogenous application in proso millet during NaCl stress.

Conclusion

Under salt stress, Se at lower concentration (1μM) functions as a mitigant to alleviate NaCl stress by regulating the synthesis of proline. Se has high binding affinities with proline biosynthesis enzymes, increasing their activities and expression, enhancing proline content and growth in proso millet.

Introduction

Millets are a diverse category of grasses that have an advantage of being capable of propagating in marginal and/or stressful environments (Cheng et al. 2018; Habiyaremye et al. 2017; Goron et al. 2015). The majority of nine millets are farmed globally and Proso millet (*Panicum miliaceum* L.) is the fourth most widely grown millet in the world. Plants are susceptible to a variety of abiotic stressors (temperature, drought, heavy metals, salt, pesticides, etc.) as well as biotic stresses (virus, bacteria, fungal, etc.). Such stresses have a negative impact on their growth and development, causing a cascade of alterations in morphological, physiological, biochemical, and molecular characteristics. Abiotic stresses can induce a variety of responses in plant life, including changes in transportation and metabolic pathways, which result in growth suppression (Jaleel et al. 2007). The primary result of abiotic stress is ion difference and hyper osmotic pressure, resulting in the increased accumulation of ROS

(reactive oxygen species). ROS scavenging by antioxidant machinery is critical. Non-enzymatic and enzymatic antioxidants molecules are involved in the scavenging of ROS (Sharma et al. 2012). Proline is an important non-enzymatic amino acid that is recognized to ensue in plants under environmental stresses (Hsu et al. 2003; Kishor et al. 2005). It is a standard amino acid with a cyclic structure and non-polar side chains. Proline is responsible for osmotic regulation by balancing cellular structures (like bio-membranes, lipids, amino acids and enzymes), removing free radicals and safeguarding cellular redox potential (Meena et al. 2019). Proline acts as metal chelator and improves plant growth, flowering, pollen/embryo/leaf enlargement. In reaction to stress, proline boosting usually take places in cytosol as it adds to the osmotic adjustment (Ashraf et al. 2007). Mild applications of proline could safeguard and mitigate the deleterious effects of abiotic stress. Proline enhances heavy metal tolerance in plants (Singh et al. 2015; Siripornadulsil et al. 2002). Plants with higher accumulation of proline confirmed improved resistance to drought or salinity stress (Hong et al. 2000). *Arabidopsis thaliana* mutants compromised in proline were highly sensitive to salt stress (Szekely et al. 2008). Owing to the positive characteristics of proline, it is worthwhile to study the role of proline in plants, so that it can be applied as a crop improvement strategy.

A method for the synthesis of proline was first elucidated in *E. coli* in the year 1952 (Vogel and Davis 1952). Housekeeping genes for proline biosynthesis were conveyed primarily in the cytosol of plants and later in the chloroplasts (Szekely et al. 2008). Proline synthesis occurs either through the glutamate pathway in mitochondria or the ornithine pathway in chloroplasts. The core proline metabolism involves two enzymes catalyzing proline synthesis from glutamate in the cytoplasm or chloroplast i.e. Pyrroline-5-carboxylate synthase (*P5CS*) and Pyrroline-5-carboxylate reductase (*P5CR*), two enzymes i.e. Proline dehydrogenase (*ProDH*) and Delta-1-pyrroline-5-carboxylate dehydrogenase (*P5CDH*) catalyzing proline catabolism back to glutamate in the mitochondria. There is also a substitute pathway for proline synthesis via ornithine and Ornithine aminotransferase (*OAT*) has a major role in this route. An increase in proline synthesis from glutamate and down regulation of proline catabolism during unfavorable conditions are known to control proline levels and help in the mitigate stress. In plants, proline is biosynthesized in diverse sub-cellular partitions based on environmental cues from (Szabados and Savoure.2010) glutamate or glutamic acid via Δ^1-pyrroline-5-carboxylate (P5C), in two consecutive reductions catalyzed by Δ^1-pyrroline-5-carboxylate synthase (*P5CS*) and Δ^1-pyrroline-5-carboxylate reductase (*P5CR*). P5CS and P5CR use NADPH and NADH cofactors, respectively. P5CS is encoded by two genes and P5CR is encoded by only one in most plants (Armengaud et al. 2004). The production of proline by means of ornithine as an originator is facilitated by ornithine δ-aminotransferase (*δOAT*) however it is suggested that *δOAT* is more vital for nitrogen recycling (Funck et al. 2008).

Considering the role of proline in salt stress tolerance, the present study evaluated proline status in *Panicum miliaceum* L. under salt stress and exogenous application of Se. We performed 3D structure modeling and validation of the target protein models of *P5CS*, *ProDH*, and *OAT*. Ligand preparation (NaCl and Se in the form of selenite) was performed. In addition, a molecular docking study was performed to determine the binding conformation and structure specificity of the selected proteins with sodium

chloride and Se separately. After performing the *in-silco* analysis, *in-vitro* parameters were also assessed. First of all, the plants were grown and NaCl treatments were given. Exogenous Se was applied to mitigate the NaCl stress. The gene expression of *P5CS*, *ProDH*, and *OAT* was also studied.

Material And Methods

Sequence retrieval and analysis:

Panicum miliaceum L. (Proso millet) *P5CS, ProDH*, and *OAT* amino acid sequences were retrieved from the SwissProt database (https://swissmodel.expasy.org/repository). For *P5CS* UniProtKB AC name A0A3L6SZ65 was selected, where as for *ProDH*, and *OAT* A0A3L6TP97 and A0A3L6TJF0 was selected respectively. ProtParam (https://web.expasy.org/protparam) was used to predict the primary structure. The physicochemical properties were calculated and included the molecular weight of the molecule, the theoretical isoelectric point (pI), the composition of amino acids and atomic composition, the total number of positive and negative residues, the extinction coefficient, the half-life estimate, the instability index, the aliphatic index, and the grand average of hydropathicity.

Secondary structural analysis:

The sequences of *P5CS, ProDH*, and *OAT* was retrieved in the form of FASTA format and used for secondary structure analysis. PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred) and the Chou-Fasman methods/tools (http://www.biogem.org/tool/chou-fasman) were used to predict the secondary structure of the protein. We calculated secondary structural features of the protein sequence, such as alpha helixes, sheets, coils, and turns.

Three dimensional Structure modeling:

Homology modeling was used to determine the three dimensional structures of the proteins *P5CS, ProDH* and *OAT* from *Panicum miliaceum* L. using Swiss modeling software (https://swissmodel.expasy.org). A Swiss repository was searched for the target sequence of interest (https://swissmodel.expasy.org/repository). The templates of *Panicum miliaceum* L. were selected. Using homology modeling, the target sequence and sequences of known structures similar to the query sequence were aligned. The sequence alignment and template structure are then used to create a structural model of the target. A sequence with the highest sequence identity score was preferred from the obtained BLAST results.

Model Assessment:

In Swiss modeling, the in-build structure assessment tool was used to evaluate the models (https://swissmodel.expasy.org/assess). For analysis of possible conformations of dihedral angles Ψ and Φ of amino acid residues in protein structures, the modeled structure was verified using the Ramachandran plot. We calculated MolProbity Score, Clash Score, Ramachandran favoured percentage,

Ramachandran outliers, Rotamer outliers, C-beta deviations, bad angles and Q mean Z score. ProSA (https://prosa.services.came.sbg.ac.at/prosa.php) was used for energy criteria analysis calculations.

Enzyme (Receptor), ligand processing and Molecular docking:

All the models prepared and validated in Swiss modeling tool (https://swissmodel.expasy.org), were downloaded in .PDB format for further requirement. Ligands (NaCl and Se in the form of selenite) were obtained from NCBI Pub chem. (https://pubchem.ncbi.nlm.nih.gov). Both models and ligands were processed and repaired for errors prior to molecular docking experiments. Open Babel GUI 3.1.1 program was used to convert ligands to other formats when ever required. To determine the best orientation and energy of interaction between ligand molecules and target proteins, molecular docking studies were executed. The docking program like CBDock (http://clab.labshare.cn/cb-dock/php) and Dock Thor (http://www.dockthor.lncc.br) was used for carrying out blind docking. Several poses were generated for each ligand based on the docking calculation. Choosing of the best pose was done keeping in view the interaction energy among ligand and the protein relations with the key residues of the protein.

Plant growth and treatments:

The seeds of *Panicum miliaceum* L. were collected from Ladakh region (India) and identified at Centre for Biodiversity and Taxonomy, University of Kashmir. The seeds were sterilized using 70% (v/v) ethanol for 1 minute and washed with sterile distilled water. Surface sterilization of seeds was performed using 0.1% $HgCl_2$ (Merck, India) solution (w/v) for 3 min followed by rinsing with sterilized distilled water. The seeds were sown in pots having 20 cm diameter and containing autoclaved sand. A controlled environment with a 26 ± 1°C temperature and a 16-h photoperiod was maintained as per Desoky et al. 2019; Hussain & Ashraf 2008. Three sets of plants were grown with three replicates each. Concentrations viz., 0, 150 and 200 mM of NaCl were added to the pots and 1 µM, 5 µM, 10 µM selenite (Se) was applied as Se source. All treatments were given as per Shah et al. 2020; Rasool et al. 2020. For the growth of the seedlings, Hoagland nutrient medium (pH 6.5) containing all macro and micro nutrients was used. The plants were harvested after 22 days of sowing. Plant growth-specific parameters were calculated as per Kausar et al. 2012; Singh & Kumar 2008. Total proline was calculated in control, NaCl treated and Se treated set according to Zhu et al. 2020.

Proline content estimation:

The ninhydrin reaction was used to assess the proline content of the leaves as per Zhu et al. 2020 with some modifications. Leaves and roots (0.5 g) collected were extracted in 3 percent (w/v) sulfosalicylic acid. The leaves/roots were weighed and finely grounded using liquid nitrogen. The mixture was kept as such for few minutes and was centrifuged at 12,000 g for 10 min. The supernatant obtained after centrifuge was used to estimate proline content. The supernatant was combined with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid and placed in a 100^{o}C water bath for 1 hour. The reaction was stopped by immersing the test tubes in an ice bath. Further 4 ml toluene was added to the mixture and

the absorbance at 520 nm was measured with a spectrophotometer. The content of proline was measured using a proline standard curve made with different concentrations.

Determination of plant growth parameters and tolerance index (TI):

Following the experiment, 10 plants were taken at random from each treatment and gently cleaned four times with deionized water to remove adherent sand from the root surfaces. The morphological parameters were examined as per Dikobe et al. 2021; Kausar et al. 2012; Singh & Kumar 2008. To evaluate the capacity of plants to thrive under high saline environments, the tolerance index (TI) was calculated by the equation given by Wilkin's 1957:

Tolerance Index (TI %) = MLT / MLC × 100

MLT = Mean length (root, shoot) of the longest root in treated plants, MLC = Mean length (root, shoot) of longest root in control.

Gene expression studies of *P5CS*, *ProDH* and *OAT* genes:

Molecular characterization of these genes of proline pathway in *Panicum miliaceum* L. was performed. Samples in triplicates were prepared in a mortar using liquid nitrogen. Total RNA was isolated from plant material using Trizol reagent (Invitrogen) according to the modified protocol of Deepa et al. 2014. RNase free DNase (Promega) was added to remove genomic DNA contamination. The quality of RNA was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies). For validation of these genes of proline pathway, cDNA was synthesized using reverted aid cDNA synthesis kit (Thermo scientific) by manufacturer's protocol and gene expression studies were carried out by transcriptomic analysis.

Data analysis:

Two way analysis of variance (ANOVA) and significance of data ($P < 0.0001$) was calculated statically. All these tests were performed with XLSTAT 2021 and graph pad prism 6.

Results

Sequence retrieval and analysis, secondary structural analysis

For *P5CS* UniProtKB AC name A0A3L6SZ65 (*A0A3L6SZ65_PANMI*) was selected, where as for *ProDH*, and *OAT*, A0A3L6TP97 (*A0A3L6TP97_PANMI*) and A0A3L6TJF0 (*A0A3L6TJF0_PANMI*) was selected respectively. The physicochemical parameters of each protein computed for primary structure are given in tables below (Tables 1 & 2). The calculated results from secondary structure analysis are shown in Table 3; Fig. 1; Fig. 2. The P5CS, OAT and ProDH proteins have 716, 474 and 484 amino acid residues with an estimated molecular weight of 77625.95, 51350.39 and 51414.87 respectively. For *P5CS*, the maximum number of amino acids present in the sequence was found to be Leu (10.9%) and least was that of Trp

(0.5%). Similarly, for both *OAT* and *ProDH* maximum number of amino acids present in the sequences was found to be Ala (11.4%) and (17.8%) and least was that of Trp (0.4% and 0.6%) respectively. The theoretical isoelectric point of *P5CS, OAT* and *ProDH* was 6.25, 6.65 and 7.62 respectively. The instability index and grand average of hydropathicity for these proteins is 33.19, 27.79, 39.91 and − 0.072, -0.095 and 0.046 respectively. Secondary structure analysis revealed that in *P5CS, OAT* and *ProDH*alpha helix was predominant (81.1%, 71.9%, and 75.4%).

Table 1
Predicted physiochemical properties of enzymes

Parameters	**Values for *P5CS***	**Values for *OAT***	**Values for *ProDH***
Number of amino acids	716	474	484
Molecular weight of the molecule	77625.95	51350.39	51414.87
Theoretical isoelectric point (pI)	6.25	6.65	7.62
The total number of negatively charged residues (Asp + Glu)	92	60	52
The total number of positively charged residues (Arg + Lys)	85	58	53
Molecular Formula	$C_{3412}H_{5571}N_{961}O_{1052}S_{23}$	$C_{2260}H_{3641}N_{639}O_{666}S_{29}$	$C_{2266}H_{3652}N_{664}O_{671}S_{15}$
Total no. of atoms	11019	7235	7268
Extinction coefficient M^{-1} cm^{-1}, at 280 nm measured in water (assuming all pairs of Cys residues form cystines)	36370	25285	33390
Half-life estimate	30 hours (Mammals) > 20 hours (Yeast). > 10 hours (E.coli)	30 hours (Mammals) > 20 hours (Yeast). > 10 hours (E.coli)	30 hours (Mammals) > 20 hours (Yeast). > 10 hours (E.coli)
Instability index	33.19	27.79	39.91
Aliphatic index	101.73	91.27	96.38
Grand average of hydropathicity	-0.072	-0.095	0.046

Table 2: Amino acid composition in enzyme

Parameter	Values for *P5CS*	Values for *OAT*	Values for *ProDH*
Ala (A)	9.2 %	11.4 %	17.8 %
Arg (R)	5.4 %	5.9 %	8.7 %
Asn (N)	3.2 %	2.5 %	1.7 %
Asp(D)	7.5 %	6.1 %	4.3 %
Cys (C)	1.1 %	3 %	1.9 %
Gln (Q)	2.8 %	2.7 %	2.5 %
Glu (E)	5.3 %	6.5 %	6.4 %
Gly (G)	7.3 %	9.1 %	7.6 %
His (H)	2.5 %	2.5 %	2.1 %
Ile (I)	6.3 %	6.5 %	3.9 %
Leu (L)	10.9 %	9.7 %	11.8 %
Lys (K)	6.4 %	6.3 %	2.3 %
Met (M)	2.1 %	3.2 %	1.2 %
Phe (F)	2.5 %	3.4 %	2.9 %
Pro (P)	3.1 %	5.3 %	5.6 %
Ser (S)	8.4 %	5.5 %	6.2 %
Thr (T)	4.9 %	2.3 %	4.3 %
Trp (W)	0.4 %	0.4 %	0.6 %
Tyr (Y)	1.8 %	1.9 %	2.3 %
Val (V)	8.8 %	5.7 %	6 %
Pyl (O)	0.0 %	0.0 %	0.0 %
Sec (U)	0.0 %	0.0 %	0.0 %

Table 3
Secondary structure analysis of *P5CS, OAT* and *ProDH*.

Parameter	Values for *P5CS*		Values for *OAT*		Values for *ProDH*	
	Total residues	%	Total residues	%	Total residues	%
Helix (H)	581	81.1	341	71.9	365	75.4
Sheet (E)	218	30.4	253	53.4	260	53.7
Turn (T)	98	13.7	59	12.4	56	11.6

Three dimensional Structure modeling and Model Assessment

Homology modeling of *P5CS, ProDH*, and *OAT* was predicted using BLAST search. From the BLAST results, the selected template for *P5CS* protein was *2h5g.1.A*. This template has 47.37% sequence identity with the target protein. The model of *P5CS* was built on the basis of sequence alignment of *2h5g.1.A* and *P5CS*. In similar way the selected template for *ProDH* and *OAT* was *5kf6.1.A* and *5eav.1.A* respectively. These templates have 23.51% and 46.94% sequence identity with the target proteins. These models were built in a same manner as previously done. The three dimensional models predicted are shown in Fig. 3 (A C and E). The three dimensional models made were assessed through Ramchandran plot analysis. ProSA revealed energy criteria of the model. The analysis by ProSA revealed Z score of -9.12 for *P5CS*, -10.15 for *OAT* and − 7.32 for *ProDH*. The parameters revealed by Ramachandran plot analysis are show in Table 4.and the corresponding Ramachandran plot for each protein is shown in Fig. 3 (B, D and F). Ramachandran plot revealed that 93.82%, 95.15% and 89.49% of residues are in favoured region for *P5CS*, OAT and *ProDH* respectively. *P5CS* and *OAT* have QMEAN value of -1.6 and 0.59. The low quality QMEAN value of -6.73 was observed for *ProDH*.

Table 4: Ramachandran plot analysis of 3D proteins models

Parameter	Values for *P5CS*	Values for *OAT*	Values for *ProDH*
MolProbity Score	1.53	1.67	2.3
Clash Score	1.23	6.13	8.95
Ramachandran Favored	93.82 %	95.15 %	89.49 %
Ramachandran Outliers	0.95 %	0.36 %	3.74 %
Rotamer Outliers	2.29 %	0.74 %	2.16 %
C-Beta Deviations	2	6	23
Bad Angles	64/8770	60/8718	66/4468
Cis Non-Proline	2/818	1/782	2/408
QMEANDisCo Global	0.73±0.05	0.81±0.05	0.54±0.05
QMEAN	-1.6	0.59	-6.73
Torsion	-1.5	0.37	-5.46
Solvation	0.37	0.73	-2.50

Molecular docking

In an effort to find the possible role of Se in mitigating NaCl stress and its affect on key genes of proline pathway, we aimed to study molecular docking affinities of these enzymes with NaCl and our mitigant Se. The study reveled that Se has greater binding affinities with *P5CS* and *OAT* which might result in the hyper accumulation of proline in salt stress conditions and in turn mitigation of this stress. To cross validate whether this hyper accumulation is due to NaCl or Se, docking affinities of NaCl with these enzymes were also studied. This result also discloses that in comparison to NaCl, the binding affinities of Se with these enzymes are higher. The docking affinities of *P5CS*, *OAT* and *ProDH* with NaCl were similar at -1.6 and with Se the affinities were − 3.2, -3.5 and − 3.4 respectively revealing the higher binding affinities of these enzymes with Se (higher negative value). The ligand receptor binding and molecular docking is shown in Fig. 4; Supplementary Fig. 1 respectively.

Proline content, plant growth parameters and tolerance index

In order to further validate the role of Se in mitigating NaCl, *In vitro* experiments were performed. Plants (*Panicum miliaceum* L.) were grown and treatments were given as mentioned. Proline content analysis revealed that Se up regulates proline content as shown in Fig. 5. Plant morphological parameters like shoot length (SL), root length (RL), leaf length (LL), leaf width (LW) and tolerance index (TI) were calculated (Fig. 5; Table 5) and the principal component analysis (PCA) of all parameters is shown in Fig. 6. The leaf proline content (LPC) in proso millet under salt stress increased by 176.15% at 150mM NaCl and by 97.01% at 200mM NaCl. The application of Se (1 µM, 5 µM and 10 µM) had a positive impact on

LPC by increasing it by 107.72%, 49.19% and 202.26% in comparison to control. The application of Se (1 µM) increased LPC by 6.46% however it decreased at Se (5 µM and 10 µM) by 3.88% and 12.39% with respect to 150mM NaCl. However, with Se application (1 µM, 5 µM and 10 µM) decreased it by 1.67%, 17% and 14.92% with respect to 200mM NaCl. The root proline content (RPC) in proso millet under salt stress increased by 21.87% at 150mM NaCl and by 72.5% at 200mM NaCl. The application of Se (1 µM)) had a positive impact on RPC by increasing it 17.42% in comparison to control. However Se (5 µM and 10 µM) decreased RPC by 2.83% and 27.94%. The application of Se (1 µM) increased it by 2.99% however it decreased at Se (5 µM and 10 µM) by 6.97% and 42.21% with respect to 150mM NaCl. The application of Se (1 µM) increased RPC by 1.4% however decreased at Se (5 µM and 10 µM) by 1.64% and 0.46% with respect to 200mM NaCl. The shoot length(SL) in proso millet under salt stress decreased by 12.7% at 150mM NaCl and by 24.15% at 200mM NaCl. The application of Se (1 µM, 5 µM, 10 µM) had a positive impact on SL by increasing it by 17.22%, 3.71% and 4.70% respectively in comparison to control. The application of Se (1 µM, 5 µM, 10 µM) increased by 20.5%, 7.9% and 16.7% with respect to 150mM NaCl. However, with Se application (1 µM, 5 µM) it increased by 8.9% and 4.2% but decreased at Se 10 µM by 10.9% with respect to 200mM NaCl. The root length (RL) in proso millet under salt stress decreased by 21.01% at 150mM NaCl and by 23.67% at 200mM NaCl. The application of Se (1 µM, 5 µM, 10 µM) had a positive impact on RL by increasing it by 27.12%, 23.67% and 16.48% respectively in comparison to control. The application of Se (1 µM, 5 µM, 10 µM) increased by 18.5%, 14.81% and 4% with respect to 150mM NaCl. However, with Se application (1 µM, 5 µM) it increased by 8.01% and 12.5% but decreased at Se 10 µM by 1.74% with respect to 200mM NaCl. The total length (TL) in proso millet under salt stress decreased by 15.6% at 150mM NaCl and by 23.16% at 200mM NaCl. The application of Se (1 µM, 5 µM, 10 µM) had a positive impact on TL by increasing it by 2%, 0.94% and 10.24% respectively in comparison to control. The application of Se (1 µM, 5 µM, 10 µM) increased by 20.44%, 11.21% and 2.77% with respect to 150mM NaCl. However, with Se application (1 µM, 5 µM) it increased by 7.68% and 7.82% but decreased at Se 10 µM by 8.98% with respect to 200mM NaCl. The leaf height (LH) in proso millet under salt stress decreased by 1.57% at 150mM NaCl and by 11.02% at 200mM NaCl. The application of Se (1 µM, 5 µM) had a positive impact on LH by increasing it by 22.02% and 11.02%, however at Se 10 µM it decreased by 5.5% in comparison to control. The application of Se (1 µM, 5 µM, 10 µM) increased by 15.2%, 8.8% and 4% with respect to 150mM NaCl. However, with Se application (1 µM) it increased by 15.04% but decreased at Se 5 µM and 10 µM by 16.8% and 17.7% with respect to 200mM NaCl. The leaf width (LW) in proso millet under salt stress decreased by 32% at 150mM NaCl and by 36% at 200mM NaCl. The application of Se (1 µM, 5 µM) had a positive impact on LW by increasing it by 8% and 12%, however at Se 10 µM it decreased by 20% in comparison to control. The application of Se (1 µM) increased by 35.29%, however it decreased at Se (5 µM, 10 µM) by 4.34% and 18.18% with respect to 150mM NaCl. However, with Se application (1 µM) it increased by 43.75% but no change was observed at Se 5 µM and decreased at Se10 µM by 18.75% with respect to 200mM NaCl. The leaf area (LA) in proso millet under salt stress decreased by 8.6% at 150mM NaCl and by 39.3% at 200mM NaCl. The application of Se (1 µM, 5 µM) had a positive impact on LA by increasing it by 215.05% and 149.16%, however at Se 10 µM it decreased by 0.78% in comparison to control. The application of Se (1 µM and 5 µM) increased by 72.3% and 70.47%, however it decreased at Se (10 µM) by 35.35% with respect to 150mM NaCl.

However, with Se application (1 µM) it increased by 126.35% but decreased at Se 5 µM and10 µM by 29.6% and 47.22% with respect to 200mM NaCl. Tolerance index (TI) improved in the plants treated with Se in comparison to NaCl treatments only. The TI in shoots decreased by 5% at 150mM NaCl and 16.40% at 200mM NaCl with respect to control. The increase in TI of shoots was 21.31%, 10.65% and 14.75% at Se (1 µM 5 µM and 10 µM) respectively. The application of Se (1 µM and 5 µM) increased by 14.75% and 3.1%, however it decreased at Se (10 µM) by 4.1% with respect to 150mM NaCl. With Se application (1 µM, 5 µM) it increased by 8.2% and 2.46% but decreased at Se 10 µM by 9.83% with respect to 200mM NaCl. The TI in roots decreased by 6.25% at 150mM NaCl and 12.50% at 200mM NaCl with respect to control. The increase in TI of shoots was 40.62%, 34.37% and 23.43% at Se (1 µM, 5 µM and 10 µM) respectively. The application of Se (1 µM, 5 µM and 10 µM) increased by 3.12%, 1.56% and 1.56% with respect to 150mM NaCl. However, with Se application (1 µM) there was no change but it increased at Se (5 µM) by 9.39% and decreased at Se (10 µM) by 3.13% and with respect to 200mM NaCl.

Table 5
TI (root TI %), shoot tolerance index (shoot TI %), of proso millet

Treatments	TI % Shoot	TI % Root	Treatments	TI % Shoot	TI % Root
C	100	100	150 NaCl + 5 Se	99.18	95.31
1Se	140.62	140.62	150 NaCl + 10 Se	91.8	95.31
5 Se	134.37	123.43	200 NaCl	83.6	87.5
10 Se	123.43	123.43	200 NaCl + 1 Se	91.8	87.5
150 NaCl	93.75	93.75	200 NaCl + 5 Se	86.06	96.37
150 NaCl + 1 Se	96.85	95.31	200 NaCl + 10 Se	73.77	84.37

Transcriptomic studies

One specific goal was to investigate gene expression of *P5CS*, *OAT* and *ProDH* under NaCl stress and with applications of Se. The transcriptomic studies revealed that Se (1µM) up-regulated the expression *P5CS*, *ProDH* and *OAT*. The expression of *P5CS* was 1.28 folds (in 150 mM NaCl treated plant) and 1.63 folds (in plants treated with 150 mM NaCl and 1µM Se) as compared to control. Similarly, the expression of *OAT* gene was 0.87 folds (in 150 mM NaCl treated plant) and 1.2 (folds in plants treated with 150 mM NaCl and 1µM Se) as compared to control. *ProDH* was 1.0 folds more (in 150 mM NaCl treated plants) and 1.3 folds up-regulated (in plants treated with150 mM NaCl and 1µM Se).

Discussion

Salt stress has negative consequences on plant growth and plants respond to this stress by number of ways (Ibrahimova et al. 2021). These responses vary from plant to plant and include regulation of osmolytes, transcriptional factors, over expression or down regulation of certain genes, regulation of biochemical parameters and modification of signaling cascades (Alnusairi et 2021; Shah et al. 2021; Jin

et al. 202; Ahmad et al. 2021; Zhang et al. 2021). The ultimate role of these changes is to withstand the harmful environment. The regulation of proline is one among these changes as it has a positive role under salt stress (Ibrahim et al. 2019; Alzahrani et al. 2019). In this study exogenous Se was applied for mitigating NaCl stress in *Panicum miliaceum* L. The role of key genes of the proline biosynthetic pathway under salt stress and Se applications were explored. To accomplish these objectives, a combination of *in-silico* approaches, biochemical, gene expression and morphology analysis were applied. Results showed that amongst P5CS, OAT and ProDH proteins P5CS is the largest with molecular weight of 77625.9. Higher percentage of Ala, Val, Leu and Ile in an enzyme increases its aliphatic index (Negi et al. 2017). The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains, it may be regarded as a positive factor for the increase of thermo stability of globular proteins (Ikai 1980). The aliphatic index indicates that these proteins are thermo stable. For *P5CS*, the maximum numbers of amino acids present in the sequence were found to be Leu and least was that of Trp. Similarly, for both *OAT* and *ProDH* maximum number of amino acids present in the sequences was found to be Ala (11.4%) and (17.8%) and least was that of Trp (0.4% and 0.6%) respectively. This may perhaps be the basis that, *P5CS* being most stable, followed by ProDH and OAT. An enzyme or protein's N terminal residues play an important role in its stability (Negi et al. 2017). The N-end rule based half life of all the enzymes was studied. The half life indicated that every enzyme have half life of more than 10 hours. On the basis of total number of positive and negative residues, P5CS and OAT were negatively charged whereas ProDH is slightly positively charged. The theoretical isoelectric point of *P5CS, OAT* and *ProDH* was 6.25, 6.65 and 7.62, revealing the basic nature of these proteins. According to Guruprasad et al. 1990, a protein whose instability index is smaller than 40 is predicted as stable, a value above 40 predicts that the protein may be unstable. The instability index for these proteins were 33.19, 27.79 and 39.91 respectively, which classify these proteins as stable. Negative grand average of hydropathicity values indicates the hydrophilicity of the protein (Baloji et al. 2019). The calculated grand average of hydropathicity for *P5CS, OAT* and *ProDH* was − 0.072, -0.095 and 0.046 respectively. This indicates that only *P5CS* and *OAT* are hydrophilic in nature. Secondary structure analysis revealed that in *P5CS, OAT* and *ProDH* alpha helix was predominant (81.1%, 71.9%, 75.4%) followed by random sheets and turns. The 3D structure assessment scores were calculated and models were validated by means of Ramachandran plot. MolProbity Score is the combined protein quality score and should be as low as possible. From the results it is clear that the calculated values for MolProbity are very low. Among all enzymes the *P5CS* have lowest score i.e. 1.53, followed by *OAT* and *ProDH* indicating model validities. Similar trend was observed for clash score. Ramachandran plot revealed that 93.82% of residues are in favoured region for *P5CS*, 95.15% for *OAT* and 89.49% for *ProDH*. This also indicates the stability of all the models. QMEAN Z-scores around 0.0 reflect a native-like structure and, a QMEAN Z-score below 4.0 indicates a model with low quality (Benkert et al. 2011). *P5CS* and *OAT* have QMEAN value of -1.6 and 0.59, indicating there native like structure. The low quality QMEAN value of -6.73 was observed for *ProDH* and the primary possible reason for this can be the low sequence similarity of this enzyme with template. Molecular docking affinities of *P5CS, OAT* and *ProDH* with NaCl and the mitigant Se (in the form of selenite), reveled that Se has greater binding affinities with *P5CS* and *OAT* which might result in the hyper accumulation of proline in NaCl stress conditions and in turn mitigating the NaCl stress. Proline is essential for plants to adapt to their

environment variations, droughts, and soil salinity (Sekhar et al. 2007). During salinity adjustments, proline acts as a compatible solute that plays a vital role in osmotic adjustment and adaptation (Wu et al. 2021). Our results suggest that more proline is accumulated under the application of exogenous Se. We found that the proline content in NaCl-exposed plants increased significantly. Further increases in proline content were found by the application of Se to the salt-treated plants as reported earlier in Switchgrass (Guan et al. 2020). In various plants proline is being used as mitigant to reduce stress (Mushtaq et al. 2021). An increase in the proline content was found in comparison with control when exogenously Se was added as a mitigant in *Panicum miliaceum* L. (Shah et al. 2020). The pretreatment of rice seeds with proline resulted in neutralization of the detrimental effect of stress. Similarly lower concentration of proline used externally helped in improving the antagonistic effects of salinity stress in rice and tobacco cells (Roy et al. 1993, Ashraf et al. 2007). Additionally in culture of *Arachis hypogea* it successfully improves fresh weight and reduced the peroxidative harm to lipid membranes (Jain et al. 2001). Upon examining the effects of NaCl on various growth and morphological parameters in proso millet, it was observed that NaCl (150, 200 mM) treatments significantly affected growth i.e., root length, shoot length, leaf height, width and area. The results revealed that decline in the growth parameters of plants under stress and the decline was much greater for plants treated with 200 mM than for those treated with 150 mM. Similarly, this effect on growth parameters was previously observed in *Phaseolus vulgaris* L. (Taibi et al. 2016), maize (Ahmad et al. 2021), Wheat (Ashraf & Ashraf 2012) and Soyabean (Nigam et al. 2022) under salt stress. We observed that the application of Se relieved the salt induced symptoms with 1µM Se treatments found to be more effective in mitigating NaCl stress. As reported earlier the application of Se on olive under salt stress improved the growth and development (Regni et al. 2021). Similar low concentrations of Se prevented salt induced damage in wheat and cucumber (Elkelish et al. 2019; Hawrylak 2009). These results strongly indicate that low levels of Se alleviated the NaCl stress by modulating the proline biosynthesis. The transcriptomic analysis of the proline biosynthesis enzymes *P5CS, OAT* and *ProDH* under these conditions revealed that their expressions were upregulated under NaCl stress and the application of Se resulted in more expression which suggests that there is a correlation between *P5CS, OAT* and *ProDH* expression and NaCl stress tolerance. Similarly, Over expressing heterologous *P5CS* genes from *Lolium perenne* and *Puccinellia chinampoensis* in switch grass improved salt tolerance by dropping the electrolyte escape and ROS levels (Guan et al. 2019). *Arabidopsis thaliana* plantlets showed enhanced proline content, *P5CS* mRNA and *OAT* under salt stress (Roosens et al. 1998). Over expression of *Arabidopsis δOAT* gene in tobacco and rice had amplified proline content and increased stress tolerance (Roosens et al. 2002).Thus our study confirms that proline biosynthesis enzymes are closely linked to stress tolerance revealing their stability under NaCl stress and the data further confirms that Se is involved in the improvement in protein stability and structure by acting as the mitigant.

Conclusion

Under NaCl stress, Se functions as a mitigant and regulates the synthesis of proline. The study examined the interaction of Se with proline biosynthesis related genes viz. *P5CS*, *OAT* and *ProDH*. Se showed high

binding affinities with these enzymes, which in turn may be responsible for the increased expression of their corresponding genes in proso millet. In this study, it was found that Se (1µM) promotes proline synthesis and thereby imparts better stress adaptability. As a result Se can act as a suitable candidate for the improved performance of plants under saline conditions. Further research is warranted for a better understanding of molecular mechanisms governing salt stress alleviation by Se application in plants.

Declarations

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Figures

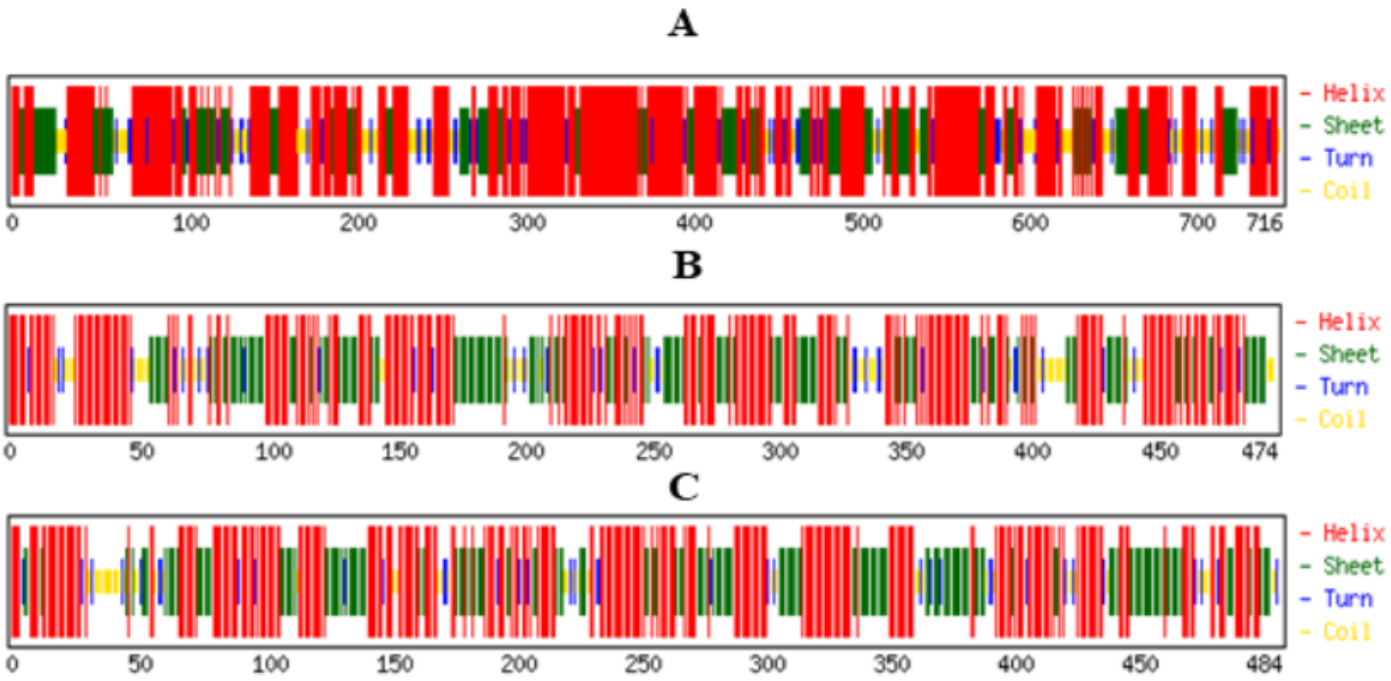

Figure 1

Secondary structure analysis (A) *P5CS* (B) *OAT* (C) *ProDH*

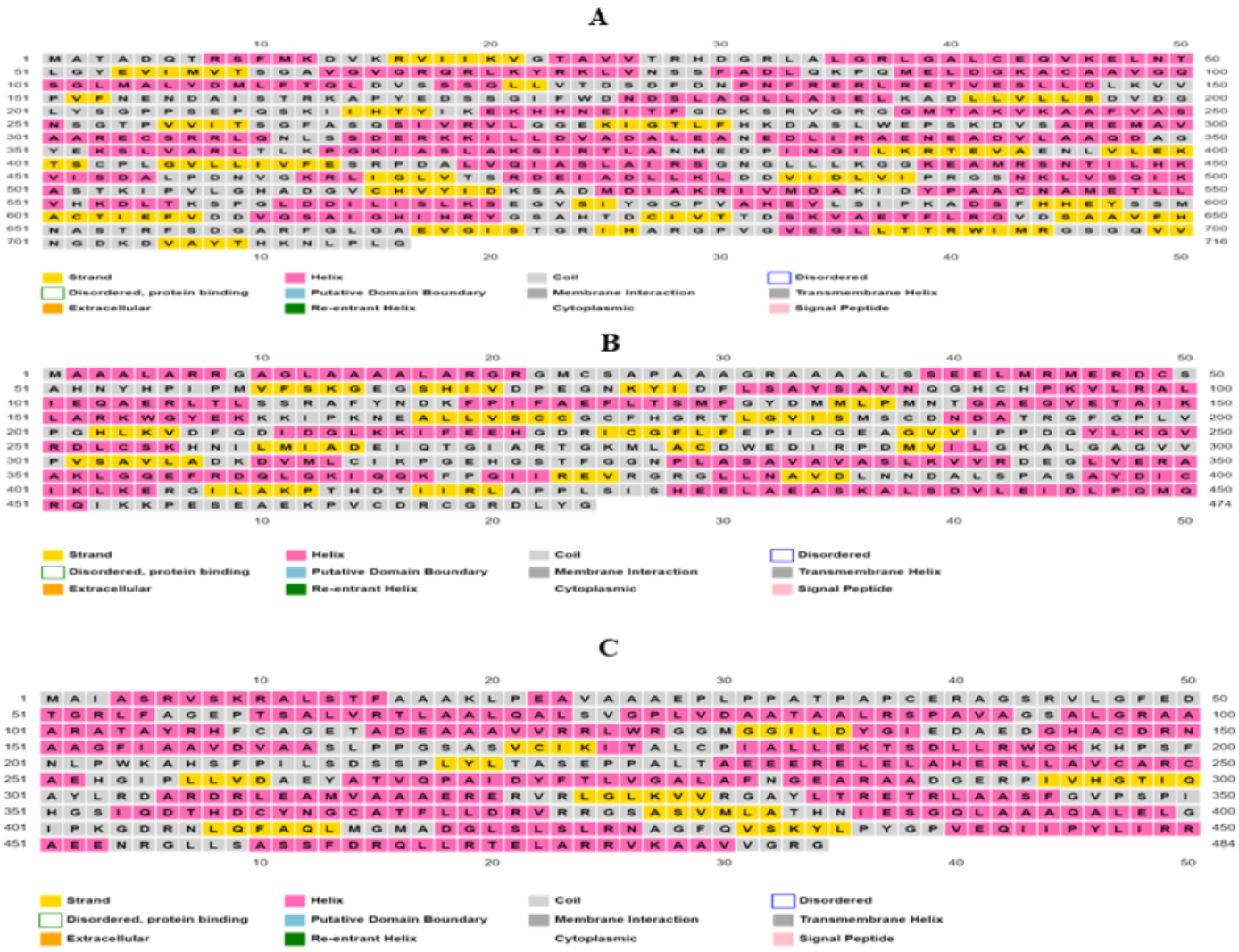

Figure 2

Secondary structure analysis: Psipred sequence Plot (A) *P5CS* (B) *OAT* (C) *ProDH*

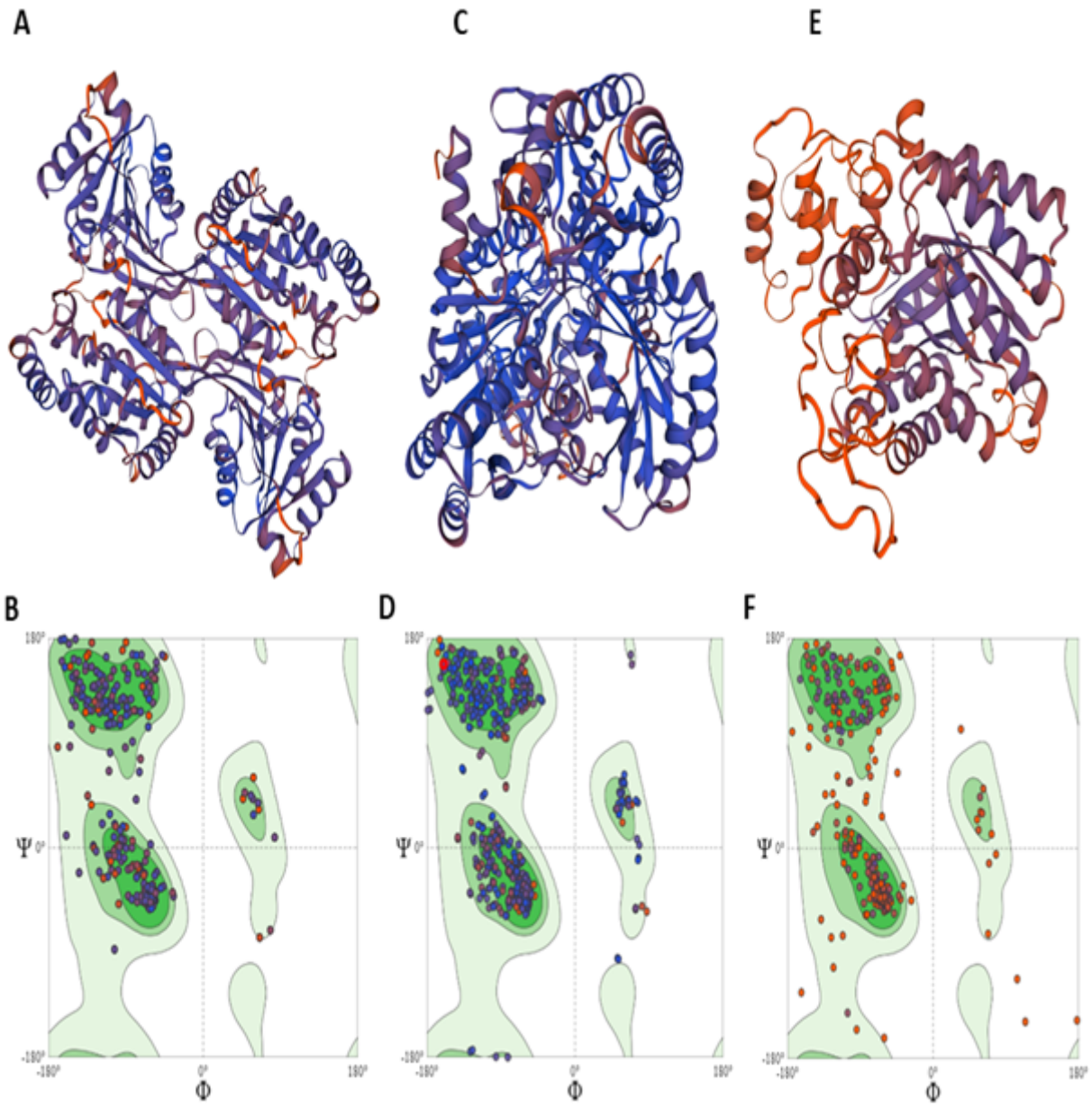

Figure 3

Modeled structures: (A) *P5CS* (C) *OAT* (E) *ProDH* (B) Ramachandran plot of *P5CS* (D) Ramachandran plot of *OAT* (F) Ramachandran plot of *ProDH.*

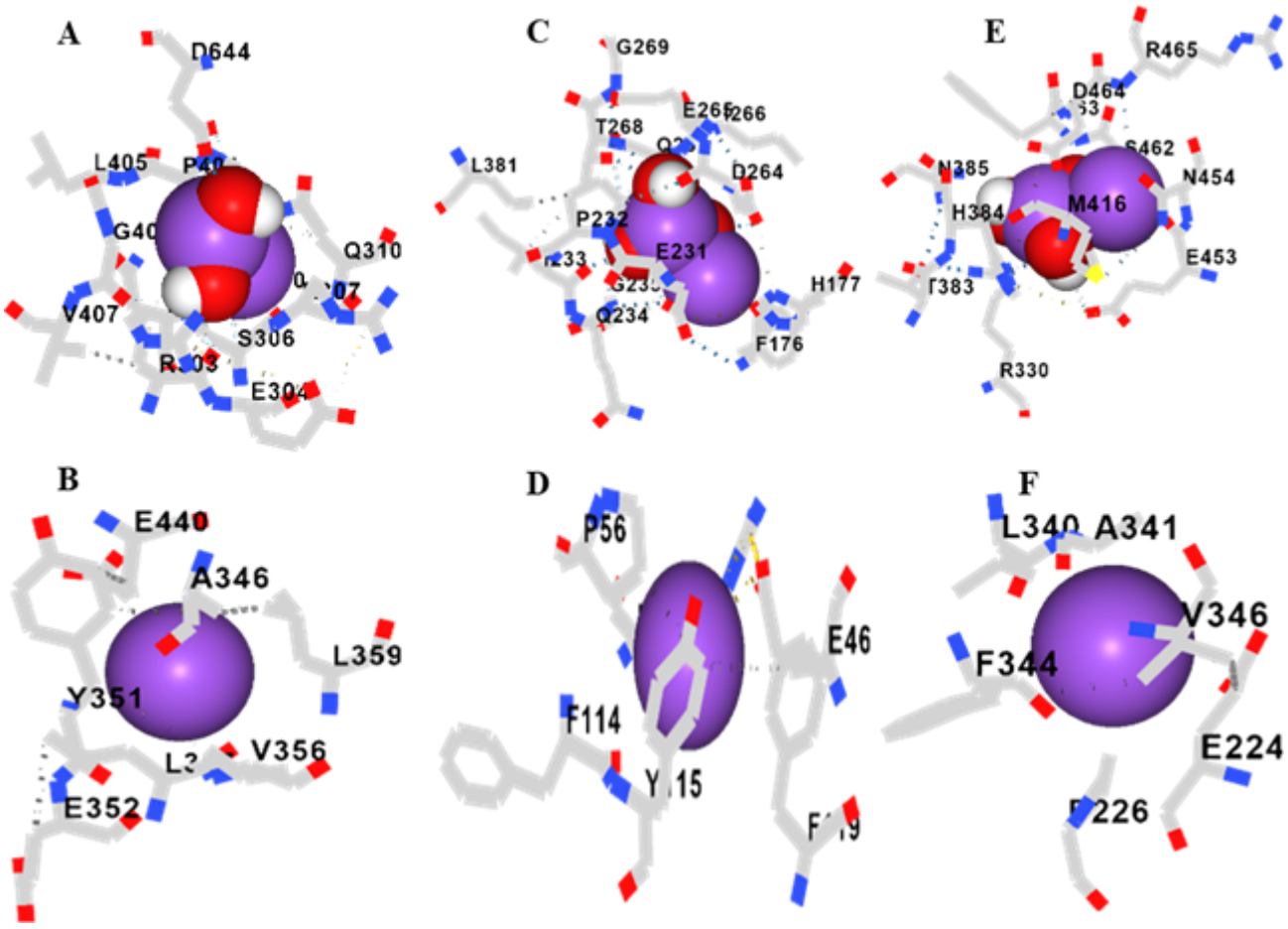

Figure 4

Receptor ligand interactions (A) *P5CS* with Se (B) *P5CS* with Nacl (C) *OAT* with Se (D) *OAT* with Nacl (E) *ProDH* with Se (F) *ProDH* with NaCl.

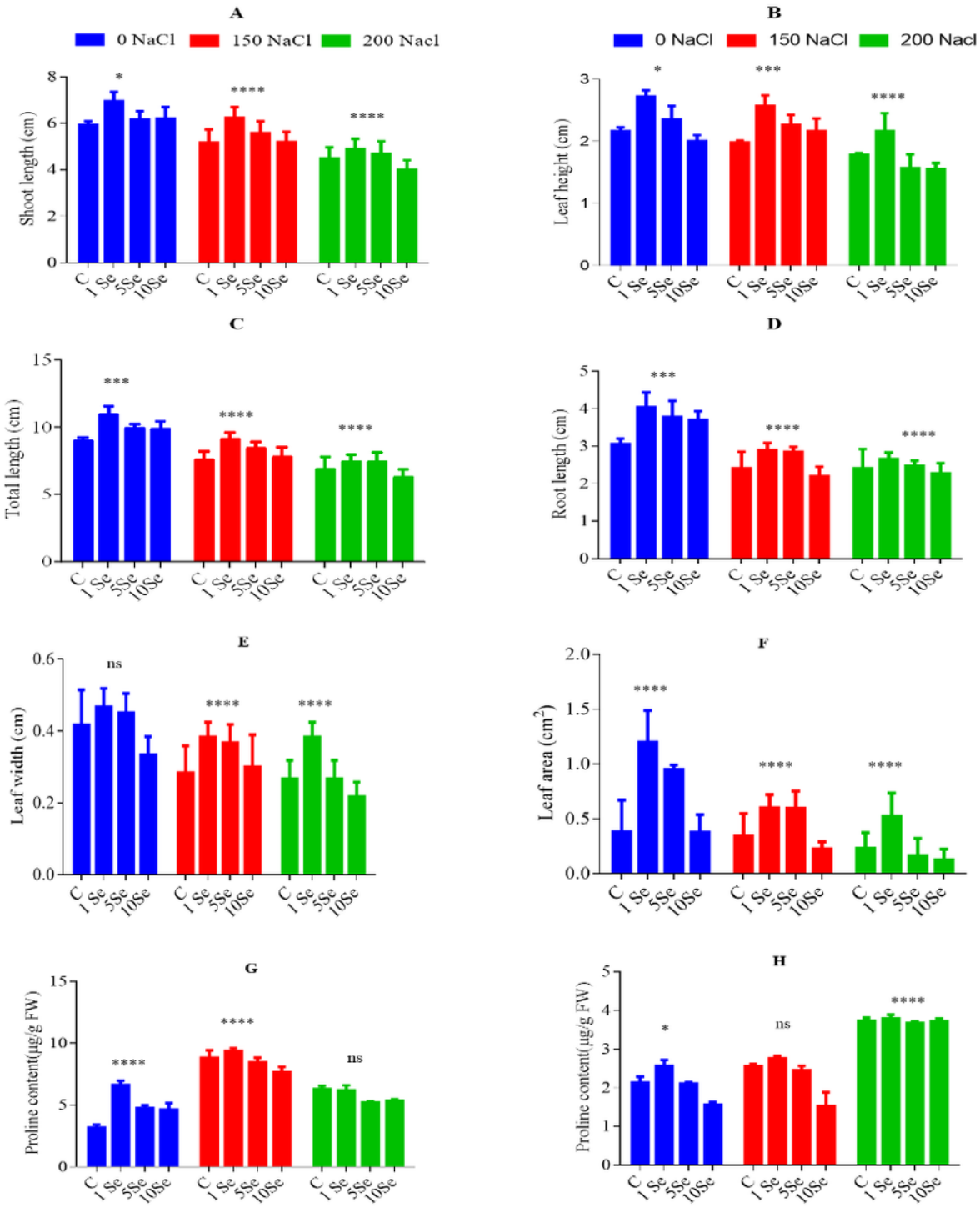

Figure 5

Effects of Nacl and Se on morphological parameters (A) Shoot length (B) Leaf height (C) Total length (D) Root length (E) Leaf width (F) Leaf area (G) Proline content in shoot (H) Proline content in root.

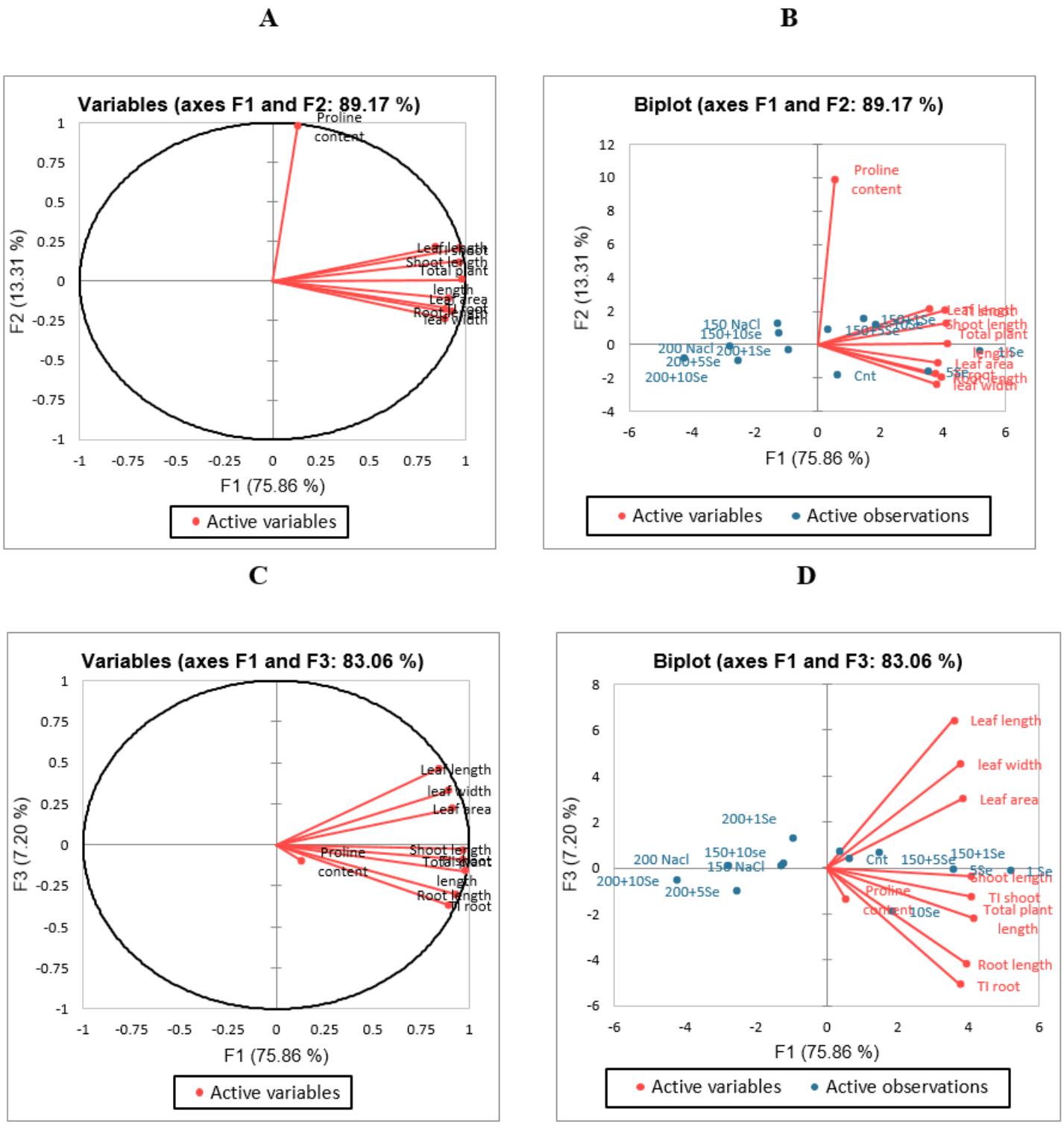

Figure 6

PCA between different variables and actives observations. (A) Component analysis between F1 &F2 (B) Bi plot analysis between F1 &F2 (C) Component analysis between F2 and F3 (D) Bi plot analysis between F2 and F3.

Supplementary Files

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