



RESEARCH ARTICLE

Evaluation of proteome complexes normalizing osmoregulation in salt stressed *Luffa acutangula* (L.) Roxb.

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ARTICLE HISTORY

Received: 16 May 2021
Accepted: 03 August 2021
Available online
Version 1: 05 September 2021
Version 2: 05 February 2022

KEYWORDS

Luffa acutangula
Salinity stress
LC-MS/MS
Antioxidants
Osmoregulators

ABSTRACT

Modern-day agriculture is facing the challenge of sustaining global food security. However, the rapid increase in salinity stress among arable areas poses a major threat to crop health and yield. Salinity stress is one of the most common and rapidly spreading stress that has a detrimental effect on the productivity of edible plant family i.e. *Cucurbitaceae*. The present study endeavors to evaluate the Osmoregulators (anti-oxidants and proteins), that supports the growth of two varieties of *Luffa acutangula* (L.) Roxb. under salt stress. The 2-3 weeks old saplings were exposed to salt stress (up to 200 mM NaCl) for one week. Post-treatment the osmoregulatory metabolites like Trehalose, Proline & enzymic anti-oxidants like peroxidase (POD), Superoxide dismutase (SOD) and proteins using LC-MS/MS were analyzed. In both the varieties, Trehalose increased with increasing salt concentration, while the level of Proline increased in Variety 1 and decreased in Variety 2. With increasing salt concentrations, the POD activity decreased in both varieties whereas that of SOD levels increased in Variety 2 and decreased in Variety 1. The protein identified by LC-MS/MS and functional annotation analysis employing Uniport database & BlastP algorithm, aided in the detection of differentially expressed proteins in response to salt stress. This was followed by metabolic interaction annotation enrichment analysis by FunRich 3.0 tool, enabling characterization of proteins to be involved in the Calvin cycle, amino acids biosynthesis, carbohydrate and energy metabolism, ROS defence, hormonal biosynthesis and signal transduction. The augmentation of the metabolic activities of the Calvin cycle, biosynthesis of amino acids, carotenoids and peroxisomes, glycolytic pathway and the tricarboxylic acid cycle will conceivably influence the photosynthetic capacity in *L. acutangula* varieties under salt stress. The upsurge of key enzymes involved in these above described biological processes possibly appears to play an important role in the enhancement of salt tolerance.

Introduction

Salinity; prominent abiotic stress, influences adversely plant growth, development and yield values. A large part of the Asian continent (21.5 million ha) is affected by salinity, of which 9.5 million ha are alkaline/ sodic (1). Vegetation in these areas not only demonstrate lower growth rates but also dwindled leaves and chlorophyll content under salinity stress (1). Manifestation of excess salt during the growth phase; leads to the deposit of Na⁺ and Cl⁻ in different plant organs (2). Several researchers have acknowledged that; ion toxicity, deficiency of water in elder leaves and the deficiency of carbohydrates in young leaves are some of the consequences of long-term salinity stress (2–4). The sustenance of the plant under the saline condition mostly confides in the competencies of the plant to progress in its tailor-made adaptive approach under stress conditions. *Cucurbitaceae* encompasses species consumed as food worldwide

that flourishes well in tropical, subtropical arid deserts and temperate zone. The most vital are viz; *Cucumis* (Cucumber – *Cucumis sativus*; Muskmelon – *Cucumis melo*), *Cucurbita* (Squash, pumpkin, Zucchini - *Cucurbita pepo* and other gourds), *Lagenaria* (bottle gourd / Calabash - *Lagenaria siceraria*), *Citrullus* (watermelon - *Citrullus lanatus*) and many others (7).

In arid and semi-arid regions, sodic soils are indicative of hot and dry weather combined with scant water resources (5). In India, domesticated agricultural patterns along with the rigorous application of fertilizer and manure contribute to intensifying soil salinity (6). With approx. 130 genera and 800 species (7), genetic diversity within the *Cucurbitaceae* family is tremendous, and hence means to thrust salinity stress may differ greatly among the species in the family.

Most of the Cucurbits are reasonably sensitive to salt stress, including the Cucumber (*Cucumis sativus*

L.), Musk melon (*Cucumis melo* L.) and Squash (*Cucurbita pepo* L.). The salinity helps in enhancing the quality of musk melon by boosting its dry matter, total sugars, total soluble solids and pulp firmness (8). The fruit quality of Zucchini has been demonstrated to improve through augmentation of physical (fruit firmness) and chemical properties (total soluble solids) (9). Similarly, a commonly grown cucurbitaceous vegetable, bitter melon (*Momordica charantia* L.) extensively diagnosed for its hypoglycaemic properties also shows greater salinity tolerance. Cucurbit displays higher susceptibility to NaCl in comparison to CaCl₂, indicating its high Na specific - salinity effects (10). Further, due to the high Na- salinity effect within the cells, it may permit incompetent partition of Na within the cells, and may primarily force the plant to expel out the Na from the leaf. However, watermelon (*Citrullus lunatus* L.), widely known for its moderate sensitivity to salinity shows a decline in its yield (13) due to salinity (0% at 2.5 mmhos cm⁻¹ to 100% at 10 mmhos cm⁻¹).

Among the Cucurbits, *Luffa acutangula* (L.) Roxb. (ridge gourd) is predominant in the subtropical region of Asia and India is considered as a centre of origin (11). The ethnic groups in India, particularly Maharashtra and some tribes of Madhya Pradesh have extensively used different parts of this plant for therapeutic purposes like treatment of jaundice, skin infections, haemorrhoids, diabetes, leprosy, conjunctivitis etc (12).

The Indian germplasm of cucurbits exhibits rare salt-tolerant lines. Meanwhile, the research in the arena of crop improvement under the stress condition on cucurbits is very low. In the present study, a vegetal species of *Cucurbitaceae*, *L. acutangula* (L.) Roxb. has been investigated for its potency to sustain under induced stress of NaCl. To establish the accurate salinity induced delimitations, the study attempted integrating data analysis using tools of proteome along with the routine antioxidant osmoregulatory characterization.

Materials and Methods

Plant and Reagents Used

Two varieties of plant *L. acutangula* (L.) Roxb. namely Mumbai Local (Variety 1) and Jaipuri Long (Variety 2) were used for the experiment. The seeds used were of "Ratanshi Agro-Hortitech" bought from Bombay Seeds Supply. CO., Navi Mumbai and Namdeo Umaji Seeds Pvt. Ltd., Mumbai. The seeds were imbibed in water for 48 hrs before sowing them in pots containing normal garden soil at room temperature for growth. The plants were maintained in a normal garden environment for 2-3 weeks and were watered once daily. No other media was used. After 2-3 weeks of growth, salinity stress of varying concentrations of NaCl was induced. All analytic grade chemicals and reagents used were from SRL Lab Pvt. Ltd., HiMedia Laboratories Pvt. Ltd., Molychem Pvt. Ltd., S D Fine Chem Ltd.

Biochemical Estimation of Osmoregulators Peroxidase (POD) Estimation

For each sample, one part of tissue was homogenized with five parts (W/V) of 0.1M Phosphate buffer (pH 6.5). The supernatant collected from centrifugation of the homogenate mixture (15 min at 500 rpm) was considered as an enzyme source. 3 ml of 0.05M Pyrogallol solution and 20 µl of supernatant was mixed to which 0.5 ml of 1% H₂O₂ was added and change in the absorbance was recorded for 3 min in every 30 sec interval at 430 nm (13).

Superoxide Dismutase (SOD) Estimation

For estimation of SOD, in a tube, 0.1 ml of supernatant, 1 ml of 125 mM Sodium carbonate, 0.4 ml of 25 µM NBT and 0.2 ml of 0.1 mM EDTA was added. To this assay mixture, 0.4 ml of 1 mM Hydroxylamine hydrochloride was added and absorbance was recorded at 560 nm for 90 min at every 1-min interval. Inhibition of 50% NBT reduction by a specific amount of enzyme in one min was measured as one unit of an enzyme (14).

Proline Estimation

For estimation of proline; 1 ml of supernatant was added to a mixture containing 2% of Ninhydrin solution and 1 ml of Glacial Acetic acid. This mixture was incubated in a boiling water bath for 1 hr. This was followed by stopping the reaction by keeping the tubes in an ice bath for 10 min. To this reaction mixture, 4 ml of Toluene is added and mixed vigorously so that the chromophores get mixed in the organic phase. The optical density of these toluene containing chromophores is measured at 520 nm. The amount of Proline is determined by using a spectrophotometer (UV 1601, UV Visible spectrophotometer, Shimadzu) (15).

Trehalose Estimation

Quantification of Trehalose was carried out using an Anthrone reaction. The reaction mixture (0.5 of Trehalose solution, 5 ml of 66% sulphuric acid containing 0.05% of Anthrone) was incubated for 15 min in a boiling water bath. To quantify the amount of trehalose in the respective treated samples, the absorbance was measured at 620 nm and compared with the standard curve of Trehalose (16).

Extraction of Proteins

For the extraction of proteins, 0.5 g tissue sample was homogenized in a pre-chilled mortar and pestle using liquid nitrogen (17). To this homogenate, 5 ml of extraction solution (0.5 M of Tris-Cl pH 6.8, 10% SDS and 20 mM of DTT) was added. The tubes were then centrifuged for 15 min at 10000 rpm at 4 °C. For proteome studies, the supernatant containing soluble proteins were used for further analysis.

Protein Identification by LC-MS/MS

All the protein samples obtained from the aforementioned techniques were separated using 1D Electrophoresis after which in-gel trypsinization was carried out. The trypsinized samples were then analyzed using Orbitrap Fusion Mass Analyzer (LC-MS/MS, IIT-Bombay, (BT/PR13114/INF/22/206/2015; 2018) to obtain the proteome mass spectrometry data

which was further analyzed using Proteome Discoverer 2.2 software (18) developed especially for Orbitrap Fusion Mass Analyzer.

In-silico Functional Annotation of Proteins

For functional annotation of these proteins, the proteins were searched for their homologs in the Uniprot KB database using the BlastP algorithm (19) keeping only reviewed the record as an output in the list. The outcomes obtained were manually filtered out for plants only. Homology threshold was kept for greater than 60% identity and 60% query coverage. In each case, the top 20% higher side results from the homologs were selected. Furthermore, the functional annotation using Funrich tool 3.0 with a background data of Uniprot was also evaluated (20, 21) (Fig. 1).

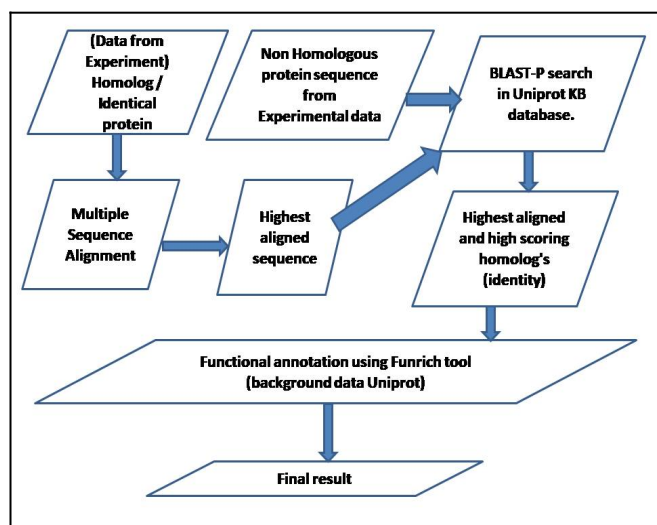


Fig. 1. Schematic representation of methodology adopted for functional annotation of proteins.

Results and Discussion

Osmoregulation fluxes in L. acutangula under salinity stress

The luffa varieties exposed to varying salt concentrations (up to 200 mM NaCl) were analyzed for various enzymic and non-enzymic osmoregulatory metabolites and proteins that may enhance the sustenance of plants under salt stress.

The proline content of Variety 1 increased from 14.19 $\mu\text{g/ml}$ at 0 mM of salt to 21.21 $\mu\text{g/ml}$ at 50 mM of salt. It decreased slightly at 100 mM of salt (17.8 $\mu\text{g/ml}$) and increased again to 23.84 $\mu\text{g/ml}$ at 200 mM of salt. The proline content of Variety 2 decreased from 10.45 $\mu\text{g/ml}$ at 0 mM of salt to 6.78 $\mu\text{g/ml}$ at 50 mM of salt, 6.51 $\mu\text{g/ml}$ at 100 mM of salt and 4.94 $\mu\text{g/ml}$ at 200 mM of salt (Fig. 2). Thus, it can be observed that the proline content of Variety 1 was more in salt-treated samples than in control samples, with the highest proline content in the sample treated with 200 mM salt, whereas the proline content of Variety 2 decreased with increasing salt concentration, with the highest proline content in the control sample. Together with proline, the Trehalose content of Variety 1 increased from 110.46 $\mu\text{g/ml}$ at 0 mM of salt to 219.04 $\mu\text{g/ml}$ at 50 mM of salt and 257.27 $\mu\text{g/ml}$ at 100 mM of salt, but it decreased to 168.75 $\mu\text{g/ml}$ at 200

mM. The Trehalose content of Variety 2 thus followed a similar trend as that of Variety 1, i.e it increased from 181.21 $\mu\text{g/ml}$ at 0 mM of salt to 204.78 $\mu\text{g/ml}$ at 50 mM of salt and 274.48 $\mu\text{g/ml}$ at 100 mM of salt, but it decreased to 228.69 $\mu\text{g/ml}$ at 200 mM (Fig. 3). Thus, in both varieties, an increase in accumulation of Trehalose with increasing salt concentration demonstrated that plant's defensive features increase as a response to salinity stress. The observations recorded in the present study were statistically evaluated using Anova (on MS excel) and were found to be under the level of significance ($p\text{-value} \leq 0.1$).

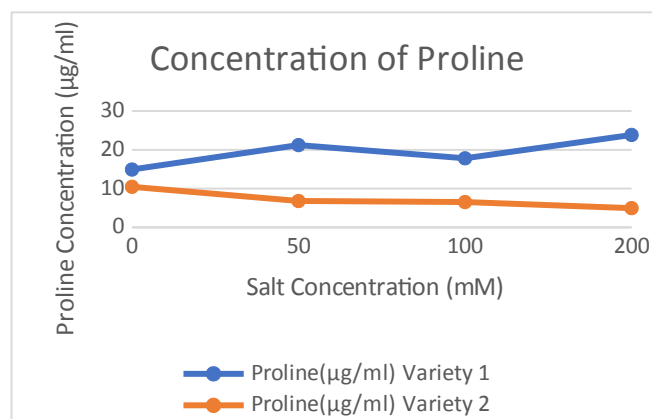


Fig. 2. Concentration of proline at different salt concentrations.

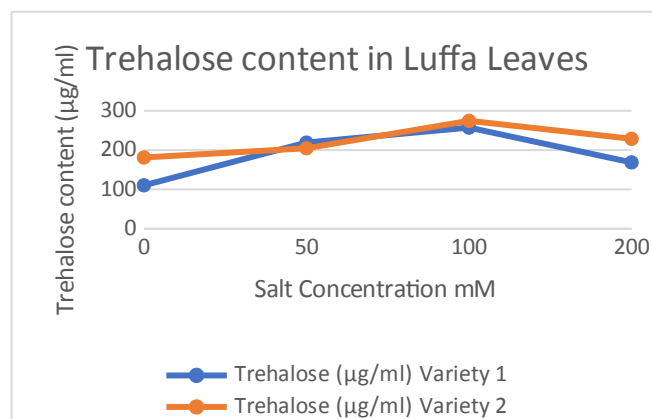


Fig. 3. Trehalose content in *Luffa* leaves at different salt concentrations.

Salt stress instigates cellular dehydration by diminution of turgor pressure among plant cells. Plants, therefore, commission osmoregulation as a mechanism to tolerate salt stress (22). Similarly, in the present study, the changes in fluxes of major osmoregulatory molecules in both varieties of *L. acutangula* indicated its preliminary shield pattern alongside increased Na^+ . The factors that significantly affect the osmoregulation fluxes in any abiotic stresses may be contributed either individually or collective effects of salt stress, plant age and the metabolite or chemical concentration responsible to promote a stress condition. As per Shavrukov (23), the concentration of the salt that stimulates the salt stress is directly proportional to the nature of damage caused due to the salt stress in the plant. The effect may range from a drop in osmotic pressure due to lower salt concentration to osmotic shock and an imbalance in proteome and metabolome under high salt concentration. Numerous studies exhibited a wide

strategy of utilizing juvenile plants with a concentration of NaCl from 100 to 200 mM and its efficiency in analysis and studying the diverse molecular pathways allied with the response of sugarcane cultivars to the salt stress (24, 25). Considering these methodologies as a scaffold, we have employed a wide range of NaCl concentrations ranging from 0 to 200 mM in the present study to unveil the protein complexes enhancing/deteriorating the growth of *L. acutangula* varieties.

The upsurge of Na⁺ and Cl⁻ in soil declines the water potential of the soil. To neutralise the lower water potentials, the plants maintain a constructive gradient for water flow from the soil into roots, by the virtue of the accumulation of osmolytes, such as proline, betaine, polyamines, sugars, organic acids, amino acids and trehalose (26). Previous studies have demonstrated that proline accumulates during drought stress, salinity, low temperature and other environmental stresses (27). Proline has also been reported to play a key role in maintaining membrane structures and scavenging ROS under stress. The significant association between proline and trehalose accumulation and osmotic stress tolerance in leaves has been reported earlier (14, 28), suggesting that the accumulation of these osmolytes not only play a role in osmoregulation but also contributes by scavenging ROS, as a source of energy for the repair process and as a signal molecule to modulate the cellular redox homeostasis. Studies have also reported that with increasing salt stress, the levels of proline is higher in the leaves which protects photosynthetic activity by maintaining chlorophyll level and cell turgor (29).

In this study, comparative analysis of control in both the varieties indicates a spike in Trehalose concentration at 100 mM salt stress. Previous studies in potato, *Arabidopsis*, maize, rice tomato have also indicated accumulation of non-reducing disaccharides like trehalose when exposed to salinity, drought and heavy metal stress (30).

Salinity also induces the production of ROS in plants. The plants often show a variation in the expression of enzymic oxidants like POD, SOD which negate the detrimental effects of ROS by scavenging them (31). In this study, POD activity for Variety 1 was recorded to be reduced drastically from 14.19 units/ml at 0 mM, 6.44 units/ml at 50 mM and 0.57 units/ml at 100 mM of salt concentration; while in Variety 2, the POD activity dropped drastically from 13.06 units/ml at 0 mM, 9.51 units/ml at 50 mM and 0.45 units/ml at 100 mM of salt concentration (Fig. 4). Additionally, the SOD activity in Variety 1 showed skewed results. It decreased from 0.197 units/mg at 0 mM to 0.087 units/mg at 50 mM of salt; further increased to 0.123 units/mg at 100 mM of salt and decreased again to 0.064 units/mg at 200 mM of salt. Whereas, SOD activity in Variety 2, increases from 0.093 units/mg at 0 mM to 0.192 units/mg at 50 mM of salt but it decreases back to 0.118 units/mg at 100 mM of salt and increases to 0.204 units/mg at 200 mM (Fig. 5). Salinity stress impacts gas exchange with soil negatively, resulting in less amount of CO₂ for photosynthesis which is followed by a drastic decrease in electron cycle transportation which further leads to the formation of ROS in plants. The majority of the plants

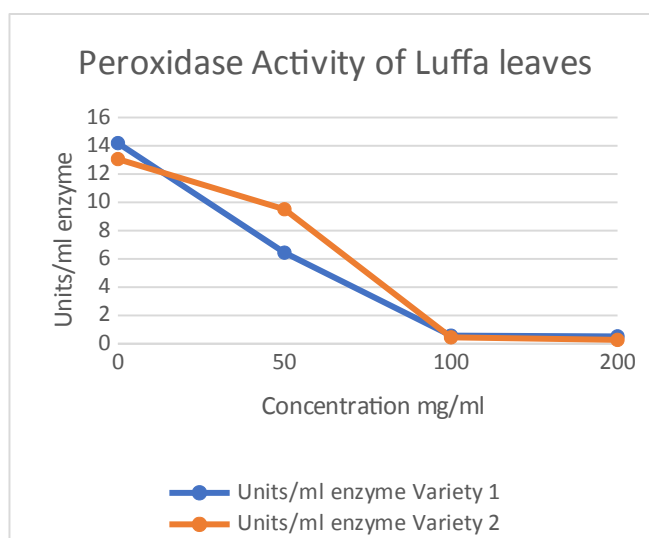


Fig. 4. Peroxidase activity at different salt concentration.

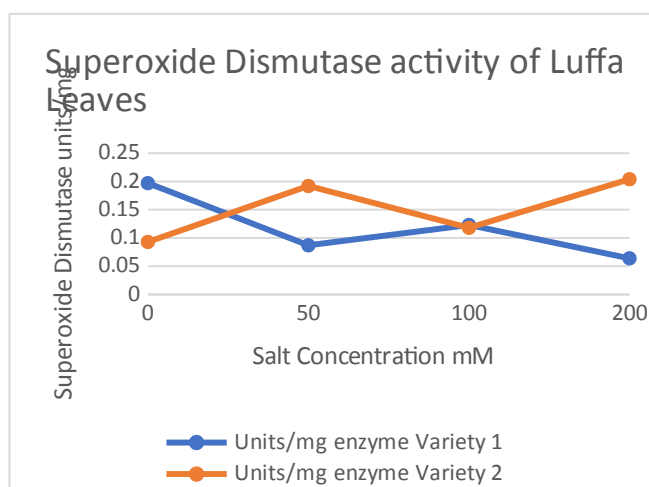


Fig. 5. Activity of Superoxide dismutase at different salt concentrations.

scavenge the ROS generated by having various mechanisms and enzymes like SOD, POD, APX, etc. Of these, SOD plays a valuable role by scavenging O₂-radicals, resulting in the formation of H₂O₂ and O₂. Earlier studies reported a decrease in SOD activity from 0 mM to 250 mM of salt concentration in leaves of *Aeluropus littoralis* (32) and *Broussonetia papyrifera* from 0 mM to 150 mM of salt (33). The observations recorded were in concordance with our parallel studies in seedlings of *L. acutangula* (14).

LC-MS/MS and Functional annotation of Identified proteins

The protein conformational modifications are known to respond for regulation and recovery of homeostasis among plants, under environmental cues including salinity stress. But the information in relation to macromolecular structural modifications is still inadequate and the molecular mechanisms underlying the contributory roles will take a progressive time in dissecting the event (34). To determine the proteomic fluxes in response to salinity stress, proteins of Variety 1, at 0 mM (Control) and 100 mM (treated) were resolved on 15% PAGE. Quantification of the bands was carried out using Biorad's Image Lab software (Fig. 6). The bands which showed visible variation in

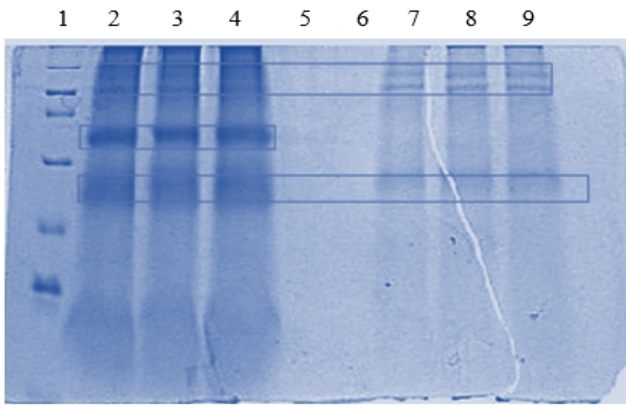


Fig. 6. Bands excised from control and treated samples (L-R: 1: High Range Protein molecular marker, 2-4: *Luffa* variety 1 control, 7-9: *Luffa* variety 1; 100 mM salt).

their intensities and bands which are absent in either of the sample but present in other are excised and subjected to MS-MS analysis. The raw file generated from MS analysis was compared against a Database compiled from amino acids sequences of genus *L. acutangula* obtained from Uniprot that comprised of 20 proteins; which were identified using Proteome Discoverer 2.2 software developed by Thermo Scientific Pvt. Ltd. (Fig. 7). The structural plasticity of proteins against salinity stress among assembled

protein sequences was examined by aligning them with their homologs in the UniprotKB database, along with the BlastP algorithm and the metabolic interaction annotation enrichment analysis, by FunRich 3.0 tool. All the proteins mapped to different *L. acutangula* species (Table 1) and their potential role in the adaptability of *L. acutangula* to salinity stress was thus examined *in-silico*. It was observed that salinity had induced changes in the integrity and functionality of chloroplasts it had eventually obstructed cell function as a whole. Furthermore, due to the salinity, the alteration in the abundance of the functional group the proteins are noted that are essentially involved in the light-harvesting complexes, photosynthetic Calvin-Benson Cycle and electron transfer pathway.

Based on our observation, we searched for the proteins/enzymes that catalyse the synthesis of ATP, NADPH, sucrose, starch etc., which in turn enhance the plant biomass, yield, and also exhibit resistance to stresses. The modulation in localization of such potentially vital proteins under salinity stress are discussed below:

Catalase

Comparing of the Catalase sequence (Swiss-prot-id: A0A159BPP6 (*Luffa aegyptiaca*) and A0A0U2DAT2 (*L. aegyptiaca*)) exhibited a 27 % - 97% and 35% - 95%

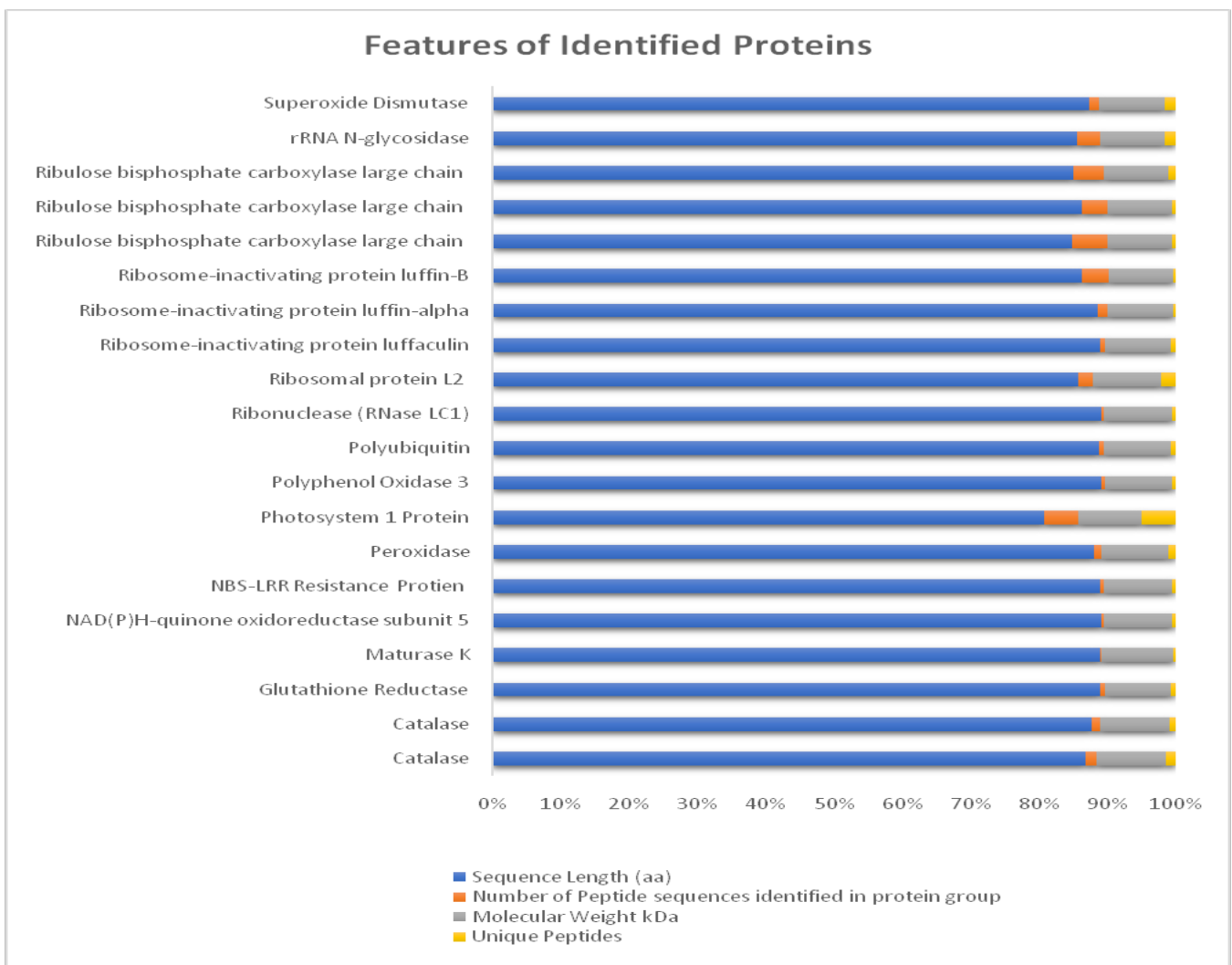


Fig. 7. The proteins Identified by LC-MS/MS analysis.

Table 1. The proteins identified from LC-MS/MS analysis in *Luffa acutangula* using *in-silico* tools

S. No.	Accession ID	Protein Name	Gene name	Uniprot_name	Matched Speices
1	A0A159BPP6	Catalase	CAT2	A0A159BPP6_LUFAE	<i>Luffa aegyptiaca</i>
2	A0A0U2DAT2	Catalase	NA	A0A0U2DAT2_LUFAE	<i>Luffa aegyptiaca</i>
3	A0A1L5JHV6	Glutathione Reductase	NA	A0A1L5JHV6_LUFAE	<i>Luffa aegyptiaca</i>
4	A5X4W3	Maturase K	matK	A5X4W3_LUFAC	<i>Luffa acutangula</i>
5	W8W166	NAD(P)H-quinone oxidoreductase subunit 5	ndhF	W8W166_LUFAC	<i>Luffa acutangula</i>
6	H9CZL5	NBS-LRR Resistance Protien	NA	H9CZL5_LUFAE	<i>Luffa aegyptiaca</i>
7	A0A0U2D7M1	Peroxidase	NA	A0A0U2D7M1_LUFAE	<i>Luffa aegyptiaca</i>
8	Q6TK99	Photosystem 1 Protein	psaC	Q6TK99_9ROSI	<i>Luffa echinata</i>
9	A0A01L5JHU3	Polyphenol Oxidase 3	NO entry	NO entry	<i>Luffa aegyptiaca</i>
10	A0A161CD95	Polyubiquitin	NA	A0A161CD95_LUFAE	<i>Luffa aegyptiaca</i>
11	Q40115	Ribonuclease (RNase LC1)	NA	Q40115_LUFAE	<i>Luffa aegyptiaca</i>
12	Q6TK80	Ribosomal protein L2	rpl2	Q6TK80_9ROSI	<i>Luffa echinata</i>
13	P84530	Ribosome-inactivating protein luffaculin	NA	RIP_LUFAC	<i>Luffa acutangula</i>
14	Q00465	Ribosome-inactivating protein luffin-alpha	NA	RIPA_LUFAE	<i>Luffa aegyptiaca</i>
15	P22851	Ribosome-inactivating protein luffin-B	NA	RIPB_LUFAE	<i>Luffa aegyptiaca</i>
16	R4I6V0	Ribulose bisphosphate carboxylase large chain	rbcl	R4I6V0_LUFAC	<i>Luffa acutangula</i>
17	A0A0U4DPP9	Ribulose bisphosphate carboxylase large chain	rbcl	A0A0U4DPP9_LUFAC	<i>Luffa acutangula</i>
18	Q32537	Ribulose bisphosphate carboxylase large chain	rbcl	Q32537_9ROSI	<i>Luffa quinquefida</i>
19	B0EVM6	rRNA N-glycosidase	RIP1	B0EVM6_LUFAC	<i>Luffa acutangula</i>
20	A0A1L5JHV8	Superoxide Dismutase	NA	A0A1L5JHV8_LUFAE	<i>Luffa aegyptiaca</i>

identity to the protein sequence of catalase from species/genera/plants of *C. pepo*, *Arabidopsis thaliana*, *Nicotiana glauca*, *Nicotiana glauca*, *Glycine max*, *Gossypium hirsutum*, *Soldanella alpina*, *Vigna radiata* var. *radiata*, *Avicennia marina*, *Ipomoea batatas*, *Helianthus annuus*, *Oryza sativa* subsp. *indica*, *Ricinus communis*, *Oryza sativa* subsp. *japonica*, *Zea mays*, *Pisum sativum*, *Solanum melongena*, *Hordeum vulgare*, *Triticum aestivum*. The gene enrichment analysis revealed a higher occurrence of catalase in a cellular component such as plasma membrane, peroxisome, glyoxysome etc. Catalase is highly involved in molecular functions such as metal ion binding, heme binding, cobalt ion binding, mRNA binding and catalase activity. It also affects the biological process such as nitric oxide homeostasis, response to abscisic acid, cellular response to sulfate starvation, response to xenobiotic stimulus, circadian rhythm, response to light stimulus, response to salt, response to hydrogen peroxide, response to oxidative stress, hydrogen peroxisome catabolic process (Fig. 8). Only the catalase domain is identified from the submitted dataset.

Glutathione Reductase

Study of Glutathione reductase (Swiss-prot id: A0A1L5JHV6 (*Luffa aegyptiaca*)) exhibited an identity from 27% - 79% to the protein sequence of cytosolic, chloroplastic, mitochondrial Glutathione reductase from *Pisum sativum*, *Arabidopsis thaliana*, *Brassica rapa* subsp. *pekinensis*, *Spinacia oleracea*, *Oryza sativa* subsp. *japonica*, *Nicotiana glauca*, *Glycine max*. The gene enrichment analysis revealed a higher occurrence of Glutathione reductase in cellular components such as cytoplasm, peroxisome and chloroplast. The enzyme is also involved in molecular function with glutathione-disulfide reductase activity, flavin adenine dinucleotide binding, NADP binding, electron transfer activity and thioredoxin-disulfide reductase activity. Further, its involvement is also in the biological

process such as glutathione metabolic process and cell redox homeostasis (Fig. 9).

Maturase K

Homologous to Maturase K (Swiss-prot id: A5X4W3 (*L. acutangula*)) were protein sequences of Maturase K from *Cucumis sativus*, *Hamamelis virginiana*, *Hamamelis japonica*, *Vitis vinifera*, *Mentzelia lindleyi*, *Hydrangea macrophylla*, *Crataegus monogyna*, *Gleditsia triacanthos*, *Liquidambar orientalis*, *Vauquelinia californica*, *Parkinsonia aculeata*, *Liquidambar formosana*, *Gymnocladus chinensis*, *Gymnocladus dioicus*, *Mentzelia laevicaulis*, *Betula papyrifera* with 67% - 93% identity. The outcome of gene enrichment analysis revealed the occurrence of Maturase K only in chloroplast, possess RNA binding as only molecular function and tRNA processing, RNA splicing and mRNA processing only 3 biological processes (Fig. 10).

NAD(P)H-quinone oxidoreductase subunit 5

Homologous to NAD(P)H-quinone oxidoreductase subunit 5 (Swissprot id: W8W166 (*L. acutangula*)) ranged sequence identity from 35% - 91.2%. The identical sequence was retrieved from the following plants viz. *Cucumis sativus*, *Manihot esculenta*, *Eucalyptus globulus* subsp. *globulus*, *Populus alba*, *Populus trichocarpa*, *Carpenteria californica*, *Carica papaya*, *Vitis vinifera*, *Oenothera parviflora*, *O. glazioviana*, *O. biennis*, *O. argillicola*, *Cichorium intybus*, *Lactuca sativa*, *Atractylodes lancea*, *Morus indica*, *Athrosisma gracile*, *Adenocaulon himalaicum*, *Symphyotrichum cordifolium*, *Anisothrix integra*, *Guizotia abyssinica*, *Flaveria ramosissima*, *Mutisia acuminata*, *Nandina domestica*, *Carthamus tinctorius*, *Barbarea verna*, *Vicia faba* (*Broad bean*), (*Faba vulgaris*) etc. The gene enrichment analysis revealed the occurrence of NAD(P)H-quinone oxidoreductase subunit 5 in the chloroplast thylakoid membrane and

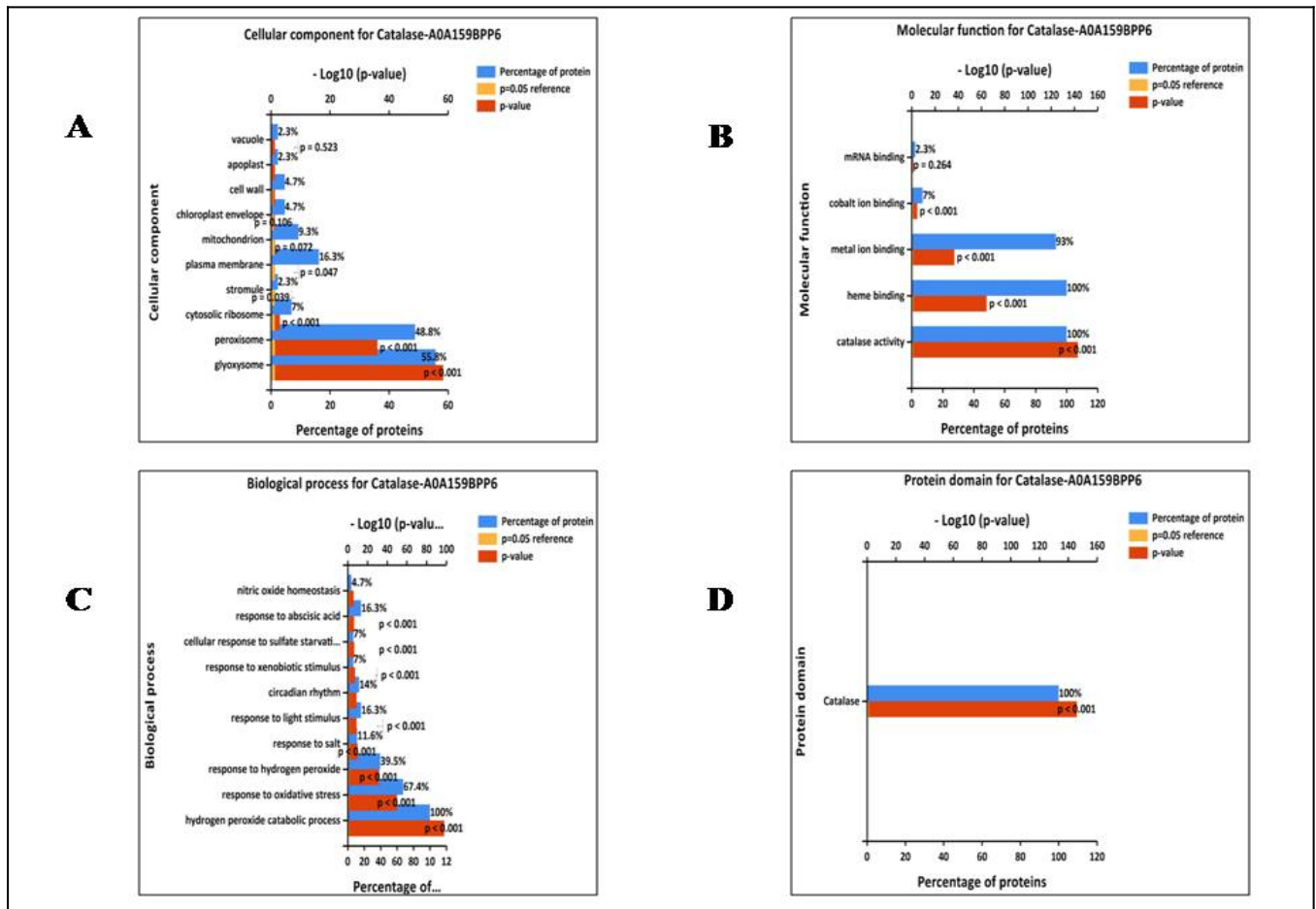


Fig. 8. Enrichment analysis for catalase : A: Cellular component, B: Molecular function, C: Biological process and D: Protein domain.

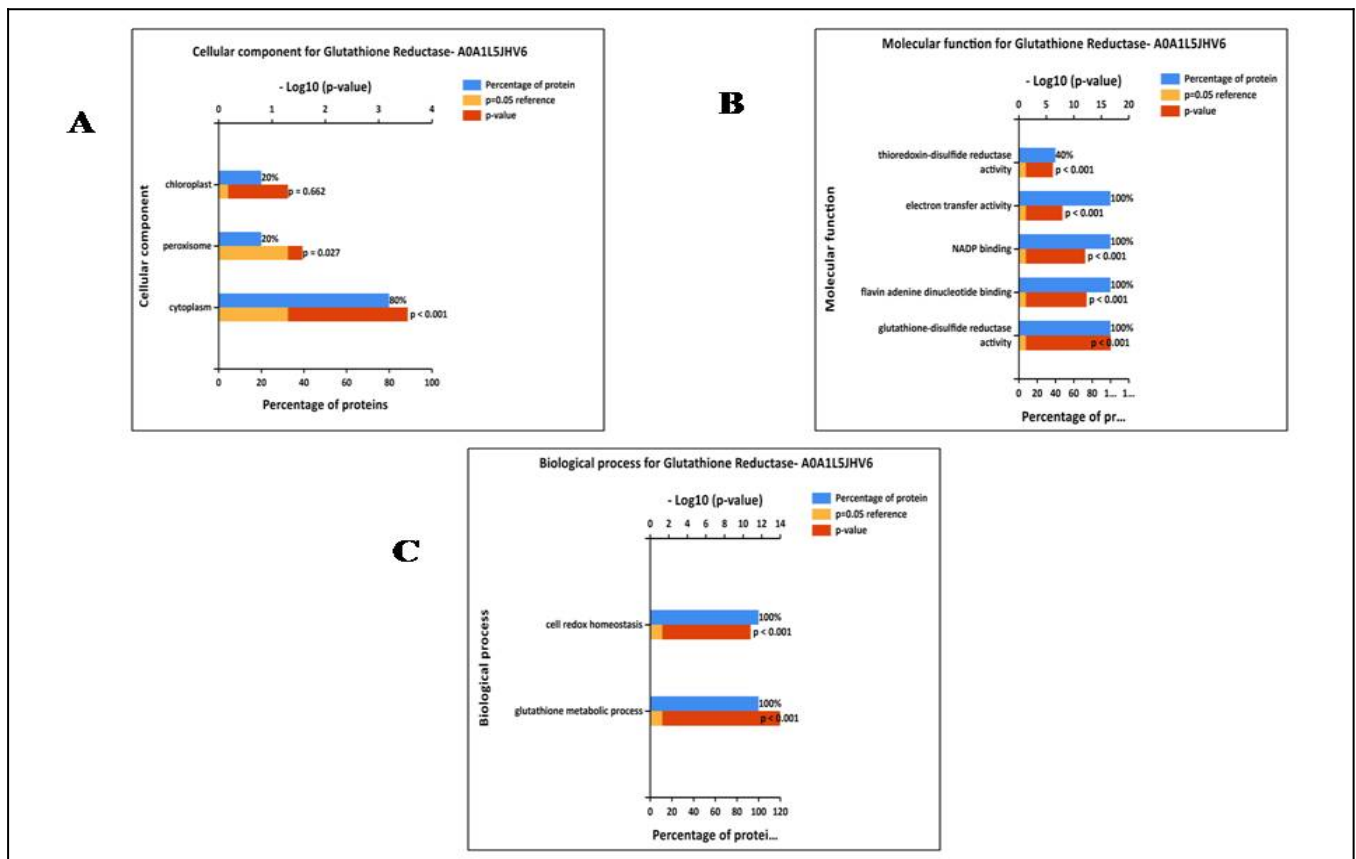


Fig. 9. Enrichment analysis for Glutathione reductase : A: Cellular component, B: Molecular function and C: Biological process.

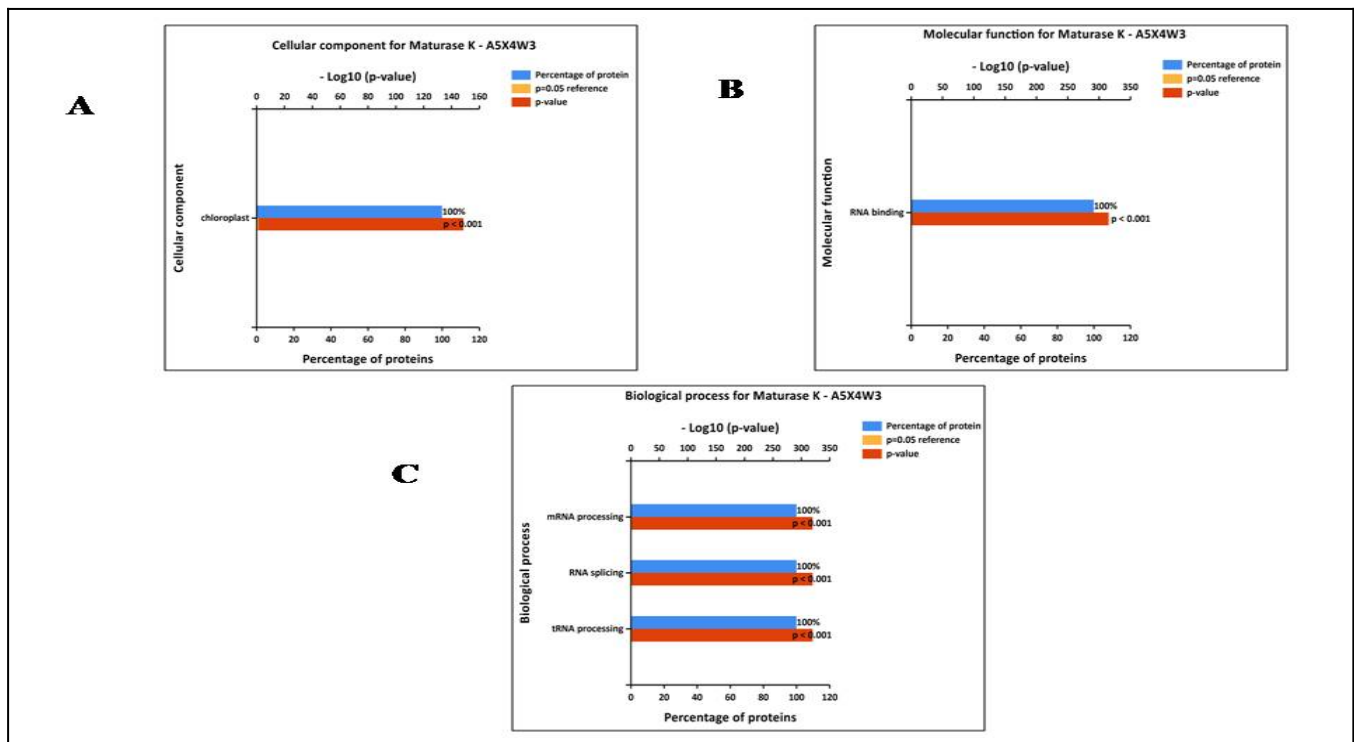


Fig. 10. Enrichment analysis for Maturase K : Fig A: Cellular component, B: Molecular function and C: Biological process.

integral component of membrane, the molecular functions such as NADH dehydrogenase (ubiquinone) activity and quinone binding and possess only ATP synthesis coupled electron transport as the biological process (Fig. 11).

Peroxidase

Protein sequence of Peroxidase from *Luffa aegyptiaca* (Swissprot id: A0A0U2D7M1) showed a sequence identity from 21% to 63.7%, within the following plant protein sequences: *Cucumis sativus*, *Ipomoea batatas*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *Armoracia*

rusticana, *Arachis hypogaea*, *Oryza sativa* subsp. *japonica*, *Vitis vinifera*, *Nicotiana glauca*, *Zea mays*, *Hordeum vulgare*, *Brassica rapa* subsp. *rapa*, *Zinnia violacea*, *Sorghum bicolor* (*Sorghum*), (*Sorghum vulgare*), *Solanum lycopersicum*, *Lupinus polyphyllus*, *Hordeum vulgare*, *Triticum aestivum*, *Pisum sativum*, *Cycas revoluta* etc. The enrichment analysis depicted Peroxidase in the extracellular region, vacuolar membrane and Golgi apparatus of the cellular component, exhibited with peroxidase activity, heme binding and metal ion binding molecular functions followed by biological processes such as defence

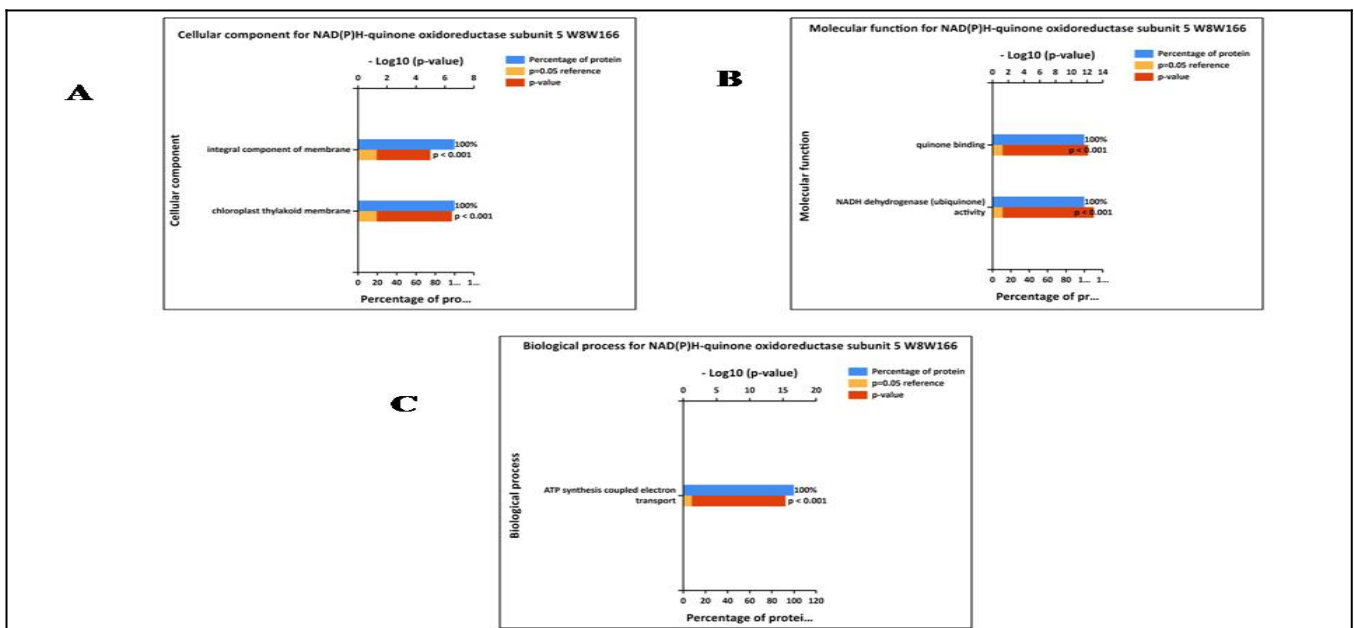


Fig. 11. Enrichment analysis for NAD(P)H-quinone oxidoreductase subunit 5: Fig A: Cellular component, B: Molecular function and C: Biological process.

response to pest, hydrogen peroxide and other oxidative stress, or even flower development (Fig. 12).

Polyubiquitin

Protein sequence of Polyubiquitin from *Luffa aegyptiaca* (Swissprot id: A0A161CD95) has shown sequence identity from 31.8% - 99.3% with the following plants: *Arabidopsis thaliana*, *Nicotiana sylvestris*, *Petroselinum crispum*, *Oryza sativa* subsp.

Biological processes reported are RNA catabolic process, anthocyanin-containing compound, biosynthetic process, cellular response to phosphate starvation and response to wounding (Fig. 14)

Ribosomal protein L2

Protein sequence of Ribosomal protein L2 from *Luffa echinata* (Swissprot: Q6TK80) retrieved identical sequence from plants such as *Populus trichocarpa*,

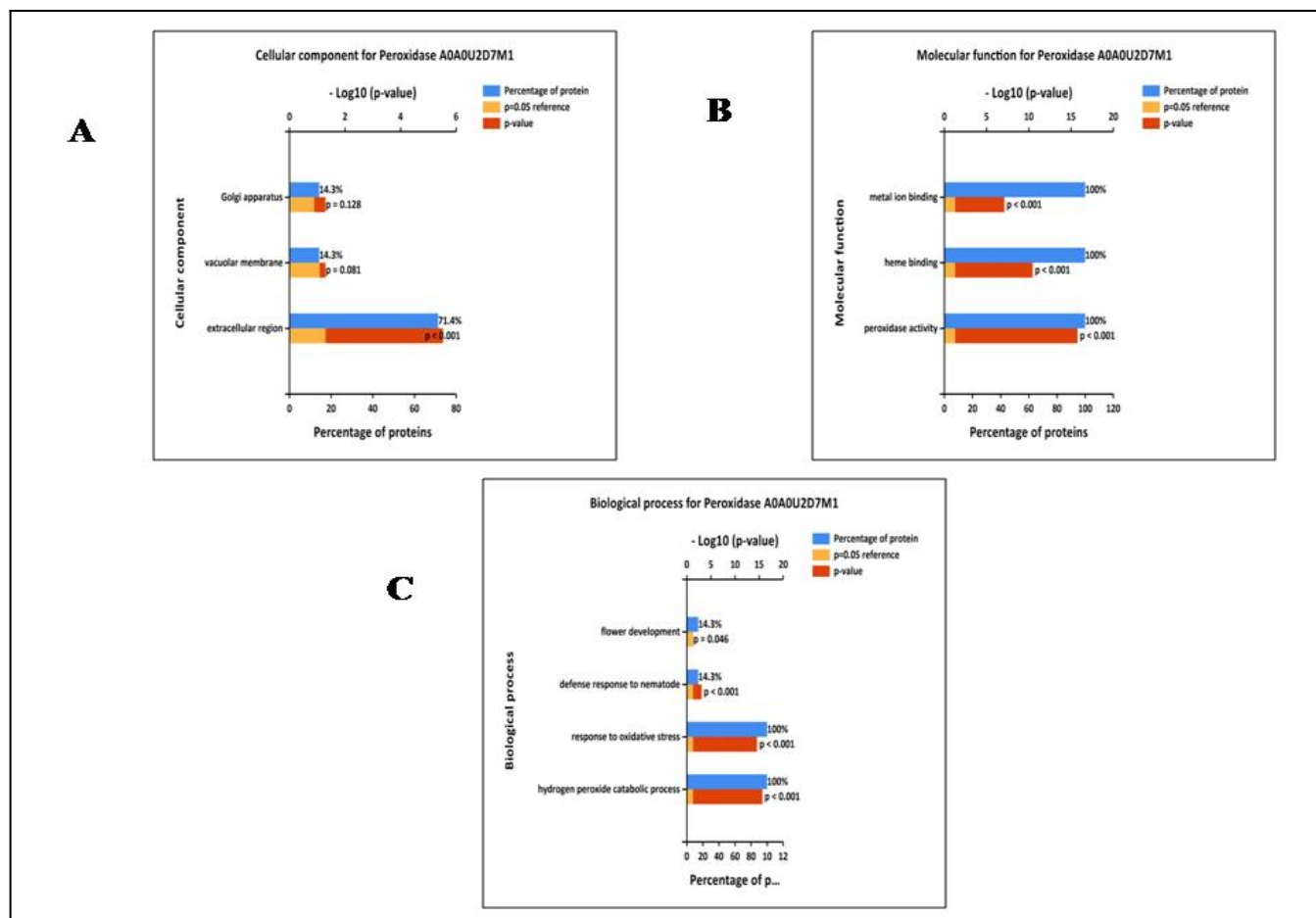


Fig. 12. Enrichment analysis for Peroxidase : Fig A: Cellular component, B: Molecular function and C: Biological process.

japonica, *Zea mays*, *Daucus carota* etc. The gene enrichment analysis exhibited Polyubiquitin in the nucleus and cytoplasm within the cellular component, a molecular function such as ubiquitin-protein ligase binding and protein tag function. Reported biological processes are a response to salicylic acid and protein (modification, ubiquitination, ubiquitin-dependent and modification-dependent) catabolic processes. Only one UBQ protein domain was identified (Fig. 13).

Ribonuclease (RNase LC1)

The protein sequence of Ribonuclease (RNase LC1) from *Luffa aegyptiaca* (Swissprot: Q40115) has exhibited a sequence identity from 30.0% - % 56.0% from plants such as *Momordica charantia*, *Arabidopsis thaliana*, *Solanum lycopersicum*, *Nicotiana glauca* etc. The enrichment analysis Ribonuclease (RNase LC1) shows its presence in the cell wall, extracellular region, extracellular space and plasma membrane of the cellular components, molecular function with ribonuclease T2 activity, RNA binding, endoribonuclease activity and ribonuclease activity.

Gossypium hirsutum, *Cucumis sativus*, *Carica papaya*, *Populus alba*, *Manihot esculenta*, *O. parviflora*, *O. glazioviana*, *O. elata* subsp. *hookeri*, *Vitis vinifera*, *Nicotiana tabacum*, *Solanum tuberosum*, *S. lycopersicum*, *S. bulbocastanum*, *Panax ginseng*, *Daucus carota*, *Atropa belladonna*, *Coffea arabica* etc. with an identity from 90% - 100%. The outcome of Ribosomal protein L12 enrichment analysis reported chloroplast, mitochondrial large ribosomal subunit and large ribosomal subunit for cellular component, molecular function with transferase activity, structural constituent of ribosome and rRNA binding. It is reported in biological processes such as translation and mitochondrial translation with protein domain Ribosomal_L2 and Ribosomal_L2_C (Fig. 15).

Ribosome-inactivating protein luffaculin

Protein sequence of Ribosome-inactivating protein luffaculin from *L. acutangula* (Swissprot id: P84530), has an identity from 24% - 94.6% from plants *Luffa aegyptiaca*, *Momordica charantia*,

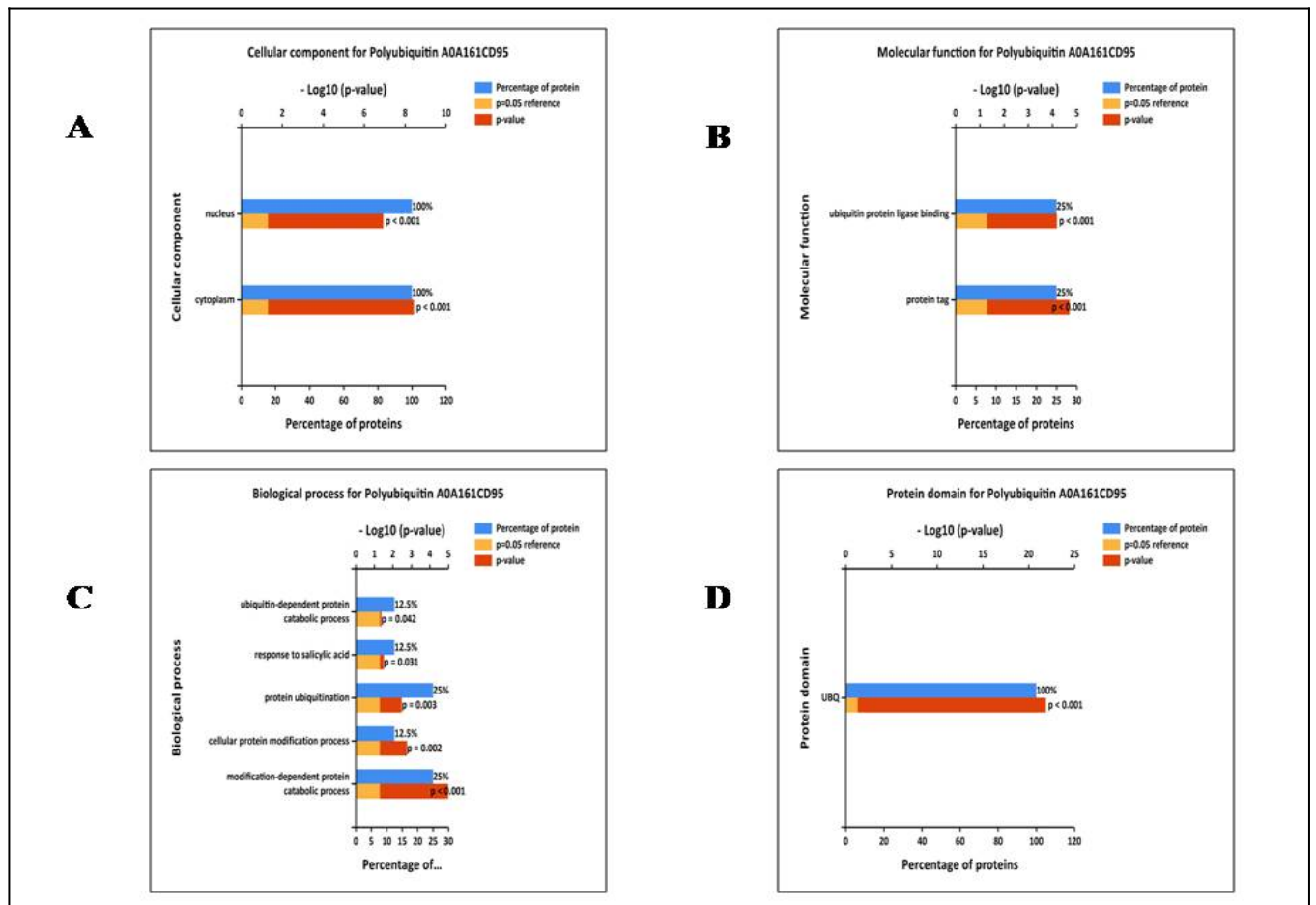


Fig. 13. Enrichment analysis for Polyubiquitin: Fig A: Cellular component, B: Molecular function, C: Biological process and D: Protein domain.

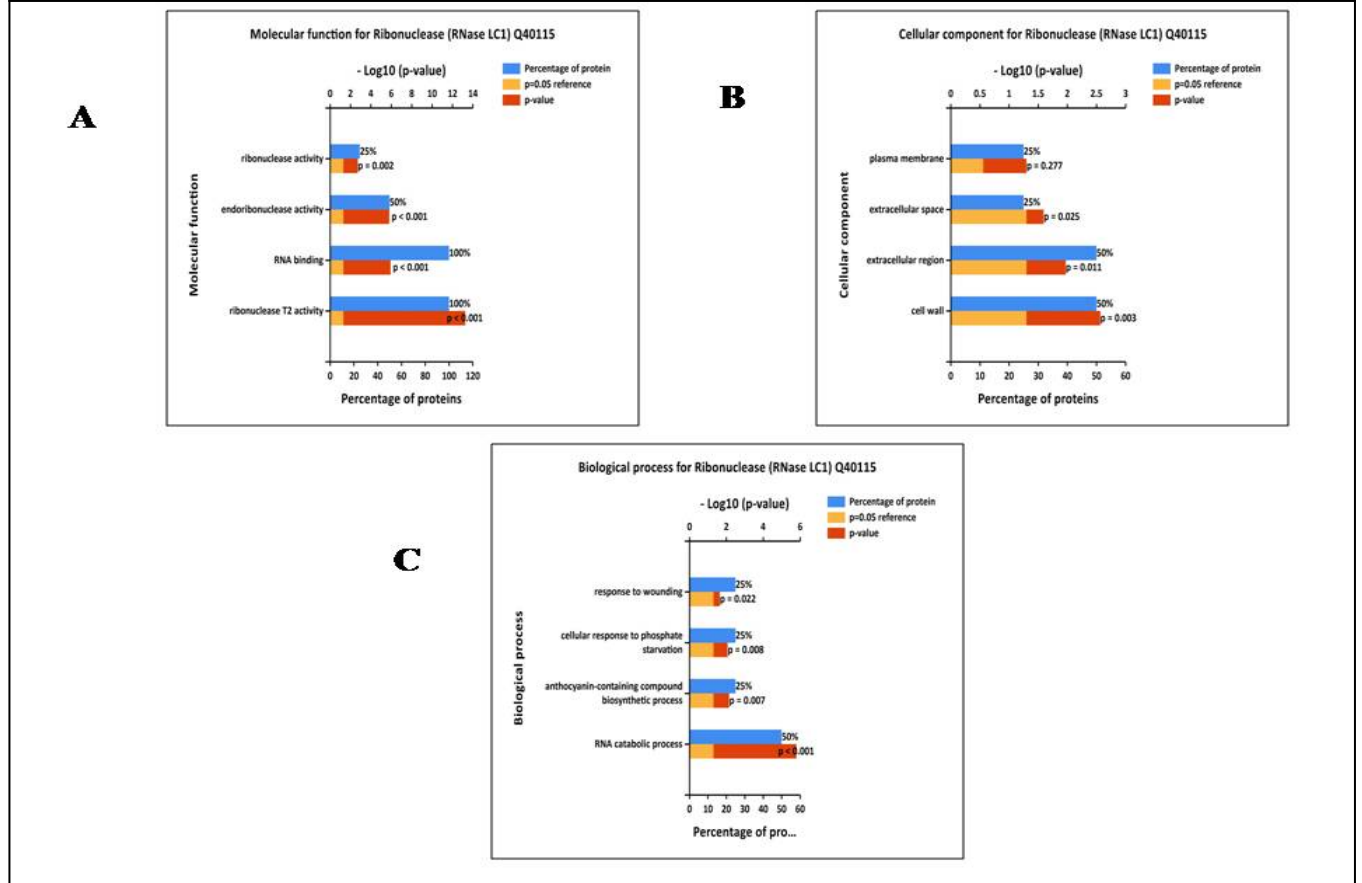


Fig. 14. Enrichment analysis for Ribonuclease (RNase LC1): Fig A: Cellular component, B: Molecular function and C: Biological process.

Cucumis ficifolius, *Bryonia dioica*, *Trichosanthes kirilowii*, *Cucurbita moschata*, *Momordica*

carboxylase activity, magnesium ion binding and nucleotide binding, while, the biological process such

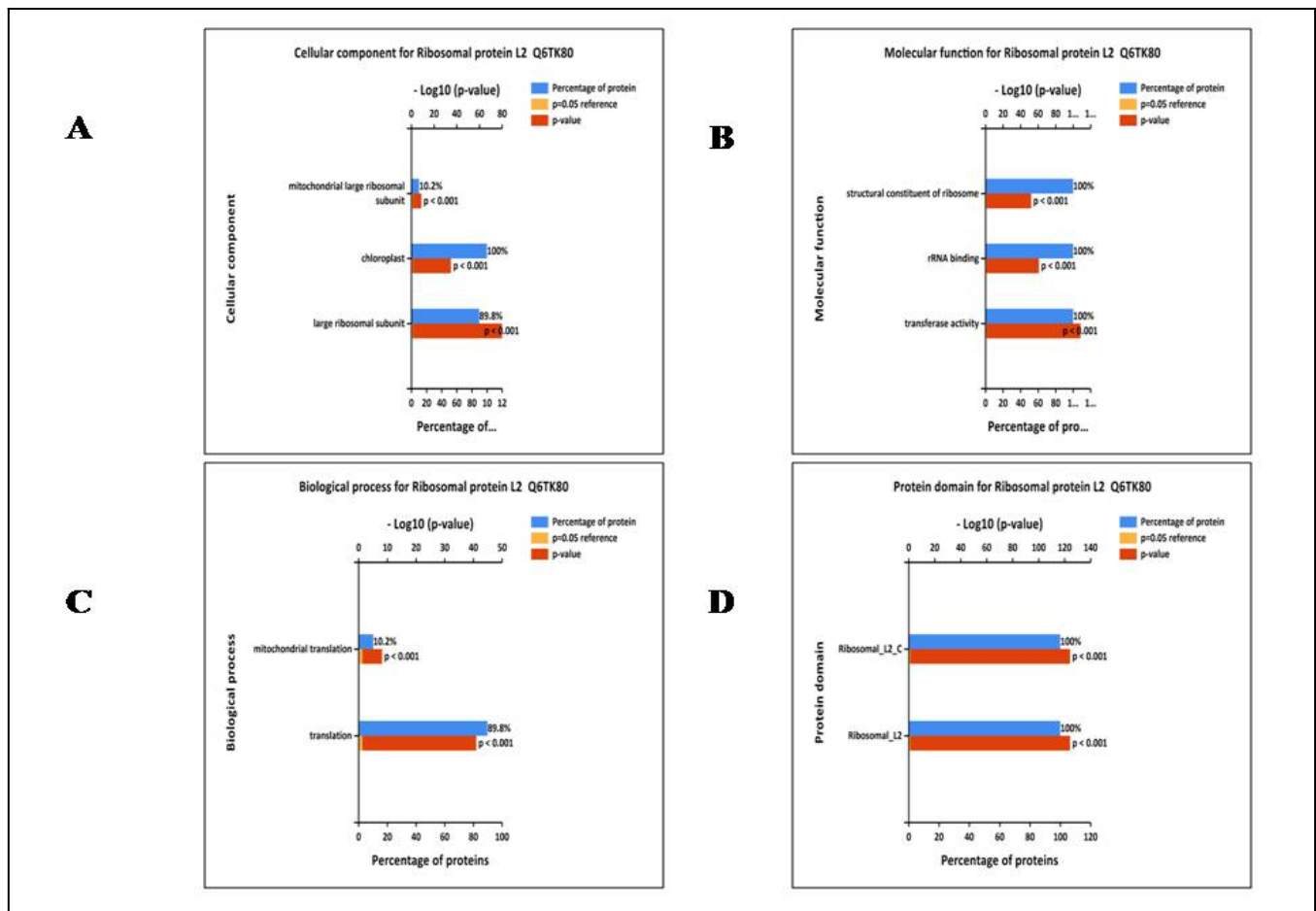


Fig. 15. Enrichment analysis for Ribosomal protein L2: Fig A: Cellular component, B: Molecular function, C: Biological process and D: Protein domain.

charantia, *Momordica balsamina*, *Trichosanthes anguina*, *Bryonia dioica*, *Gynostemma pentaphyllum*, *Sambucus nigra*, *Ricinus communis* etc. The enrichment analysis for Ribosome-inactivating protein luffaculin exhibited for molecular function such as rRNA N-glycosylase activity and toxin activity with biological processes such as negative regulation of translation, defence response to the virus, defence response and regulation of defence response to the virus (Fig. 16).

Ribulose biphosphate carboxylase large chain

Protein sequence of Ribulose biphosphate carboxylase large chain from *L. acutangula* (Swissprot id: R4I6V0) had exhibited an identity from 96% - 99% with plants: *Lactuca sativa*, *Cucumis sativus*, *Cucurbita pepo*, *Cichorium intybus*, *Flaveria pringlei*, *Glycine max*, *Sesbania sesban*, *Bartlettina sordida*, *Guizotia abyssinica*, *Flaveria bidentis*, *Barnadesia caryophylla*, *Pelargonium hortorum*, *Helianthus annuus*, *Oenothera glazioviana* etc. The enrichment analysis of the Ribulose biphosphate carboxylase large chain reveals a cellular component for chloroplast, plastoglobule, cytosolic ribosome, thylakoid, plastid, chloroplast envelope, apoplast, cell wall, chloroplast stroma, membrane and chloroplast thylakoid membrane. It is involved in molecular functions such as monooxygenase activity, ribulose-biphosphate

as photorespiration, reductive pentose-phosphate cycle, carbon fixation, response to cadmium ion and abscisic acid (Fig. 17).

rRNA N-glycosidase

Protein rRNA N-glycosidase from *L. acutangula* (Swissprot id: B0EVM6), the blast outcome resulted with a sequence identity from 32% - 96% with plants: *Luffa aegyptiaca*, *L. acutangula*, *Momordica charantia*, *Cucumis ficifolius*, *Trichosanthes kirilowii* etc. The enrichment analysis of rRNA N-glycosidase exhibited molecular function such as rRNA N-glycosylase and toxin activity, biological processes such as negative regulation of translation, defence response and regulation of defence response to the virus (Fig. 18).

Superoxide Dismutase

Protein Superoxide Dismutase from *Luffa aegyptiaca* (Swissprot id: A0A1L5JHV8) exhibited a sequence identity from 71% - 81% from plants: *Hevea brasiliensis*, *Prunus persicum*, *Nicotiana plumbaginifolia*, *Arabidopsis thaliana*, *Capsicum annum*, *Zea mays*, *Oryza sativa* subsp. *japonica*, *Pisum sativum* etc. The enrichment analysis for Superoxide dismutase reveals cellular components such as mitochondrial matrix and mitochondrion. The molecular function includes superoxide dismutase activity, metal ion binding, manganese ion binding

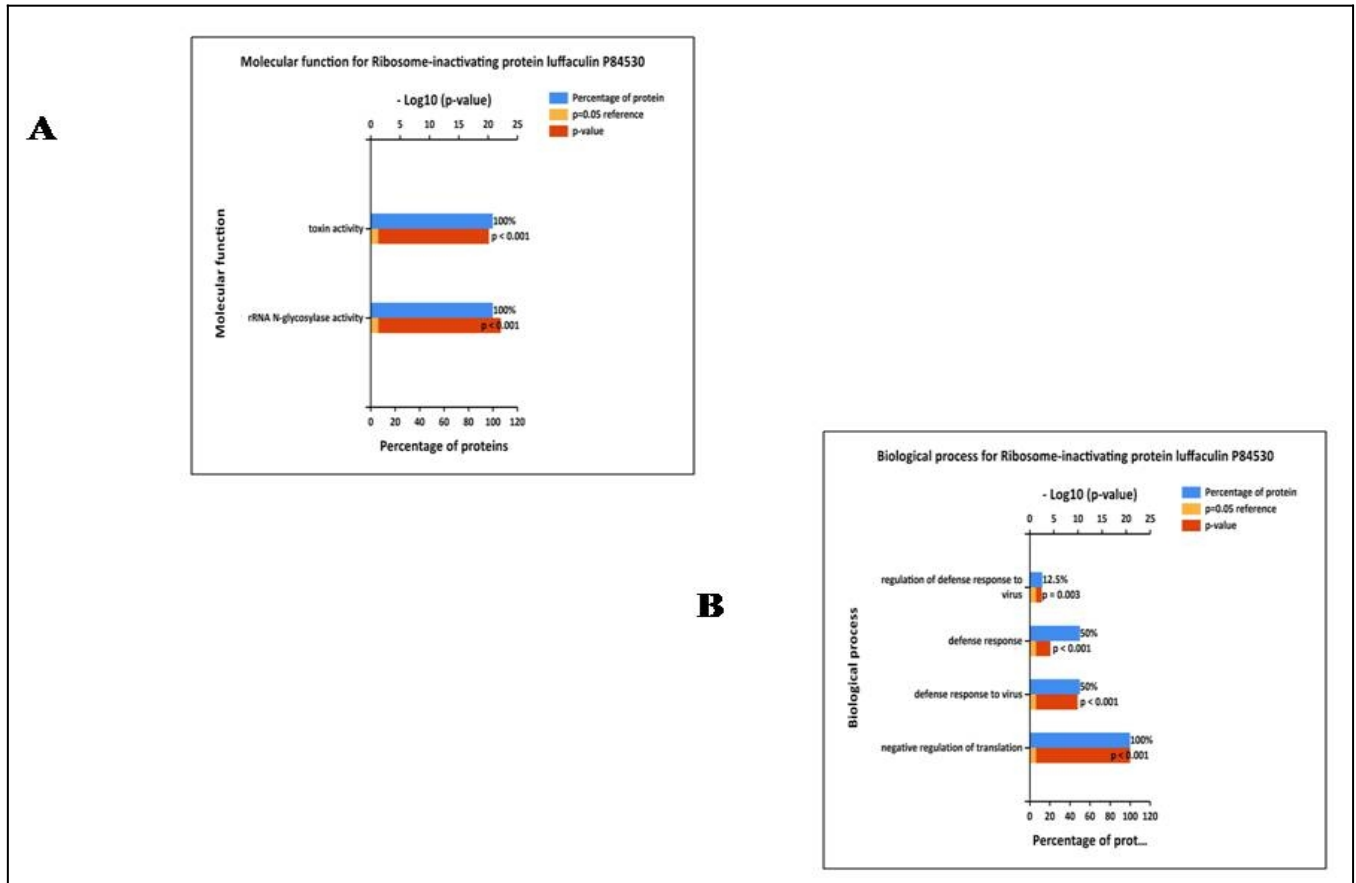


Fig. 16. Enrichment analysis for Ribosome-inactivating protein luffaculin: Fig A: Molecular function and B: Biological process.

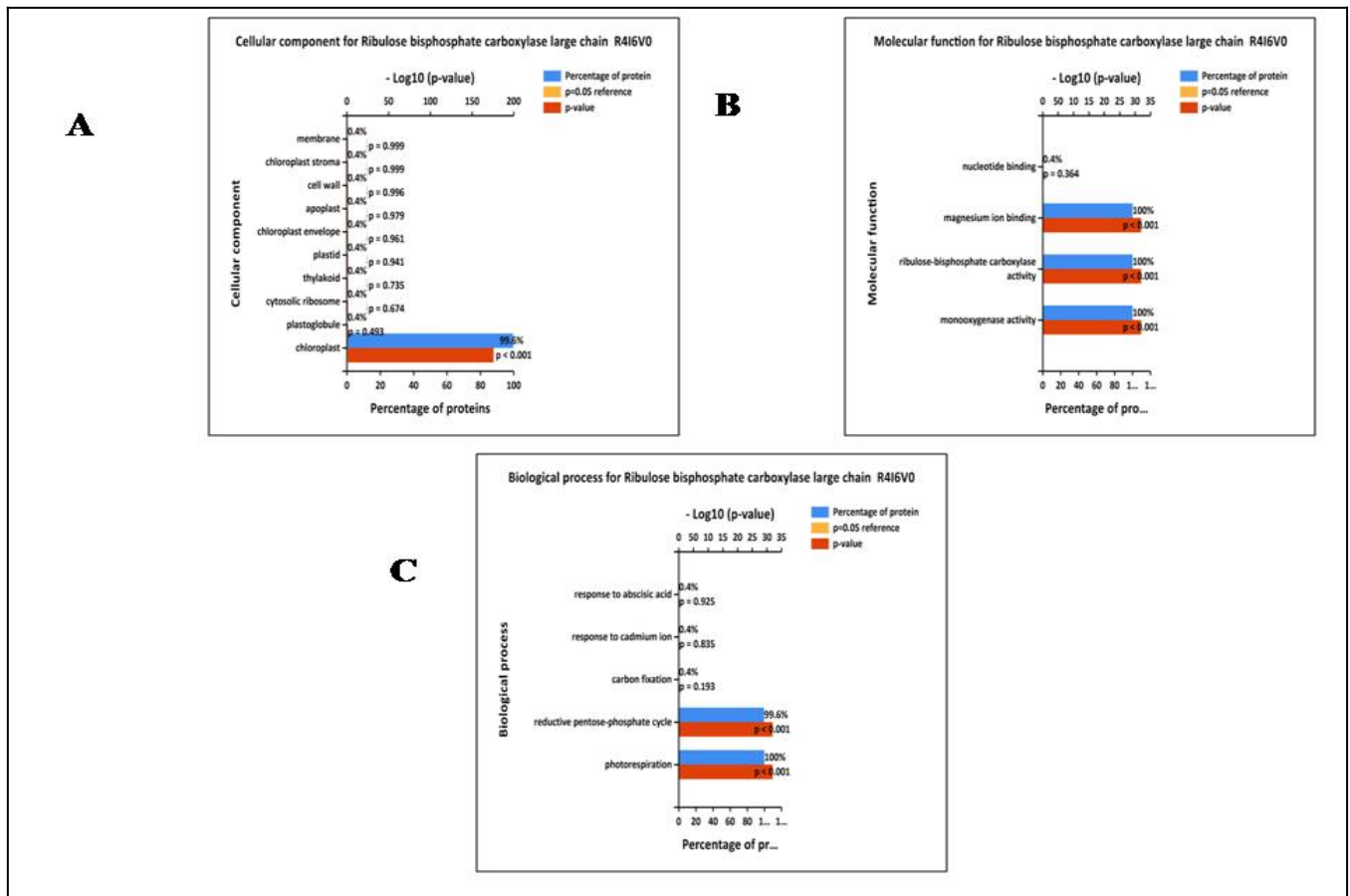


Fig. 17. Enrichment analysis for Ribulose biphosphate carboxylase large chain : Fig A: Cellular component, B: Molecular function and C: Biological process.

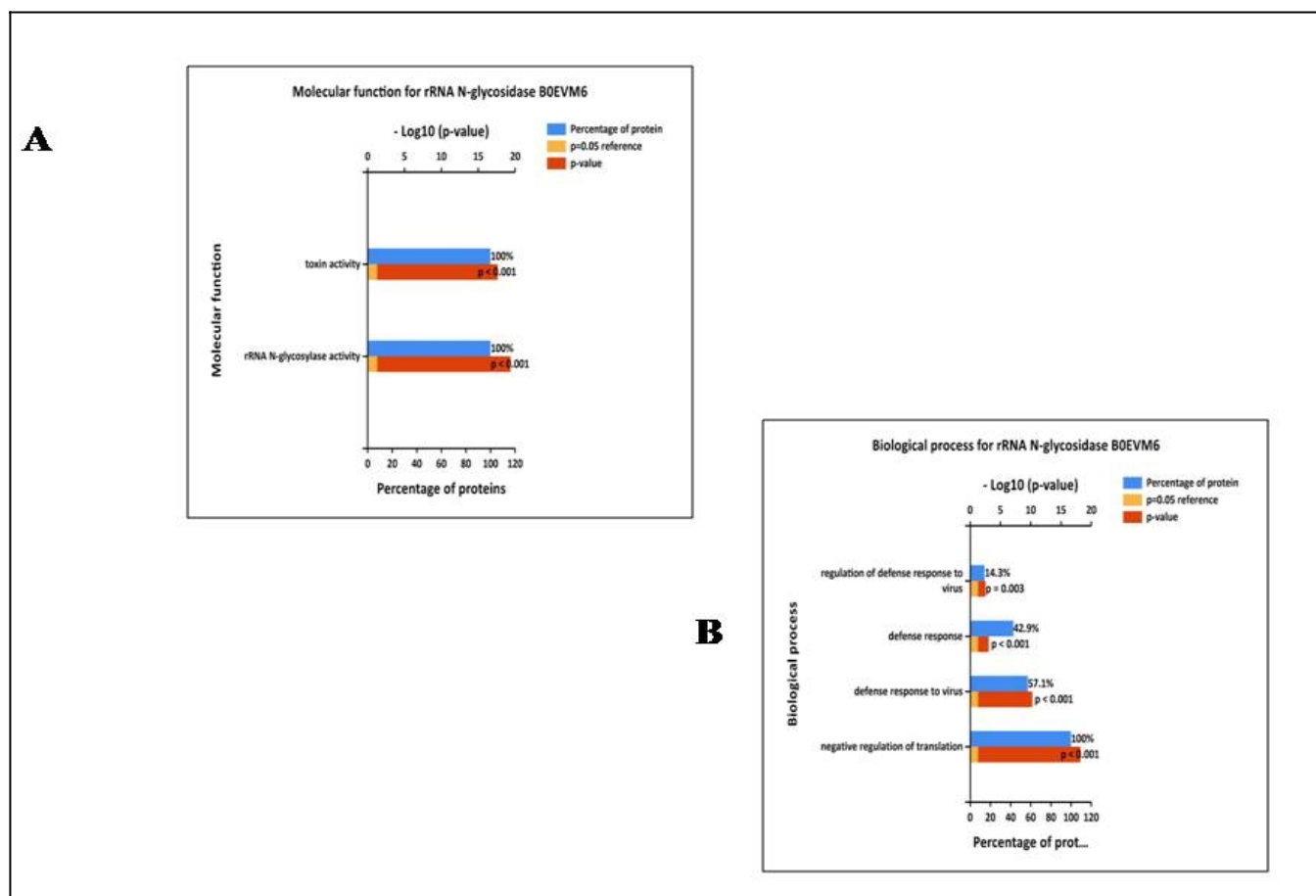


Fig. 18. Enrichment analysis for rRNA N-glycosidase: Fig A: Molecular function and B: Biological process.

and copper ion binding. Biological processes such as response to osmotic stress, salt stress, abscisic acid, xenobiotic stimulus, floral organ abscission, protein homo-tetramerization. Other reported responses might include zinc ion, reactive oxygen species, herbicide, seed dormancy, oxidative stress and defence to bacterium/ pest infestation (Fig. 19).

The differential annotation of genes and their enrichment in cell and metabolic pathways thus suggested that salt stress plays a pivotal role in stimulating a significant difference at both transcript levels as well as in the gene function among the two varieties of *L. acutangula*. The identification and location of salinity stress-responsive proteins discussed above to cellular components like chloroplast, mitochondrion, apoplast underlines the putative function of these proteins in stress signalling, ion uptake and signal transduction. A similar observation was recorded in Tibetan barley, grown under low nitrogen conditions (35) and by the Witzel research group (2009) among barley roots where downregulation of key regulatory proteins was recorded (36). Thus, the accumulation of free amino acids, proteins, soluble carbohydrates and osmotic solutes are induced by salinity stress (37).

With the development in proteomic technology and availability of sequence information from various protein databases, several research groups have used these approaches for the identification of salt-responsive proteins in

different plants. Extensive studies on proteome analysis for drought-responsive genes among cereal crops (38) however, comparatively less information is available on other crops, legumes and vegetables. In 2002, root proteome studies by Salekdeh and team on salt sensitive and tolerant rice varieties indicated the presence of salt responsive proteins, such as ABA, ascorbate peroxidase, stress-responsive protein etc. (39). Numerous proteins were reported with modification in their expression by salt concentration in a synchronised mode (40). These reported proteins were highly involved in molecular and biological functions such as control of ion channels, metabolism regulation, oxidative stress defence, photorespiration, photosynthesis process, protein folding and signal transduction. Over ~200 and ~100 proteins were observed with their altered expression level due to NaCl (150 and 200 mM) from proteome analysis of Arabidopsis roots and wheat genotypes (39, 41). The above-studied species are fairly sensitive to salinity (42) and the selected salt concentration are prospective to stimulate the unspecific stress reactions, instead of projecting in the discovery of gene products that are precisely associated with salt tolerance.

A detailed analysis of 466 salts responsive *A. thaliana* proteins retrieved from UniPort and analysed by bioinformatics tools like PANTHER, DAVID, KEGG etc. correlated the involvement of salt responsive proteins both in abiotic and several biotic conditions (43).

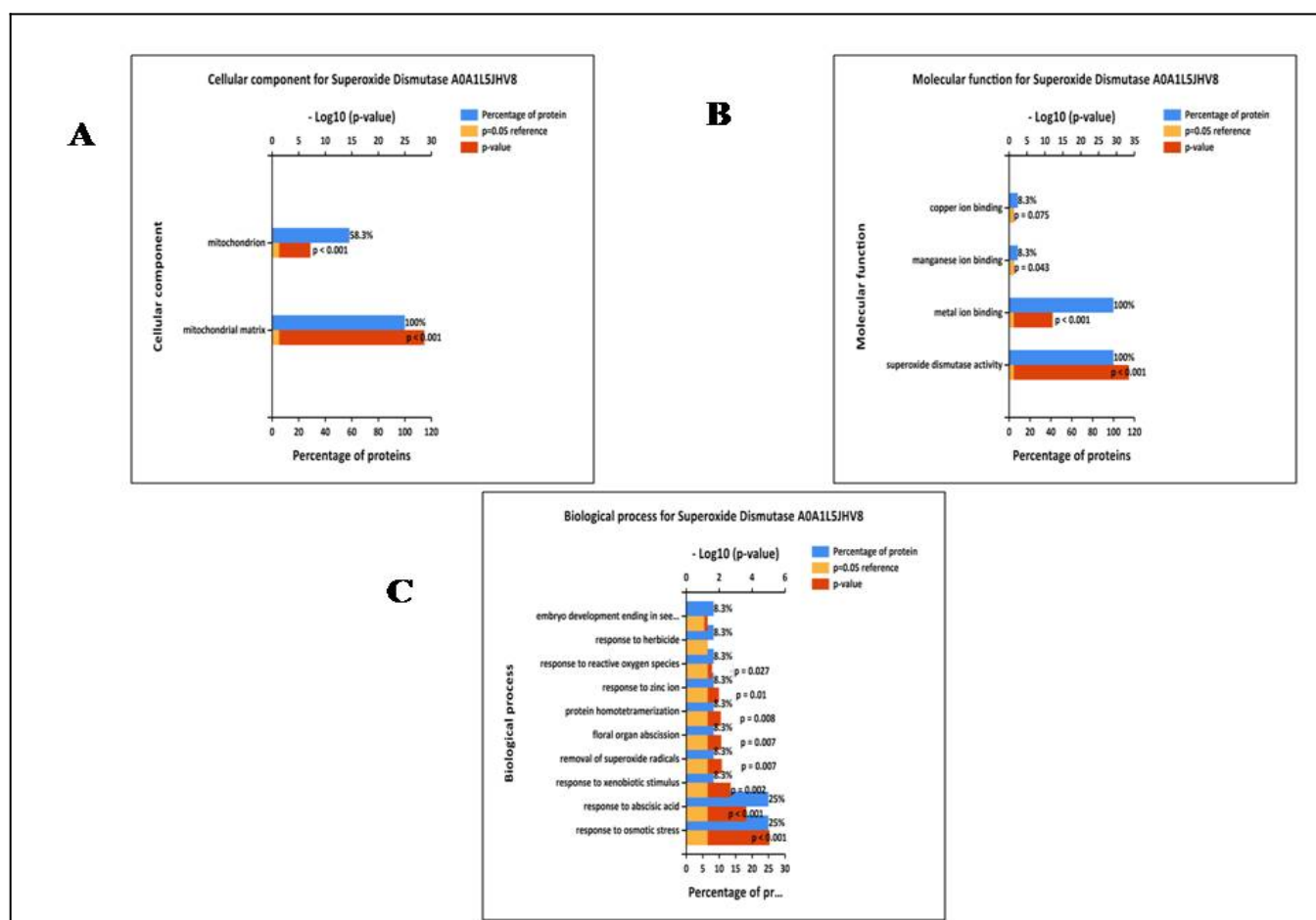


Fig. 19. Enrichment analysis for Superoxide Dismutase: Fig A: Cellular component, B: Molecular function and C: Biological process.

A presence of protein (26 kDa band), linked with salt tolerance in rice was reported by Rani and Reddy (1994) (44). A high number of differentially regulated proteins from maize shoots and roots are reported after the treatment of NaCl. In case of moderate level of salt stress with 25 mM NaCl, 31% of shoot and 45% of root proteins exhibited differential regulation but no effect is reported with alterations in plant morphology and the intracellular ionic concentrations of sodium and chloride ions. Whereas in presence of a high-stress level with 100 mM NaCl exhibited an unconventional variation and reported more than 80% of the separated proteins (45). It was (43) reported that the osmotic effect comprised with restricted water absorption owing to salinity in the rhizospheric region and due to the excess ions may lead to intracellular unevenness or toxicity which may gradually damage the structural stability of the protein and thus its functionality (46). In high salinity condition, the accumulation of ROS takes place that chiefly causes the damage or misfolding of the protein (47).

Peroxidase, pivotal in ROS scavenging, has been reported to be overexpressed under salt stress conditions among different plant species (48). H_2O_2 are primarily reduced by peroxidases through exhausting numerous molecules at the cell in the form of substrate. These substrates are viz. auxin, lignin precursors, phenolic compounds and related compounds involved in polymerization reaction like

cross-linking of cell wall proteins, lignification and suberization (49).

The substitution of impaired proteins with newly activated ones is significant for stress management. However, excessive metabolomic fluxes may eventually lead to down-regulation of these regulatory metabolites (50). In Banana, (51) increase in salt concentration led to down-regulation of thioredoxin, APX while upregulation in POD, CAT, AOC, allene oxidase, glutathione S-transferase. This consequently explains the observation of current research work, wherein an initial upregulation of osmoregulatory enzymes and proteins was followed by down-regulation with an increase in the salt concentration. Additionally, the modulations in the abundance of peroxidase and its inducibility observed in the present study on *L. acutangula* varieties; thus, supports that the influx of the ions was ameliorated by adopting modifications in the cell wall. A similar observation has also been recorded in two genotypes of chickpea (47). A change in molecular conformation of proteins, due to change in protein folding patterns due to salinity stress (52), may also be a key factor for differential expression of proteins recorded in the present study. A similar type of conduct is apparent among proteins from the same functionality. Also, many abiotic stress factors instigate the aggregation of proteins with similar functions and properties. Rice (53), soybean (54), durum wheats (55), *A. thaliana* (43, 56) and tobacco (57) are widely and majorly studied crops to understand the effect of salt stress on their

proteome compositions. The change in the proteome in these plants resulted in the build-up of enzymes that are implicated in glycolysis and carbohydrate metabolism indicates an enhanced requisite for energy (56). Moreover, foremost increased proteins have ROS scavenging enzymes like APX, DHAR, Trxh, peroxiredoxin, SOD, suggesting oxidative stress (58).

Enhanced amassing of GB and proline under saline conditions can be accredited to an amplified abundance of enzymes crucial for biosynthesis of compatible solutes such as glutamine synthase, glycine dehydrogenases etc. (57). Also, the vital proteins involved in the process like membrane stabilisation, ion homeostasis, and signal transduction have been reported by several researchers to undergo alternations in their genomic expressions. Similarly, in rice (53) a new salt-responding leucine-rich-repeat type receptor-like protein kinase, OsRPK1 is reported. Furthermore, a diminution in some glycolytic enzymes (GAPDH), along with a decrease in abundance of several proteins involved in CO₂ assimilation (a decrease in Rubisco SSU, increased fragmentation of Rubisco LSU, a decrease in PGK catalysing a reduction step in Calvin cycle, PRK catalysing regeneration of primary CO₂ acceptor RuBP in PPP) among varieties of durum wheat have been elucidated (55). In current study, a similar response among proteome components was recorded in both varieties of *L. acutangula*.

However, the knowledge on proteome fluxes ameliorating the damaging effects of any stress is fragmental, as reports are based on a comparison of observed parameters amidst the control and stressed plantlets. Also, the information on deviations in cellular metabolism along with shielding proteins under stress is available, while the role of less abundant regulatory proteins involved in stress signalling and regulation of gene expression is yet to be unveiled. In this research study quantitative changes among subcellular metabolome, along with mitochondrial and other plastid proteomes have been studied and analysed using MS analysis. The studies encompassing interactomics shall complement detailed protein functional characterization and will surely, enhance the understanding of acclimatization, stress tolerance acquisition in *Luffa* and eventual development of protein molecular markers as well as probes for *L. acutangula* and other *Cucurbitaceae* families.

Conclusion

Salt stress-responsive proteins in *Luffa acutangula* (L.) Roxb. were identified in the present study. An alteration in the expression of some proteins displayed a clear response to salt stress among luffa varieties. The stress-induced damage was observed to be mitigated by major metabolic conversions. Proteins with altered fractions of abundance disclosed differential mechanisms to respond to salinity among *Luffa* sp. The present investigation focussed on functional analysis of key proteins related to these responses distinguished a few differentially expressed proteins in *L. acutangula* variety only during salt stress. As these proteins were observed in both

varieties of *L. acutangula* only under salt stress conditions, they could be central proteins reducing stress-induced damage and thus might be useful as biomarkers associated with salinity stress. Above all, in view of the paucity of information for such molecular responses in *L. acutangula*, the present investigation may aid the of interpretation the physiological and molecular mechanisms in response to NaCl and drive us for generating the proteome maps that clarify different signalling pathways.

Acknowledgements

This research was supported in part by research scholars Piyush M. and Savita B. The authors are thankful to School of Biotechnology and Bioinformatics, D. Y. Patil (Deemed to be) University for support and encouragement. The data reported from MASSFIITB facility is supported by Department of Biotechnology (BT/PR13114/INF/22/206/2015). The team is extremely thankful to Dr. S. Srivastava, Professor, Group Head of Proteomics Laboratory at the Indian Institute of Technology, Bombay, for his support and guidance. The authors are thankful to Dr Namdev Jagtap, Assistant Professor, School of Biotechnology and Bioinformatics, D. Y. Patil (Deemed to be) University for guiding in statistical analysis.

Authors' contributions

MP, PPG, SS and DD conceived the research, designed the experiments and wrote the main manuscript text along with acquisition and analysis of data. Dr Pramodkumar P Gupta analysed the data for *Insilco* annotation of protein. All the authors were active in writing, reviewing and approving the final manuscript.

Conflict of interests

All the authors do not have any competing interest in terms of financial or non-financial, professional or personal for this research article.

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To cite this article: Mala P, Pramodkumar P G, Sunita S, Debjani D. Evaluation of proteome complexes normalizing osmoregulation in salt stressed *Luffa acutangula* (L.) Roxb. *Plant Science Today.* 2021;8(4):1015–1031. <https://doi.org/10.14719/pst.2021.8.4.1262>

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