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Studies on the physiological and biochemical characteristics of *Salicornia brachiata:* influence of saline stress due to soaking wastewater of tannery

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ABSTRACT

Salicornia brachiata is an annual halophyte belonging to the *Chenopodiaceae* family and is distributed throughout the world. It is a green, jointed, vascular, flowering, and leafless halophytic plant that carries articulated, succulent stems. The main objectives of this study were to determine the effect of salt stress on growth characteristics, succulence, biochemical parameters, osmotic, and water relations of *S. brachiata.* The salt stress was induced by irrigating *S. brachiata* with soaking wastewater of tannery. The leather processing industry or the Tannery is known to be associated with the generation of liquid waste with high total dissolved solids (TDS), in particular, salinity. Consequently, the disposal of the tannery wastewater with high salinity has become a major concern for the tanning industry. This study evaluates the effect of tannery soaking wastewater on *S. brachiata*, which was grown in pots fed with soaking effluent at varying concentrations of TDS under laboratory conditions for a period of 90 days. Under these conditions, the harvested plants showed a significant decrease in chlorophyll, carbohydrate, lipid contents, fresh and dry weight with the increase in TDS of soaking effluent. However, it has been observed that accumulation of sodium, chloride, protein, and proline increased with an increase in TDS with particular reference to salinity due to osmotic stress. The optimal growth of *S. brachiata* plants has been observed at 12,500 ppm NaCl. Thus, the study paves a way to remediate the high TDS contaminated soil.

Keywords: Halophyte; Contaminated soil; Salt tolerance; Salt accumulation; Remediation

1. Introduction

Leather making involves operations like soaking, liming, deliming, pickling, tanning, post–tanning, and finishing processes [1]. Tanning process involves conversion of putrescible skins or hides to a non-putrescible material. Conventional methods employed in leather processing subject the skin or hide to wide

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variations in pH [2]. Such pH changes demand the use of acids and alkalis, which leads to the generation of salts resulting in the net increase in total solids, chlorides, and sulfates in tannery wastewater [3]. Soaking and pickling processes of leather manufacture contribute to high dissolved solids content in the effluent as it involves the use of sodium chloride salt along with sulfuric acid [4]. Dissolved solids contributed by salts are not amenable for treatment by the conventional effluent treatment methods [5].

The state of Tamil Nadu in India, which accounts for about 5% global leather production, has a stringent norm of 2,100 mg/L for total dissolved solids (TDS). There exist various tertiary treatment methods for the treatment of dissolved solids. They are electro oxidation, reverse osmosis, and thermal distillation. However, the techno-economic feasibility of these methods is yet to be established. The contamination of land due to the discharge of tannery effluent is presently a crucial problem faced by many neighborhood agricultural lands. Hence, there is a need for better strategic practices to remediate the land to decrease the TDS in the soil.

Saline soils are one of the major abiotic stresses that adversely affect the overall metabolic activities and cause plant demise [6]. Salinity is one of the key environmental factors that limit crop growth and agricultural productivity [7–8]. Several physiological pathways, such as, photosynthesis, respiration, nitrogen fixation, and carbohydrate metabolism, were observed to be affected by high salinity [9].

Salinity exerts its undesirable effects through osmotic inhibition, ionic toxicity and also by disturbing the uptake and translocation of nutritional ions [10]. Abscisic acid-mediated (ABA) signaling also plays a vital role in plant responses to environmental stress [11]. It is widely accepted that ABA–mediated root signals limit the availability of water to the plant cells, which leads to lower plant growth during salinity stress [12–14]. This effect can disturb the physiological and biochemical functions of the plant cells, leading finally to cell death [15]. Osmotic balance is certainly crucial for the survival of a plant under salinity stressed condition. Under various environmental stresses, plant cells have experienced the accumulation of some organic solutes such as sucrose [16], glycinebetaine [17], mannitol [18], trehalose [19–20], and proline [21–22] and these organic solutes contribute to the maintenance of turgor pressure. The accumulation of such compatible osmolytes involved in osmoregulation allows additional water to be taken up from the environment, thus buffering the immediate effect of water shortage within the plant [23]. It is well documented that proline plays a predominant role in protecting plants from osmotic stress [24].

In the present study, the physiological and biochemical changes in *Salicornia brachiata* under saline stress conditions are evaluated. The phytoremediation efficiency of *S. brachiata* is studied at laboratory scale.

2. Experimental

2.1. Collection of S. brachiata

S. brachiata, a halophyte, found in salt marsh Creek lands of Ennore, Chennai, Tamil Nadu, was collected and used for the study.

2.2. Analysis of soaking wastewater

Wastewater from commercial leather processing tannery was collected, filtered, and analyzed for NaCl as per the standard procedures [25]. The wastewater was suitably diluted to obtain 2,500, 5,000, 7,500, 10,000, 12,500, and 15,000 ppm of NaCl and used for the study.

2.3. Pot scale study

S. brachiata cuttings were initially grown in pots containing sandy soil and organic manure in the ratio of 1:1 (w/w). These cultivated mother plants were used for experiments. Cuttings were planted in individual plastic pots (11 cm in diameter) filled with sterilized and washed soil and placed in a glasshouse with minimum-maximum temperatures of 21–25°C with 40–60% humidity and natural daylight. Plants were irrigated with different concentration of soaking wastewater (2,500, 5,000, 7,500, 10,000, 12,500, and 15,000 ppm of NaCl) once in two days. The experiments were conducted for a period of 90 days and at every 30 days interval, the harvested plants were analyzed.

2.4. Determination of fresh and dry weight

The plants were allowed to grow for 90 days in different concentration of soaking effluent containing NaCl. The plants were harvested, washed in commercial water and were quickly and carefully blotted dry with tissue paper for determining the fresh and dry weight. The samples were oven dried at 60°C for 48 h.

2.5. Pigment estimation

Photosynthetic pigments such as Chl *a*, Chl *b*, and total chlorophyll of *S. brachiata* plant samples were extracted and estimated by the method of Witham [26]. About 1 g of fresh *S. brachiata* green tissues were

ground with 5 mL of 80% (1:5, w/v) acetone and kept overnight in dark environment at 4°C. The sample was centrifuged at 5,000 rpm for 10 min. The supernatant was collected, and its optical densities at 663 and 645 nm were measured using CARY 100 UV–visible spectrophotometer. Chlorophyll content was estimated and expressed as mg/g fresh weight of *S. brachiata*.

2.6. Evaluation of total carbohydrate content

About 500 mg of the fresh plant tissues were taken and ground in 10 mL of 0.1 M sodium phosphate buffer (pH 7) and then autoclaved at 120°C at 15 psi for 1 h. The solution was cooled and centrifuged at 5,000 rpm for 10 min, and the supernatant was estimated for total carbohydrate content. For 1 mL of extracted sample, 1 mL of 5% phenol and 5 mL of concentrated sulfuric acid (1:1:5, v/v/v) were added. After 10 min, the contents were mixed thoroughly and measured at 490 nm using CARY 100 UV–visible spectrophotometer. Total carbohydrate was calculated from the standard graph prepared from 10 to 100 μg/mL D-glucose. The values were expressed as mg/g fresh weight of plant [27].

2.7. Estimation of total lipid content

Lipid content present in the fresh plant sample was measured according to the method described by Ganai et al. [28]. About 1 g sample was taken and homogenized using mortar and pestle with 6 mL of chloroform: methanol in 2:1 ratio. It was then transferred to a separating funnel, and the organic phase was separated. To this sample, 2 mL of 0.88% (1:2, v/v) potassium chloride was added and mixed well. The mixture was left undisturbed overnight and the lower chloroform phase containing lipid was collected. From this mixture, 0.5 mL was collected in test tubes and the solvent was allowed to evaporate at room temperature, and the residue was collected. To the residue, 0.5 mL of concentrated sulfuric acid was added and mixed well. The samples were closed and kept in a boiling water bath for 10 min and allowed to cool at room temperature. The resulting sample was taken and 5 mL of phosphovanillin reagent (200 mg of vanillin was added to 80 mL of orthophosphoric acid and 20 mL of distilled water) was added, mixed well, allowed to stand for 30 min and the color developed was read at 520 nm. Standard graph was prepared using olive oil ranging from 10 to 100 μg/mL. The value was expressed as mg/g fresh weight.

2.8. Determination of total protein content

The fresh plants (1 g) were ground using mortar and pestle in 5 mL of 0.1 M phosphate buffer (pH 7). The extract was centrifuged at 10,000 rpm for 10 min and the supernatant was collected. The protein present in the extract was precipitated by adding an equal volume of 10% (v/v) ice- cold trichloroacetic acid. The resulting solution was centrifuged at 12,000 rpm for 10 min. The solid pellet was collected and re-dissolved in 2 mL of 1.0 N NaOH. Protein content in this re-dissolved clear sample was determined using Bradford's method [29] with bovine serum albumin as the standard. The values were expressed as mg/g fresh weight of *S. brachiata*.

2.9. Determination of Na^+ and Cl^- ions in S. brachiata

The Na^+ content of the finely ground (1 g) dry plant was assayed by microprocessor flame photometry (model 1381E, ESICO), after digesting the sample at 90°C with 0.5% HNO_3 (1:10, w/v) for 2 h. Chloride was determined using the same extract by a standard argentometric method [25].

2.10. Estimation of proline content

Proline was quantified by using ninhydrin reagent and measured according to Bates method [30]. Proline in the plant extract was determined by dissolving 0.5 g of *S. brachiata* in 10 mL of 3% sulfosalicylic acid. After addition of 2 mL acid-ninhydrin (1.25 g ninhydrin dissolved in 30 mL of glacial acetic acid and 20 mL of 6 M orthophosphoric acid) and 2 mL of glacial acetic acid, the resulting mixture was heated at 100°C for 75 min in water bath. The reaction was stopped by cooling it in an ice bath and 4 mL of toluene was added. The absorbance of toluene layer was spectrophotometrically determined at a wavelength of 520 nm. Standard graph was prepared using proline ranging from 10 to 100 μg/mL and calculated on a fresh weight basis (μmol proline/g fw). Each treatment was analyzed with at least three replicates, and the standard deviation (SD) was calculated; data are expressed in mean ± SD of three replicates.

3. Results and discussion

3.1. Effect of salt stress on growth in S. brachiata

The change in growth characteristics of *S. brachiata with* different concentration of soaking wastewater for about 90 days was studied. From Fig. 1(a), it is observed that the maximum fresh weight of 6.86 g

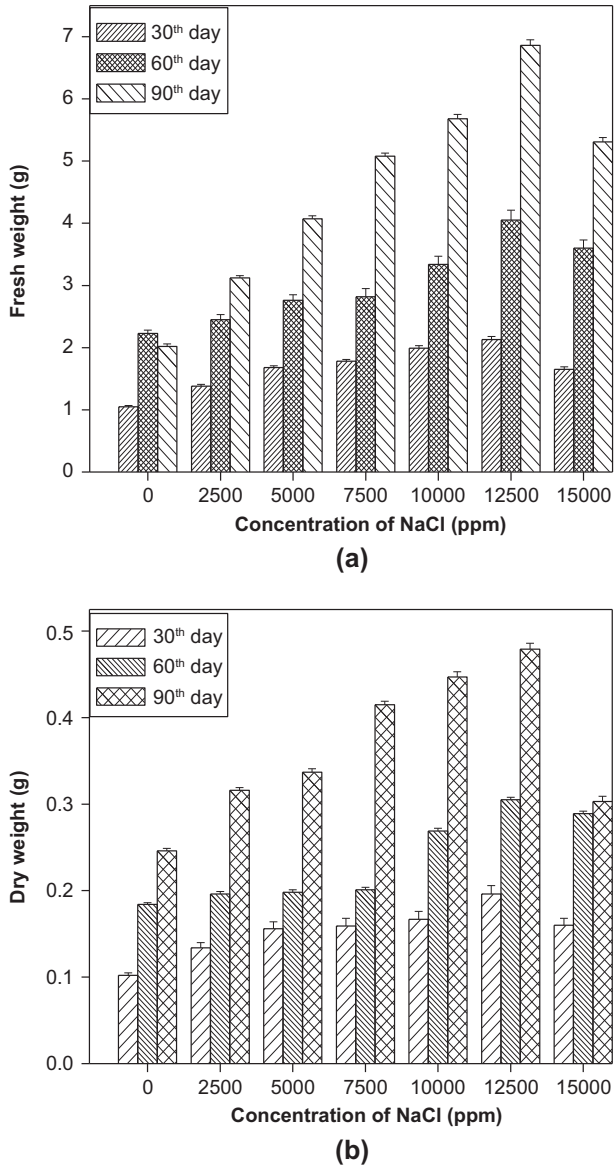

Fig. 1. Phytomass variation of *S. brachiata* grown in different Na^+ and Cl^- concentration of soaking effluent with respect to fresh weight (a) and dry weight (b).

recorded on 90th day at 12,500 ppm was more than 285% that of control. On 90th day, *S. brachiata* showed a maximum dry weight of 0.479 g at 12,500 ppm followed by 0.447 g at 10,000 ppm. The increment in dry weight on 90th day was more than 260% that of control is given in Fig. 1(a). As seen from Fig. 1(a) and (b), the fresh weight and dry weight of the plant decreased with an increase in the concentration of salt of all the experiments. This directly implies the absorption of sodium and chloride into the vacuole of the plant cells. The ability of the plant to adjust the ion balance osmotically is an important determinant for growth characteristics. Succulence is the consequence of salt accumulation in the vacuole, but does not directly contribute to salt transport [31]. The decrease in the plant growth with an increase in salinity stress can be attributed to the osmotic stress that results mainly through the restriction of solute uptake in shoot expansion. Transpiring energy could be spent by the plant in uptake of water as well as salt, which is accumulated in the vacuoles thereby reducing the energy available for the plant growth of *S. brachiata*.

3.2. Effect on chlorophyll contents under salinity stress

The effect of salinity stress on the photosynthetic pigments in *S. brachiata* is shown in Fig. 2(a) and (b). From Fig. 2(a), it is observed that the maximum concentration of Chl *a* of 0.1268 mg/g was recorded on 30th day in the control followed by 2,500 ppm (0.1192 mg/g) and 5000 ppm (0.1168 mg/g). A maximum concentration of Chl *b* and total chlorophyll was measured in control plants on the 30th day, which is shown in Fig. 2(b) and (c). A maximum concentration of 0.0968 mg/g Chl *b* was recorded in control followed by 0.0721 mg/g at 2,500 ppm and 0.0532 mg/g at 5,000 ppm. *S. brachiata* showed maximum concentrations of Chl *b* of 0.0486 mg/g in control followed by 0.0475 mg/g at 2,500 ppm and 0.0396 at 5,000 ppm on 90th day. The values of total chlorophyll measured at different concentrations of NaCl exhibited a similar trend to that of Chl *b* is shown in Fig 2(c). On 90th day, *S. brachiata* showed maximum total chlorophyll of 0.1089 mg/g in control followed by 0.1067 mg/g at 2,500 ppm and 0.1037 mg/g at 5,000 ppm. The concentrations of Chl *b* and total chlorophyll observed at 12,500 ppm on 90th day were less than 211 and 140%, respectively, of that of control. Decrease in chlorophylls level under salt stress may be due to reduction in pigment biosynthesis or enzymatic chlorophyll degradation [32,33]. Chlorophyll content is considered as one of the parameters of salt tolerance in crop plants [34]. The concentrations of Chl *a* and Chl *b* of the *S. brachiata* were maximum in control followed by 2,500 and 5,000 ppm. The concentration of Chl *a* and Chl *b* was greatly decreased at 7,500, 10,000, 12,500, and 15,000 ppm soaking effluent. The concentrations of total chlorophyll also showed similar a trend to that of Chl *a* and Chl *b*. The increment level of soaking effluent concentration may influence the reduction in photosynthetic pigments.

3.3. Influence of salinity stress in carbohydrate content

The sugar level of control and experimental plants of *S. brachiata* were shown in Fig. 3. From Fig. 3, it is seen that there was a decrease in the carbohydrate

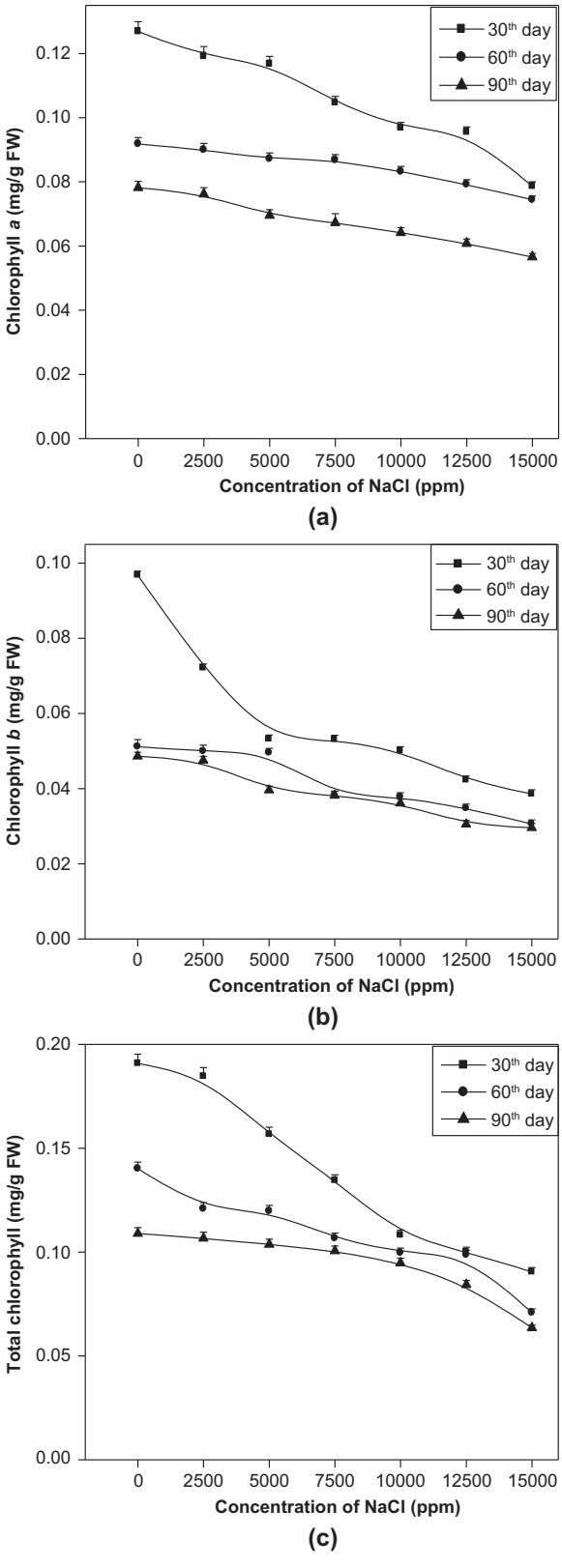

Fig. 2. Changes in pigments in *S. brachiata* under Na^+ and Cl^- stress of soaking effluent–Chlorophyll a (a), Chlorophyll b (b) and Total Chlorophyll (c).

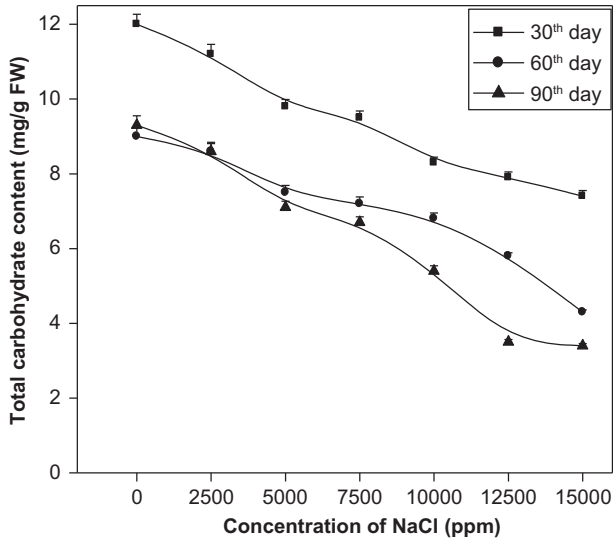

Fig. 3. Total carbohydrate content of *S. brachiata* at different concentrations of Na^+ and Cl^-.

content. The maximum total carbohydrate content of 12.0 mg/g was observed on 30th day in control followed by 11.2 mg/g at 2,500 ppm and 9.8 mg/g at 5,000 ppm. On 90th day, the amount of total carbohydrate was observed at 15,000 ppm NaCl was lower than the control values. A decrease in the level of total carbohydrate content of *S. brachiata* under salinity condition may be due to the synthesis of proline and glycinebetaine via amino acid metabolism. The precursors of betaine and proline synthesis based on the availability of amino acids which in turn depends upon the utilization of total sugars.

3.4. Variation of lipid content

The level of total lipid content was decreased in *S. brachiata* due to the different NaCl concentrations, but without any noticeable changes in control is shown in Fig. 4. On 30th day, a maximum lipid content of 6.8 mg/g was observed in control followed by 4.9 mg/g at 2,500 ppm and 4.6 mg/g at 5,000 ppm. The plants grown at 15,000 ppm NaCl contained 0.098 mg/g total lipid, which was less than 160% when compared to control.

3.5. Protein content under Na^+ and Cl^- stress

On the 90th day, the total protein of the sample grown at 15,000 ppm was 4.89 mg/g, which is more than 140% of the control sample, as shown in Fig. 5. Similarly, plants grown at 12,500 ppm contained 4.77 mg/g total protein on the 90th day, which was

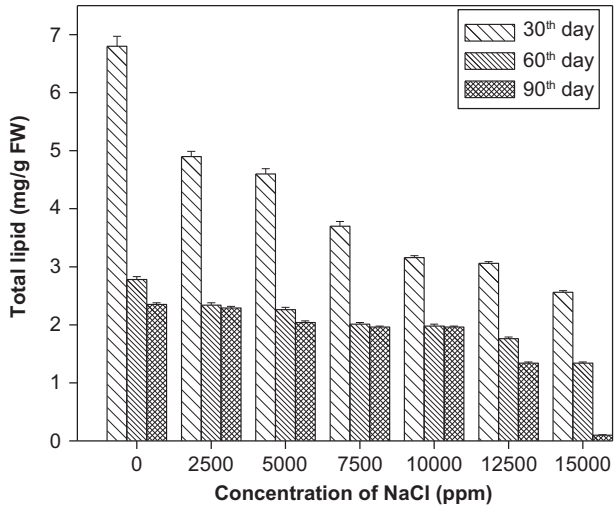

Fig. 4. Effect of lipid level in *S. brachiata* on Na^+ and Cl^- stress of effluent.

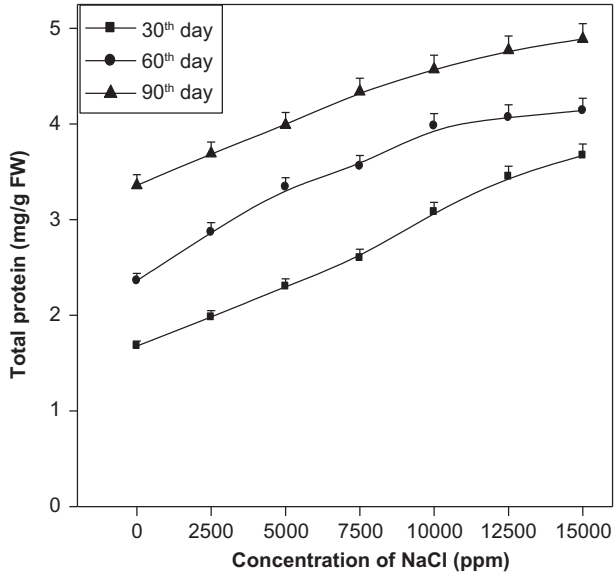

Fig. 5. Effect of Na^+ and Cl^- of soaking effluent on the protein contents in *S. brachiata.*

130% that of control. The level of protein accumulation under salinity stress was high, especially at 15,000 mM NaCl. Therefore, it is attributed that exposure of *S. brachiata* to high salinity over a period of time could induce protein accumulation. In the study, the protein content of the plants increased gradually in response to NaCl stress. Salinity promotes the fixation of inorganic nitrogen into protein, thus favoring protein synthesis. Salinity stress caused a significant increase in protein content in salt stressed plants. Protein accumulation is particularly important for cell survival under salt stress and causes membranes stabilization under NaCl stress. In response to salinity, plants produce new proteins that help them to grow and develop under saline condition. One may speculate that salt tolerant cultivars producing higher protein concentration are due to higher efficiency of an osmotic regulation mechanism in the plants which in turn causes decreasing sodium toxicity in the cytoplasm compared to susceptible ones [35].

3.6. Salinity effects on Na^+ and Cl^- contents

The accumulation of Na^+ and Cl^- was increased when the plants were irrigated with increasing concentrations of NaCl in the soaking wastewater. In general, the accumulation of Na^+ was higher than that of Cl^- in the plant that is presented in Table 1. The plants treated with 15,000 ppm NaCl of soaking wastewater had shown high amounts of Na^+ and Cl^-, which were more than five and three folds, respectively, when compared to control. However, the accumulation of Na^+ and Cl^- was only 6 and 4%, respectively, at 2,500 ppm NaCl in soaking wastewater offered. This level of high accumulation is required to maintain normal turgor dependent growth, since it leads to higher tissue NaCl concentration than it would be necessary for an osmotic adaptation to the growth medium low in salt. The contribution of Na^+ to total osmotic adjustment in *S. brachiata* was indirect through stimulation of soluble sugars and other osmolyte biosynthesis. The succulent halophytes are known to accumulate extremely high level of chloride. Halophytes utilize at least one of the three mechanisms to prevent Na^+ accumulation in the cytoplasm, reducing Na^+ entry into the cell, active Na^+ efflux from the cell, and active sequestration of Na^+ in the vacuole [36].

3.7. Proline accumulation in the development of salt tolerance

Accumulation of proline content was increased during the growth of *S. brachiata* as seen from Fig. 6. The plants grown at 15,000 ppm NaCl showed a maximum of 4.95, 5.98, and 6.29 mg/g of proline content on 30th, 60th, and 90th day, respectively, as observed from Fig. 6. On 30th day, the plant grown at 12,500 ppm had 4.65 mg/g of proline, which was more than 310% to that of control. On 90th day, the accumulation of proline content at 15,000 ppm was more than twofolds when compared to control. Proline accumulation in many plants was correlated with stress tolerance, and the concentrations were generally higher in stress-tolerant plants. One of the most

Table 1
Accumulation of Na^+ and Cl^- by *S. brachiata* grown in soaking wastewater

Concentration of NaCl (ppm)	Na^+ offered to plant (mg)	Concentration of Na^+ in plants (mg/g dry weight)		Cl^- offered to plant (mg)	Concentration of Na^+ in plants (mg/g dry weight)	
		Initial	Final		Initial	Final
0	137	28 ± 0.6	44 ± 0.9	144	19 ± 1.8	34 ± 0.6
2,500	1,477	31 ± 0.7	118 ± 1.1	2,273	21 ± 1.9	111 ± 0.9
5,000	2,955	30 ± 0.4	137 ± 1.3	4,545	18 ± 1.5	123 ± 1.9
7,500	4,432	27 ± 0.3	153 ± 1.6	6,818	22 ± 1.3	136 ± 1.7
10,000	5,910	32 ± 0.5	178 ± 2.2	9,090	17 ± 1.2	154 ± 2.3
12,500	7,387	31 ± 0.6	197 ± 4.2	11,363	20 ± 1.7	176 ± 3.8
15,000	8,865	36 ± 0.1	215 ± 4.7	13,635	20 ± 1.5	193 ± 4.9

Note: Values are means of three replicates ± SD.

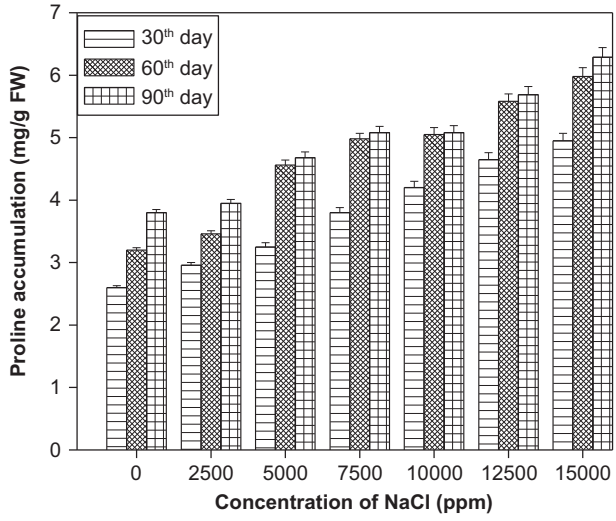

Fig. 6. Influence of Na^+ and Cl^- of soaking effluent on proline accumulation in *S. brachiata.*

important mechanisms in higher plants under salt stress is the accumulation of compatible solutes such as proline. Proline accumulation in salt stressed plants is due to the primary defense response to maintain the osmotic pressure in cells of cytosol with that vacuole and external environment.

4. Conclusions

The present study concluded that salinity stress greatly influences the growth characteristics of *S. brachiata*. The results of this study investigate that soaking effluent affects some physiological and biochemical parameters in the *S. brachiata*. Under salinity stress, the plant shows the reduction in total chlorophyll pigments about 0.0635 mg/g. Carbohydrate and lipid content in the plant reduced to 3.4 and 0.098 mg/g, respectively, due to salinity stress. The plants synthesized proteins to adapt to the saline conditions, which resulted in an increase in the protein content during the growth period. *S. brachiata* accumulates about 6% of Na^+ and 4% of Cl^-, it may help to reduce the TDS in the contaminated soils. It accumulates substantial amounts of Na^+ and Cl^- to achieve osmotic balance across the soil-water-plant gradient. Hence, the presence of proline is a marker to evaluate the osmotic stress and growth of *S. brachiata*. The study ascertains the importance of biochemical parameters as a direct measure of salinity stress. The study enabled the understanding of mechanism of saline and osmotic stress under high TDS physiological conditions. The present studies lead to the use of S. *brachiata* in phytoremediating the high saline contaminated soil.

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