

Effect of Salinity Stress on Total Amino Acids, Proline and Proteins in Salt Tolerant and Sensitive Varieties of Pigeonpea (*Cajinas cajan*, L.)

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ABSTRACT

Salt stress is the buildup of dissolved salts in water or agricultural soil at high and unfavorable concentrations for plant growth. Salinity negatively affects seed germination, plant growth, and the quantity as well as quality of yield, especially economic crops. Plants have several processes that they use to resist salinity stress. The total amino acid content exhibited significant variations when exposed to different levels of salt which played significant role in the adaptation of the plant for salinity stress. Proline is the vital amino acid which rises during salt stress, and plays the role of protecting the integrity of the membrane and energy production. Bahar and IPA-203 appear to perform better than other varieties in saline environments because of higher proline levels. Proline acts as an osmo-protectant and plays significant role in osmotic balance, protection of sub-cellular structures, enzymes and in increasing cellular osmolarity that provide the turgor essential for cell development in stress environment. Protein content markedly increased in salt-tolerant variety Bahar and IPA-203 whereas minor reduction in its content was detected in salt-sensitive variety UPAS-120 and Paras.

Keywords: Salt Stress, Pigeonpea, Salt sensitive, salt tolerant, Amino Acids, Proline, Protein.

Mathematics Subject Classification : 62P20

1. INTRODUCTION

Salinity is the most severe type of edaphic abiotic stress, accounting for huge crop losses around the world. Pigeon pea is a pulse crop that can be produced in marginal soils. Thus, it is critical to understand the metabolic processes at the systemic level under salt stress. Salinity causes an uncontrolled buildup of ROS in the tissues of pigeon pea plants. This causes lipid peroxidation and damage to protein and enzyme structures. To combat such degenerative effects, plants collect large amounts of osmolytes such as Pro, glycine betaine, and so on. These suitable solutes scavenge harmful ROS and aid to retain cellular osmotic balance during severe oxidative stress.

Amino acids, in addition to being the basic components of all living cells (Umehiya et.al., 2002, Mondal et.al., 2013, Xie et.al., 2020) are important components of root secretion (Chaparro et.al., 2013, Krafczyk et.al., 1984, Xie et.al., 2020). Amino acids play highly diverse and essential roles in plants. Plants take up amino acids directly from the soil or assimilate inorganic N into amino acids (Tegeder and Rentsch, 2010). As the building blocks of proteins and enzymes, amino acids are vital for plant structure and metabolism (Tegeder and Ward, 2012, Xie et.al., 2020). Amino acids in root secretion have also been regulated to compensate for the changes in plants under stress and have significant role in plant development, growth, and stress resistance (Pavlikova et.al., 2014). Plants induce the accumulation of compatible substances to adjust the osmotic pressure under salt-stress conditions. Proline, an amino acid, is an osmotically active organic solute in the root area (Hartzendorf and Rolletschek, 2001). Zhang et al., (2011) found that the pyrimidine and purine in tobacco decreased when treated with 50 mM sodium chloride (NaCl), but the amount of proline and aromatic amino acids increased when NaCl was increased to 500 mM. Therefore, certain previous investigations have used amino acid levels as salt stress indicator (Hartzendorf and Rolletschek, 2001, Fougère et al., 1991, Di Martino et al., 2003, Xie et.al., 2020). The level of the amino acid proline is amongst most significant biochemical elements impacted in plants in stress conditions (saline), and it is closely related to the plant resistance mechanism in stress conditions (Chutipaijit et al., 2011). Proline is amongst most important antioxidants, as it works to remove free radicals from plant tissues, and works to regulate the osmotic medium of plant tissue cells and remove the toxic effects of ammonia accumulation in cells and preserve the stability and vitality of protein and cytoplasmic membranes and not be affected by destructive stress conditions (Al-Issawi et al., 2023, AL-Taey et al., 2021). It also contributes to the protection, activation, and stability of mitochondrial enzymes in stress conditions (Ashraf and Foolad, 2007). For protecting plants from stressful situations, the amount of proline inside the cells increases. This accumulation is caused by both the increased breakdown of protein and its conversion into amino acids, including proline, and the lack of proline oxidation (Lisa et al., 2004). Proline is amongst the active substances in capturing the OH-group, and it protects the plasma membrane from oxidation (Al-Ealayawi and Al-Dulaimy, 2023). Proline accumulation is because of disturbance in breakdown of amino acid which is related to protein breakdown and aging resulting from salinity (Al-Khayri et al., 2002, Al-Falahi et al., 2022, Al-Janabia et al., 2024). Proline acts as an osmo-protectant and plays significant role in osmotic balancing, protection and in increasing of turgor essential for cell expansion (Matysik et al. 2002; Sairam and Tyagi, 2004, Surekha et. al., 2014). Proline is the single osmolyte which scavenges free radicals, like hydroxyl ions, and singlet oxygen. It also serves as redox potential regulator and protects macromolecules such as proteins, DNA and reduces enzyme denaturation due to heat, NaCl and other stresses (Matysik et al., 2002, Kumar et al., 2010, Surekha et. al., 2014). Complete elucidation of these processes requires a study of the proteins expressed by the genes because proteins are more physiologically and biochemically responsive to stress and better correlate with plant characteristics (Pandey and Mann, 2000). Salinity stress stimulates ROS generation, which results in oxidative damages of macromolecules like DNA, lipids, and proteins, which disturb vital cellular functions of plants. Stress conditions bring about qualitative and quantitative changes in plant cell

proteins. Under salt condition accumulation of protein in plant cells might be playing important role in plants salt tolerance. Salinity inhibits the synthesis of new proteins and changes gene expression. The changes in protein profiles and accumulation are dependent on plant genotypes, salt duration and the salt concentration. Sensitive genotypes did not exhibit altered protein profiles under salinity stress, whereas salt-tolerant genotypes were the only ones to show an accumulation of newly produced protein bands. Thus, a deeper knowledge of the pigeon pea's salt tolerance mechanism will come from proteomic study on the structure and function of stress-induced proteins (Chavan et al., 2018).

2. MATERIAL AND METHODS

2.1. Determination of free amino acids

Total free amino acids was estimated through the ninhydrin method (Hamilton & Vanslyke, 1943). For the estimating total free amino acids, 1ml of each sample extracted during soluble protein estimation was reacted with 1ml of 10% pyridine and 1ml of 2% ninhydrin solution. The optical densities of the colored solutions were then read at 570 nm using spectrophotometer.

2.2. Proline estimation

Extract: 20 to 50 times diluted fresh weight (w/v), typically in a 70:30 ethanol: water mixture (v/v)

Standards: proline solutions ranging from 0.04 to 1 mM, in the same medium as the one used for extraction

Reaction mix: ninhydrin 1% (w/v) in acetic acid 60% (v/v), ethanol 20% (v/v).

In 1.5 ml screw-cap tubes, 1000 µl of reaction mix was taken. For this, up to 500 µl ethanolic extract or 100 µl of 5-2-1-0.5-0.2 mM proline standard were pipetted and made up with 400µl of ethanol: water (40:60 v/v). After this, tubes were sealed, mixed and heated at 95°C in the block heater for 20 min. After this, it was centrifuged at 10000 rpm for 1 min and supernatant was taken into a 1.5 ml cuvette and readings were taken at 520 nm (Hummel et al., 2010, Carillo et al., 2011).

2.3. Protein Extraction

Protein was isolated with 0.1M phosphate buffer (6.8). 1 gram of material was grounded with mortar and pestle in 5 ml buffer and centrifuged at 10,000 rpm for 20 min. Supernatant was decanted and utilized to quantify the protein (Kar and Mishra, 1976). Protein estimation was done by the method of Lowry's (1951). For this, 0.1ml of protein samples was taken into test tubes. Reagent E was diluted to make 200µg/ml BSA stock. Standards of 20-100µg/ml were prepared from 200µg/ml BSA stock by taking different aliquots. After this, volume 1.5 ml was made up in standard and samples by 0.1N NaOH. Blank was also prepared by taking 1.5 ml 0.1 N NaOH. Then 1.5 ml reagent C was mixed and vortexed immediately. Incubation was done at room temperature for 15 min. Subsequently, 150µl of reagent D was added in test tubes and mixed immediately by vortex and incubated at room temperature under dark for 45 min. Absorbance of samples was taken at 690nm. Protein content was estimated by plotting a standard curve.

2.4. Statistical Analysis

Analysis of data was done using one-way ANOVA. Mean $\pm$ SD values were for three samples in each group and significance of the data was tested using student's *t* test ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Total Amino acid

Total amino acid content was decreased at lower salinity levels in the present study. Although it slightly increased at 75 mM and again decreased at 100 mM salt concentration in all varieties (Table 1, Fig 1). Tlahig et al. (2021) found that free amino acids reduced in the embryonic axes of *Vigna radiata* and *Vigna unguiculata* treated seedlings, while the cotyledons were unaffected by NaCl treatments, except for an increase of about 80 % in respect to control in *Vigna radiata* treated with 100 mM NaCl. Similar results were observed by Xie et al. (2020) which indicated that NaCl significantly influenced the total amino acid (Tot AA) content. Rise in Total AA was primarily because of proline accumulation (Xie et al., 2020).

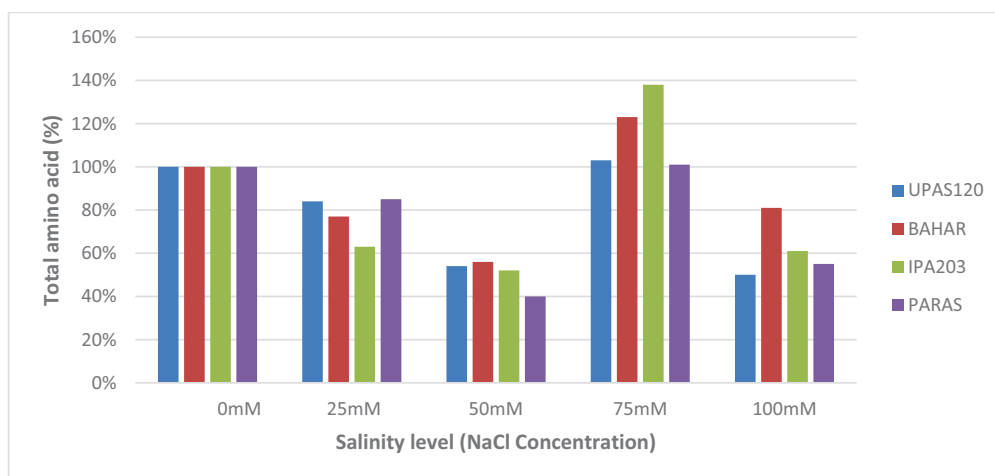


Fig-1 Effect of Salt stress on the Total Amino acid in Pigeon pea Seedlings

TABLE-1
EFFECT OF SALT STRESS ON TOTAL AMINO ACID IN PIGEONPEA SEEDLINGS

Conc.	UPAS120	BAHAR	IPA203	PARAS
0mM	0.310 $\pm$ 0.052 (100%)	0.472 $\pm$ 0.023 (100%)	0.388 $\pm$ 0.047 (100%)	0.276 $\pm$ 0.072 (100%)
25mM	0.261 $\pm$ 0.032 (84%)	0.366 $\pm$ 0.070 9 (77%)	0.247 $\pm$ 0.042 (63%)	0.235 $\pm$ 0.063 (85%)
50mM	0.168 $\pm$ 0.040 (54%)	0.267 $\pm$ 0.041 (56%)	0.204 $\pm$ 0.052 (52%)	0.111 $\pm$ 0.047 (40%)
75mM	0.321 $\pm$ 0.022 (103%)	0.586 $\pm$ 0.060 (123%)	0.536 $\pm$ 0.061 (138%)	0.281 $\pm$ 0.051 (101%)
100mM	0.157 $\pm$ 0.025 (50%)	0.385 $\pm$ 0.040 (81%)	0.240 $\pm$ 0.033 (61%)	0.153 $\pm$ 0.045 (55%)

Pigeon pea seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. Results are mean $\pm$ SE of 3 observations ($P \leq 0.05$).

3.2, Proline content

There is very sharp increase in proline level at higher salt concentration. Highest increase was observed at 75 mM NaCl concentration in all varieties. Highest rise was seen in the IPA 203 followed by other varieties (Table 2, Fig 2). Similar results were observed in tolerant chickpea genotype under salt exposure whereas in sensitive genotypes, proline levels drop during germination stage (Tutlani et. al., 2025). Proline accumulation results from the enhanced proline biosynthetic enzymes activity such as pyrroline-5-carboxylase reductase, while the proline oxidase activity, a proline degrading enzyme decreases, to mitigate oxidative damage (Tutlani et. al., 2025). Storage of proline can assist the plants for maintaining low water potentials and effectively regulate the storage of essential nitrogen and it is osmotically very active (Rains, 1981, Wyn Jones and Gorham, 1983, Misra and Gupta, 2005). It is also compatible with other cytoplasmic components and can be easily converted to glutamate that takes part in the synthesis of other essential amino acids (Rains, 1981, Waheed et.al., 2006).

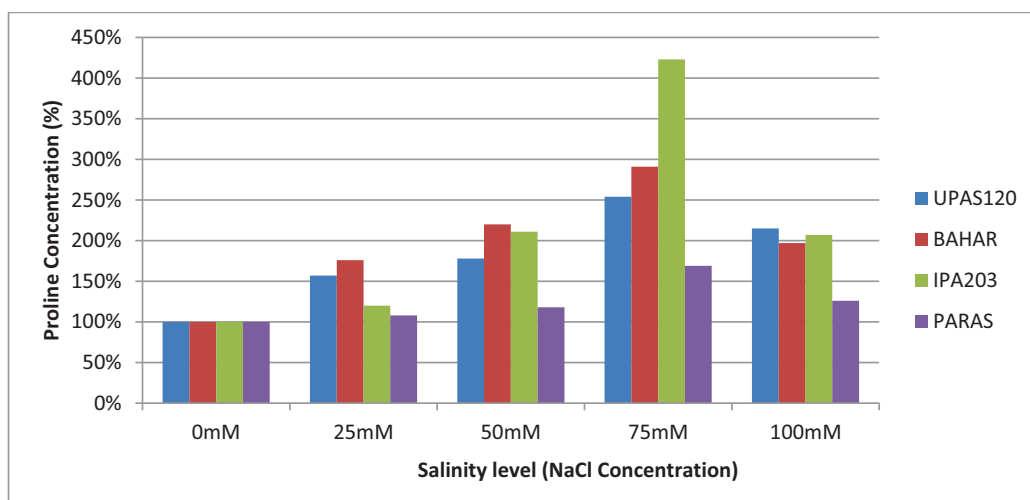


Fig-2 Effect of Salt stress on the Proline content in Pigeon pea Seedlings

TABLE-2
EFFECT OF SALT STRESS ON PROLINE IN PIGEONPEA SEEDLINGS

Conc.	UPAS120	BAHAR	IPA203	PARAS
0mM	0.220±0.02 (100%)	0.226±0.06 (100%)	0.160±0.06 (100%)	0.326±0.08 (100%)
25mM	0.346±0.10 (157%)	0.400±0.11 (176%)	0.193±0.05 (120%)	0.353±0.08 (108%)
50mM	0.393±0.11 (178%)	0.500±0.10 (220%)	0.340±0.10 (211%)	0.386±0.04 (118%)
75mM	0.560±0.12 (254%)	0.660±0.10 (291%)	0.700±0.12 (435%)	0.553±0.06 (169%)
100mM	0.473±0.10 (215%)	0.446±0.09 (197%)	0.333±0.10 (207%)	0.413±0.08 (126%)

Pigeon pea seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. Results are mean± SE of 3 observations ($P \leq 0.05$).

One of the highly significant biochemical components affected in plants under stress conditions (saline or water) is the content of the amino acid (proline), which has a close relationship in plant resistance to stress environment (Al-Issawi et.al., 2003). Proline is amongst highly significant antioxidants, as it works to remove free radicals from plant tissues, and works to regulate the osmotic medium of plant tissue cells and remove the toxic effects of ammonia accumulation in cells and preserve the stability and vitality of protein and cytoplasmic membranes and not affected by destructive stress conditions (AL-Taey et.al., 2021). It also contributes to the protection, activation, and stability of mitochondrial enzymes under stress (Ashraf and Foolad, 2007). The proline content inside the cells increases under stress to protect plants from these conditions, and the reason for the accumulation of proline is lack of its oxidation on the one hand and the increased breakdown of protein and its conversion into amino acids, including proline (Lisa et.al., 2004). There is close association between rise in proline storage and the rise in the soluble sugars, which regulate the osmosis of plant cells and tissues (Jubair and Al-Dulaimy 2023, Al-Mohammedy et.al., 2016). The accumulation of free radicals is controlled by scavengers such as Dismutase, Super oxide, Peroxidase, Catalase, Sugars, and Proline. Proline is one of the active substances in capturing the OH⁻ group, and it protects the plasma membrane from oxidation (Al-Ealayawi and Al-Dulaimy, 2023). Proline accumulation is because of disturbance in amino acid breakdown related to protein breakdown and aging resulting from salinity (Al-Khayri, 2002, Al-Falahi et.al., 2022). Glutamate and ammonia storage in plants under salinity stress proceed to the stimulation of proline formation by direct or indirect influence of the ratio of building materials to products (Hasegawa et. al., 2000, Almohammedy et.al., 2021). Lack of activity of both Proline Dehydrogenase and Proline Oxidase is another cause for proline storage in the plants. Some researchers have pointed to the importance of soluble carbohydrates in inducing proline accumulation by inhibiting proline-degrading enzymes, which leads to increased proline synthesis and accumulation (Javid et.al., 2011). Proline metabolism is controlled by two enzymes, PRODH and P5CR. The former allows for the synthesis of proline, while the latter converts it. P5CR enzymatic activity is weak during the dark and early light phases, but it is rapid during the light phase and enhanced to a maximum around six hours after the light onset. It then decreases to maintain a steady level for whole day. In contrast, PRODH enzymatic activity is weak both during the light and dark phases and is constant throughout the day. The activity of both enzymes is also highly dependent on the incubation temperature. At 10°C, PRODH is very weak and difficult to measure, while P5CR activity is distinct and clear. With increasing temperature to 60°C, the activity of both enzymes increases, then declines after this point (Sekhi et.al., 2023, Todorova et.al., 2012). From the study of the genetic map of plants that tolerate higher levels of salinity or drought, an increase in the proline has been observed, which is formed in the leaf and then transferred to the other portions of the plant (stem and roots), to accumulate starting from glutamate, which is converted to semi aldehyde and then to a proline ring (Quesada et.al., 2000, Hardan et.al., 2022). The formation of proline prevents the breakdown of proteins inside the plant and thus prevents their hydrolysis. Its presence strengthens the bonds between the amino acids that make up the protein, particularly in the early stages of plant growth. The breakdown of proteins in this sensitive phase exposes the plant to aging or death. In order to develop plant resistance to salinity and drought, the method of spraying

with a proline solution is used, with no negative effects provided that its concentration do not exceed 30 mg L⁻¹. Presence of proline in large quantities creates a defense system against tannin toxicity and prevents its inhibitory action (Burton, 1991, Al -Janabia et al., 2024).

3.3. Total Protein

Protein content markedly increased in salt-tolerant variety Bahar and IPA-203. whereas reduction in its content was detected in salt-sensitive variety Upas-120 and Paras. There is about 69% rise in protein concentration in Bahar at 100mM concentration and it almost doubled at 50mM concentration in IPA-203. In Upas-120 at 100 mM concentration highest reduction was seen in total protein concentration, followed by Paras. These results are as earlier findings of Chavan et al (2018). There was a substantial negative correlation discovered between cell membrane stability and total protein concentration. Stress causes changes in protein expression, however only a few of these proteins are likely to be directly implicated in stress tolerance. It is probable that in some circumstances, protein production signals vulnerability to a stressor rather than a tolerance mechanism. Ashraf and Waheed (1993) discovered that salt stress reduced leaf soluble proteins in all lentil lines, regardless of salt tolerance. It was already observed that salt reduced protein levels of leaves in glycophytes (Alamgir and Ali, 1999; Gadallah, 1999; Wang and Nil, 2000). When NaCl levels rise, Rhizobium's outer membrane proteins having molecular weights of 22, 38, 40, 42, 62, and 68 kDa significantly decrease (Unni and Rao, 2001, Jamil et al., 2012).

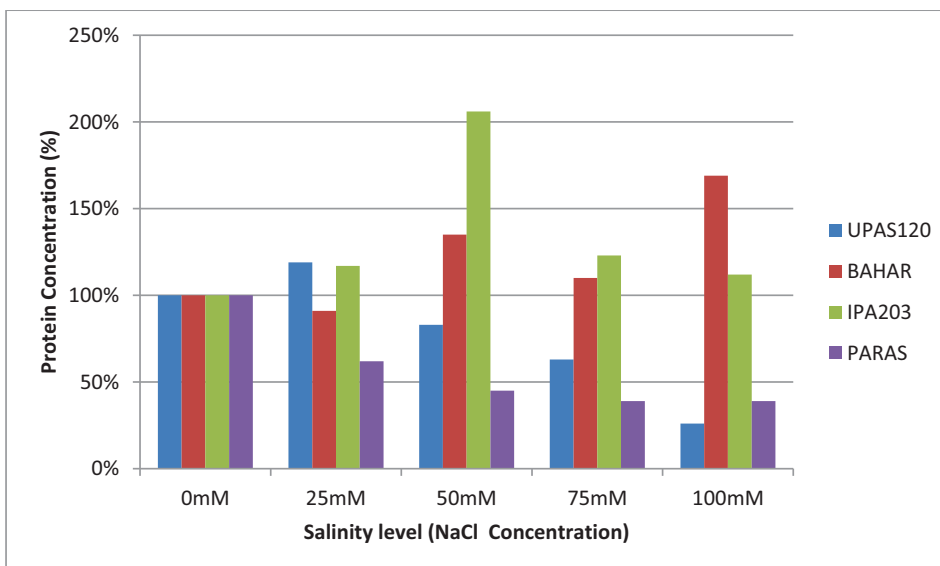


Fig-3 Effect of Salt stress on the Protein content in Pigeon pea Seedlings

TABLE-3
EFFECT OF SALT STRESS ON PROTEINS IN PIGEONPEA SEEDLING

Conc.	UPAS120	Bahar	IPA203	Paras
0mM	1.7±0.32 (100%)	1.12±0.06 (100%)	0.63±0.15 (100%)	1.16±0.16 (100%)
25mM	2.03±0.288 (119%)	1.02±0.06 (91%)	0.74±0.23 (117%)	0.72±0.28 (62%)
50mM	1.42±0.40 (83%)	1.5±0.35 (133%)	1.3±0.36 (206%)	0.53±0.19 (45%)
75mM	1.08±0.12 (63%)	1.2±0.49 (110%)	0.78±0.09 (123%)	0.45±0.05 (39%)
100mM	0.46±0.13 (26%)	1.9±0.03 (169%)	0.71±0.28 (112%)	0.45±0.14 (39%)

Pigeon pea seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. Results are mean± SE of 3 observations (P≤ 0.05).

4. CONCLUSION

It can be concluded from present study that salt stress produced by NaCl significantly influenced total amino acids. The impact of salt stress on specific total amino acids has significant role in the adaptation of the plant to salinity stress. Among them, proline was the dominant amino acid and showed relatively unique trends. Proline is a useful salt stress indicator, which can monitor the plant's health status directly. From this study, it is clear that there is high accumulation of proline. Proline is one of the active substances in capturing the OH- group, and it protects the plasma membrane from oxidation. Proline accumulation is due to disturbance in amino acid breakdown related to protein breakdown and aging resulting from salinity. It is also observed that increase in sodium chloride increases the protein concentration in tolerant varieties but there is reduction in sensitive varieties. This significant amount of variation among varieties might be because of expression of stress protein. From this study it is obvious that the underlying mechanisms for the salt tolerance in pigeon pea involve high accumulation of proline, free amino acids with proteins.

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