Effect of Exogenous Proline Application on Salinity Tolerance of *Cordia myxa* L. Seedlings. Effect on Vegetative and Physiological Characteristics.

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Abstract:
The study was conducted in the growing season of 2010 at a private orchard at Bab El Zubair area, Basra, Iraq, during the period from March, 1st, 2010 to December, 1st, 2010, to study the role of the amino acid (Proline) in salt tolerance of Lasura (*Cordia myxa* L.) seedlings (cv.Local). The experiment was designed according to Randomized Completely Block Design (R.C.B.D.) in a three factorial experiment of 27 treatments resulted from the interaction of : Three levels for the first factor (the concentrations of NaCl (0, 6 and 12 Ds. m^{-1}), three levels for the second factor (the concentrations of the amino acid proline (0, 75 and 150 mg.L^{-1}) and three replicates for each experimental unit. The results showed a significant decrease in most studied vegetative characteristics with increasing the salt concentrations, but an opposite impact occurred with increasing the proline concentrations. For physiological characteristics, the proline treatment (150 mg. L^{-1}) was of a significant superiority and possessed the highest content of chlorophyll, free proline quantity and acidity percentage. While for the interaction among the levels of NaCl and proline, the interaction treatment (12 Ds. m^{-1} NaCl + 150 mg. L^{-1} proline) showed the highest increment in the main stem height and diameter, length and diameter of secondary branch, average of leaf area, total chlorophyll and true proline.
Keywords: *Cordia myxa*, proline, salinity tolerance.

1. Introduction:

Lasura plant (Cordia myxa L.) is one of the sub-tropical plants grown in middle and south of Iraq regions. It belongs to the genus *Cordia* and the family *Ehretiaceae*. Its fruits are of high nutritive and medical value (Askar, 1994). The optimum salt concentration for typical growth of any agricultural crop is about 1500 mg. L^{-1} (or 2.5 Ds. m^{-1} NaCl), and if the plants been exposed to salt concentration higher than this, their growth and physiological functions will be affected and differ than before, where they called as salt stressed (Taiz and Zeiger, 2002). The salinity causes a deficiency in chlorophyll content in leaves by becoming smaller in size and area and less in number (Abdul Hameed, 2002). The literatures mentioned the proline as one of the means using to promote the efficiency of salt tolerance in plants to decrease the effects of salt on it (Al Saadi, 2001). The salinity has another role besides to decreasing the rate of synthesis of chlorophyll pigment, where it causes a deficiency in the necessary elements needed in the synthesis of this pigment such as Mg, Fe, and N, and it causes a decrease in carbohydrate content, and an increase in producing the Plant growth retardant hormones, such as Abssesic Acid (ABA), which accelerate the analysis of chlorophyll content (Gratten and Grieve, 1999). Later, Tawagen *et al*. (2003) confirmed that the chlorophyll content in tomato leaves irrigated with water of 8 and 12 Ds. m^{-1} salinity levels is less than those irrigated with (4 Ds. m^{-1}) level, where this case was attributed to the decrease in the number of chloroplasts. Also, the salinity decreases the swelling, flexibility and elongation of cells and the speed of photosynthesis (Al Zubaidy, 2000). Later Mohammad *et al*. (2006) found, when studied the effect of irrigation water salinity on the seedling of bitter orange, that the salinity caused a significant decrease in leaf area and plant height at the higher salinity levels. Besides, Ibrahim (2002) had mentioned that the irrigation water salinity inhibited the seedling growth by decreasing the average of plant height, foliage branches number, leaf area, leaves number, proline content and total chlorophyll concentration.

Besides to its uses in the field, proline had been tested *in vitro* along with salt stress (Al Kabby and Abdul Qader, 2007; Abbas *et al*., 2014 … and others).

The importance of amino acids been considered as a biological animator absorbed and transported rapidly inside different parts of the plant, because of its direct effects on the enzyme action of the plant.

Besides, the amino acids participate in the synthesis of nucleotides, vitamins and plant hormones, whereupon they are essential components for the living material and protoplasm; also, they take part in the synthesis of enzymes, then they participate in the enzyme reactions in the cells, and the synthesis of cellular membrane; besides, the amino acids achieve the harmonic balance which helps to stimulate the buds, the amino acids participate in the synthesis of proteins, because the amino acids are the structural units of proteins, which accomplish several functions in the plants, for example: Behave as regulators of metabolic processes, transporting and storing the nitrogen (Fowden, 1973; Bidwell, 1979).

The experiments showed that all treatments with the amino acids increased the quality of nitrogen and proteins, where the proline was considered to be one of the most amino acids that help the plant to balance the osmosis between the cell and its environment, this will enhance the plant salt stress tolerance, where the plant (under the conditions of salt stress) use some means and techniques, which one of them is the synthesis and accumulation of some compounds including proline (Popp, 1990). Also, Zeki (2000) showed that the slat tolerance in cells was related to the availability and high content of proline inside them, where the salt tolerant cells were recognized by their ability to maintain their demanded biological processes stable, in order to keep on growth despite the high salinity levels, where these cells had high proline synthesis (Pushman and Rangosamy, 2000).

While Abdul Hafid (2006) had indicated that the proline helps the plant to resist the stress conditions like salinity, high and low temperatures, water stress and bad aeration of soil. Also, Al Najjar *et al*. (2011) confirmed that the treating of date palm seedlings with proline caused an increment in leaf width, total leaf area and leaf content of nitrogen.

At last this study was conducted because of the rareness of studies on the effect of different levels of salinity, that this plant (Lasura) able to tolerate, and the extent of elevate its efficiency of salt tolerance, by treating it with the amino acid, proline. This study included the study of the effect of different levels of salinity and proline on some vegetative and physiological characteristics of Lasura (*Cordia myxa* L.) plant seedlings (cv. Local).

2. Materials and Methods:

The present study had conducted during the growing season 2012 in the period between March, 1st to December, 1st, 2012 at Bab El Zubair region, Basra, where 27 seedlings (2 years old) of Lasura plant (*Cordia myxa* L.) were used in the experiment, and been planted in loamysand soil in (10 Kgs capacity) pots. The experiment included 27 factorial treatments (3 × 3 × 3) resulted from the interaction among: three levels for the first factor (0, 6, and 12 Ds. m^{-1}NaCl), three levels for the second factor (0, 75, and 150 mg. m^{-1} Proline) and three replicates for each experimental unit. The method of application the NaCl was by applying to soil, and by spraying the foliage to apply the proline, and of course the Teen 20 were used in spraying as a spreading material. The application processes done in two dates (March, 10th and April, 10th).

2.1. Characteristics under Study:

2.1.1. Main Stem Height (cm):

Measured from the point of contact between stem and soil surface upward to the top of growing tip before starting the experiment, then during the experiment the original length of seedlings were neglected and the only new increment in the height after three months from starting the spraying were listed, this procedure done the same for the main stem diameter and the length and diameter of lateral branches.

2.1.2. Main Stem Diameter (cm):

A vernia caliper was used to measure the diameter.

2.1.3. Number, Length and Diameter of branches (the main lateral branches and their secondary branches):

Measured for each main lateral branch and its secondary branches after three months from starting of spraying.

2.1.4. Leaves Number:

After three months of the spraying, the increment in the number of completely grown leaves on the main stem only, was listed.

2.1.5. Leaf Area (cm^2):

Measured in terms of weight, where the third leaf was drawn on a transparent paper, then the drawn shapes were cut, weighted and compared with a weight of a (1 cm^2) leaf area.

2.1.6. Water Content and Dry Material (%):

In order to estimate these two characteristics, 30 grams only of the plant tissue (Leaves) were weighted after three months from starting the treatments with NaCl and Proline, thereafter, each sample were dried inside an oven at 70 °C for 48 hrs. till the stability of weight. The values of water content and dry materials (%) found from the following equations:

$$\text{Water Contents (\%)} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100$$

$$\text{Dry material (\%)} = \frac{\text{Dry weight}}{\text{Fresh weight}} \times 100$$

2.1.7. Adjustable Total Acidity (%):

Estimated according to Howrtiz (1975) method.

2.1.8. Calcium Pectate in Leaves (%):
Estimated according to Rouhani and Bassiri (1976) method.

2.1.9. Free Proline (µg.g^{-1} dry material):
Measured according to Troll and Lindsley (1955) method.

2.1.10. Total Chlorophyll in leaves (mg.g^{-1} fresh weight):
Estimated according to Howrtiz (1975) method.

2.1.11. Experiment Designation and Analysis:
The data of the experiment were designed according to the Randomized Completely Block Design (R.C.B.D.) in a factorial experiment with two factors, i.e., three levels of NaCl salinity (first factor) and three levels of Proline (second factor), and each experimental unit (treatment) was of three replicates, this resulted in 27 factorial treatment (3 × 3 × 3). The results of the experiment were analyzed using the statistical program (SPSS). And to compare among the averages of treatments, the Revised Least Significant Difference (Revised L.S.D.) were used at a probability level of (0.05) according to Al Rawi and Khalaf Allah (2000).

3. Results and Discussion:
3.1. Effect of Application of The NaCl, Proline and their interaction on some vegetative Characteristics of (*Cordia myxa* L.) Seedlings:
Data in table (1) show that the increase in NaCl level applied to soil from (0 Ds. m^{-1}) to (12 Ds. m^{-1}) caused a significant decrease in most vegetative characteristics studied. Main stem height of seedling, length of secondary branch, leaf number and leaf area, while no significant difference occurred in the main stem diameter and secondary branch diameter. Also, the control plants possessed the highest averages, in the above characteristics, compared with other treatments (16.66, 6.96, 12, 4 and 46.25) respectively. While NaCl treatment (12 Ds. m^{-1}) caused the lowest decrease in the main stem height, secondary branch length, leaves number, secondary branches number and leaf area of (12.07, 5.61, 9.1 and 17.25) respectively.
The decrease in growth, generally might be attributed to the direct effects of ions during photosynthesis, which resulted in decreasing the plant growth, or that the exposing plants to high levels of salinity caused a decrease in the absorption of Potassium, Phosphorus, Calcium, and Magnesium (Gorhon, 1992). These results are in accordance with those of Orcutt and Nilsen (2000); Abdel Hameed (2002); Ibraheem (2002); Mohammad *et al.* (2006) and Mohammad (2007).
These results might be due to the role of salinity in the inhibition of cell division and elongation (Levitt, 1980), besides to the physiological role of salinity (NaCl) in decreasing the swelling and elasticity of cells and also the role in decreasing the speed of photosynthesis (Al Zubaidi, 2000). Or that the salinity had bad effects on the physiological processes of the plants, which caused a decrease in growth and development of plant and that these bad effects, were induced by inhibiting the influx of water and nutrients into the plants through the accumulation of poisonous ions inside the plant (Tester and Davenport, 2003). For the proline alone effect on the vegetable characteristics, the table (1) also showed a semi-gradual increase in most studied characteristics compared with the control plants, where a significant effect of the highest proline concentration (150 mg. g^{-1}) occurred on most: stem diameter, secondary branch length, secondary branch diameter, leaves number and leaf area of (20.8, 1.97, 7.08, 1.32, 14 and 84.37) respectively. These results might be due to that the spraying of proline caused an increase in the capability of seedlings to get rid of these bad effects of salinity; these results are in accordance with (El Hammady *et al.*, 1999; Al Saadi, 2001 and Mohammad, 2007). Or the results may be attributed to the increase of proline content inside the plant tissues, leading to increase the plant resistance against the salt stress, the encourage the roots to absorb the nutrients and increasing their contents inside the leaves, this will promote the photosynthesis and reflect positively to increase the vegetative growth of the plant (Al Najjar *et al.*, 2011). Where the exposing of plant to salt stress increase the content of proline in branches, this accumulation of proline was considered to be one of the most dominant and repeated interaction against salt stress in plants, even more than other amino acids, the quantity of proline is available from tissue to another along with the amount and time of exposing to salinity stress (Ashraf and Foolad, 2007). Also must be noted that the existence of proline as a component of cell wall has only a protective role but not an osmotic regulator (Nanjo *et al.*, 1999), while Saqr (2008) gave a reason for all this, that the role of proline was to preserve the stability of salt stress ratio by helping the cells to preserve their selective ability during the absorption of nutrients from the environment, so this what had noticed in this present study at the concentration of proline (75 – 150 mg. L^{-1}).
For the effect of the interaction between NaCl and proline on vegetative characteristics, data from table (1) show a significant effect in all studied characteristics in exception of main stem diameter and secondary branch

diameter where they were statistically not significant, the increase in main stem height was (21.39 cm) at the interaction treatment (6 Ds. m^{-1} NaCl + 75 gm. g^{-1} proline), while the secondary branch length under (0 Ds. m^{-1} NaCl + 150 gm. g^{-1} proline) was of (9.67 cm). The leaves number had the highest value (16 leaves) under (0 Ds. m^{-1} Ds. m^{-1} NaCl + 150 gm. g^{-1} proline). This value was non-significant with that under (6 Ds. m-1 +150 gm. g-1 proline). This result was in accordance with El– Hammady *et al.* (1999), who confirmed that the interaction between salinity and proline was of significant effect on the main stem height and diameter, secondary branch length and diameter, and the leaf area, but of non-significant effect on leaves number and secondary branches number, where it was observed that the treatment with proline caused an increment in the ability of seedling to eliminate and reduce the bad effects of salinity, this result was in accordance with (Al Saeedi, 2001), who attributed the increase in salt tolerance of plants to the treatment with proline. For the effect of interaction alone on the number of secondary branches, the data in table (1) show that the treatment (0 Ds. m^{-1} Ds. m^{-1} NaCl + 75 gm. g^{-1} proline) gave a rise to (5) branches, where this result was non-significant with that under (0 Ds. m^{-1} Ds. m^{-1} NaCl + 150 gm. g^{-1} proline) of (5) branches too. While for leaf area the (0 Ds. m^{-1} Ds. m^{-1} NaCl + 150 gm. g^{-1} proline) treatment was of a significant superiority (of 73.67 cm^2) over all characteristics, but of non-significant value comparing with the treatment (6 Ds. m^{-1} Ds. m^{-1} NaCl + 150 gm. g^{-1} proline) of (71.01 cm^2).

This salt tolerance of plants might be attributed to the treatment with proline, through that the tissue tolerate the salinity when ions accumulate inside it, then distributed into compartments of vacuoles of the plant cells, so the plants control the salt concentration inside the cytoplasm of vacuole and keep the Na^+/K^+ ratio high in the cytoplasm of the cells (Chinnusamy *et al.*, 2006), in turn this will protect the cytoplasm against the poisonous ions and prevent the accumulation of salts inside cell wall, respectively this will generate a transportation of salt ions from cytoplasm toward the inside of vacuole, reflecting in high osmotic potential through its membrane (Volkamar *et al.*, 1998), this osmotic graduation will neutralize at the high ion concentration of mineral ions or organic Solutes of low molecular weight, which are not harmful to cellular metabolism (Blumwald *et al.*, 2000).

Table 1. Effect of application of NaCl, proline and their interaction on some vegetative characteristics of *Cordia myxa* L. seedlings after three months of starting the treatment.

NaCl (Ds. m^{-1})		Main stem height (cm)	Main stem diameter(cm)	Secondary branches length (cm)	Secondary branches diameter(cm)	Secondary branches Number	Leaves Number	Leaf area (cm^2)
0		16.66	0.96	6.96	0.96	4	12	46.25
6		15.26	0.83	5.67	0.97	2	10	30.13
12		12.07	0.76	5.61	0.91	1	9	17.25
R.L.S.D. (0.05)		**2.03**	**N.S.**	**0.17**	**N.S.**	**1.06**	**1.37**	**2.36**
Proline (mg. L^{-1})								
0		16.66	0.96	6.96	0.96	4	12	46.25
75		19.33	1.59	7.99	1.05	5	13	63.33
150		20.08	1.97	8.08	1.32	5	14	84.37
R.L.S.D. (0.05)		**1.27**	**0.12**	**0.22**	**0.12**	**N.S.**	**1.03**	**6.37**
Interaction								
NaCl	**Proline**							
0	0	16.66	0.96	6.96	0.96	4	12	46.25
	75	10.07	0.76	9.61	0.91	5	14	67.25
	150	11.08	0.71	9.67	0.83	5	16	73.67
6	0	14.33	0.99	6.69	1.05	2	11	43.33
	75	21.39	1.12	7.09	1.19	3	13	64.37
	150	20.51	1.21	7.13	1.33	3	16	71.01
12	0	10.27	1.08	5.11	1.36	1	8	31.29
	75	18.33	1.13	6.29	1.47	2	10	36.37
	150	19.08	1.27	6.67	1.72	3	13	40.85
R.L.S.D. (0.05)		**2.66**	**N.S.**	**0.35**	**N.S.**	**0.67**	**1.67**	**9.07**

3.2. Effect of Application of The NaCl, Proline and their interaction on some Physiological Characteristics of (*Cordia myxa* L.) Seedlings:

Data in table (2) show that the application of NaCl (0, 6 and 12 Ds. m^{-1}) resulted in a significant increment of some physiological characteristics such as Calcium pectate percentage, free proline content and dry matter percentage with increasing the salt concentrations to (12 Ds. m^{-1}), the results were (3.83 %, 83.01 ($mg.g^{-1}$) and 49.17%)respectively. But in contrary for total chlorophyll quantity, it was decreased (10.89 $mg.g^{-1}$ fresh weight) under highest salinity level (12 Ds. m^{-1}) in comparing with other treatments. The control plants distinguished by the lowest averages of Calcium pectate percentage , free proline content and dry matter percentage which were (3.16 %, 701.06mg. g^{-1} and 32.94 %) respectively, while the control total chlorophyll possessed the highest quantity of (17.03 mg. g^{-1} fresh weight). These results as explained by Fernando (2009), might be attributed to the effect of high salt levels on the total chlorophyll pigment, as seen under electron microscope when studying chloroplast, where their thylakoids were swollen, along with shortage in grana number, besides to the accumulation of H_2O_2 inside the cell. While Al Saaidi (2005) showed that the NaCl in its high concentrations affected the activity of enzyme included in the synthesis of pigment inside the plant, as well as decreasing the absorption of mineral elements needed in the synthesis of chlorophyll such as Fe and Mn. In the other hand Ziska *et al*. (1990) confirmed the accumulation of chloride ions in the leaves, which affecting the content of total chlorophyll in pear plants. This result might be due to the role of salinity in inhibiting the cell division and elongation (Levitt, 1980; Demiral, 2005), besides to the physiological role of salinity in decreasing the cell swelling and elasticity and its role in slowing down the photosynthesis (Al Zubaidi, 2002; Chartzoulakis *et al*., 2002). Or the salinity may had harmful effects on the physiological processes inside the plants leading to decrease the growth and development of plant (Tester and Davenport, 2003), these harmful effects were induced by interrupting the water and nutrients influx into the plants, by accumulating the poisonous ions inside the plant (Blumwald *et al*., 2000). As well, the statistical analysis results of this study showed no significant difference among the salinity levels for the total acidity percentage and water content percentage.

For the effect of proline on physiological characteristics, it was observed from data in table (2) that there were significant differences among the proline treatments applied (0, 75 and 150 mg. L^{-1}), where fluctuation in total acidity percentage had occurred with increasing the proline concentration. It was seen that the proline treatment (75mg. L^{-1}) had possessed the least average of acidity percentage (0.71 %) which was non – significant with (150 mg. L^{-1}) of (0.78%), while the highest percentage (0.94 %) found in control plants (0 mg. L^{-1}) concerning the total acidity percentage. The free proline content in shoot of (*C. myxa* L.) seedlings were affected by spraying of exogenous proline, the later resulted in an increment in free proline content of shoot of seedlings with increasing the concentrations of exogenous proline, where the treatment (150 mg. g^{-1}) of (903.08 µg. g^{-1}) had a superiority over the rest of treatment comparing with least value in control plants (701.06 µg. g^{-1}). For the dry matter percentage of (*C. myxa* L.) seedlings, the data in table show a semi-gradual decrease in dry matter with increasing the exogenous proline concentrations, where the treatment (75 mg. L^{-1} proline) was significantly superior over the rest of treatments of proline, where it possessed the least value of dry matter percentage (28.95%), which non- significantly differed with the treatment (150 mg. L^{-1}) of (29.68%), while the control plants had the highest value of dry matter (32.94 %). For the total chlorophyll content in (*C. myxa* L.) seedlings, as seen in table (2), there were significant differences among the treatments, where the (150 mg. L^{-1} proline) was significantly superior (18.61 mg. g^{-1} fresh weight), over the rest of treatments, while the control plants possessed the lowest content of total chlorophyll (17.03 mg. g^{-1}). This result was in accordance with Gadallah (2004), who mentioned that the accumulation of proline had positive results on plant by its role in increasing the plant efficiency to absorbe the K^+, and increasing the chlorophyll content. The data in table (2) also showed that statistically there were non-significant differences concerning the impact of proline concentrations (0, 75 and 150 mg. L^{-1}) on calcium pectate content and water content percentage in Lasura (*C. myxa* L.) seedlings.

Concerning the effect of interaction among NaCl levels and proline concentrations on the physiological characteristics, the data in table (2) showed significant effects on some characteristics. Where the interaction treatment (0 Ds. m^{-1} NaCl + 150 mg. L^{-1} proline) was of a significant superiority for free proline content (756.07 µg. g^{-1}) and water content (70.03%), this result might be due to the role of proline in maintaining the water content of cells, which resulted in a case of swelling (Ronchi *et al*., 1985), also the above treatment was a significantly superiority concerning the total chlorophyll content (18.01 mg. g^{-1} fresh weight), where this amount of chlorophyll was resulted from the accumulation of proline, which reflected in positive results in plants by the role of proline in increasing the plant efficiency to absorb the K^+ and increase the chlorophyll content (Gadallah, 2004), or the proline was more dominant and accumulated in leaves, so it had a big role to defend the plants against the harm of salt stress, reflected on the chloroplasts found in leaves, where they were not affected under low concentrations of NaCl (Carlos, 1999), while the effect was variant for the other treatments.

For the effect of the interaction between NaCl and proline on total acidity percentage, Calcium pectate percentage and dry matter percentage, the statistical analysis showed non- significant differences among their averages.

Table 2. Effect of NaCl, proline and their interaction on some physiological characteristics of *Cordia myxa* L. seedlings after three months of starting the treatment.

NaCl ($Ds.\ m^{-1}$)		Total acidity (%)	Calcium Pectate (%)	Free proline ($\mu g.\ g^{-1}$)	Water content (%)	Dry matter (%)	Total chlorophyll ($mg.\ g^{-1}$ fresh weight)
0		0.94	3.16	701.06	67.06	32.94	17.3
6		0.87	3.76	716.67	53.97	46.03	12.97
12		0.91	3.83	813.01	50.83	49.17	10.89
R.L.S.D. (0.05)		**N.S.**	**0.16**	**11.17**	**N.S.**	**1.37**	**3.97**
Proline ($mg.\ L^{-1}$)							
0		0.94	3.16	701.06	67.06	32.94	17.03
75		0.71	3.01	857.99	71.05	28.95	18.11
150		0.78	2.57	903.08	70.32	29.68	18.61
R.L.S.D. (0.05)		**0.06**	**N.S.**	**21.02**	**N.S.**	**1.03**	**0.11**
Interaction							
NaCl	**Proline**						
	0	0.94	3.16	701.06	67.06	32.94	17.03
0	75	0.77	3.07	721.61	67.91	32.09	17.13
	150	0.68	3.13	756.07	70.03	29.97	18.01
	0	0.90	3.29	631.76	57.76	42.24	16.21
6	75	0.69	3.37	696.09	59.07	40.93	16.97
	150	0.71	3.32	671.15	61.01	38.99	17.07
	0	0.97	3.67	626.08	53.42	46.58	15.17
12	75	0.76	3.28	617.28	57.47	42.53	16.77
	150	0.71	3.03	607.67	61.62	38.38	17.01
R.L.S.D. (0.05)		**N.S.**	**N.S.**	**15.35**	**3.62**	**N.S.**	**1.02**

4. Conclusions and Recommendations:

From this study it was concluded that the Lasura (*Cordia myxa* L.) seedlings can tolerate salinity to limits up to ($8\ Ds.\ m^{-1}$) after treating the plants with (75 and 150 $mg.\ L^{-1}$ proline), while their growth and production might be weakened in case of increasing the salinity to ($12\ Ds.\ m^{-1}$) or more. That's to say, the application of amino acid proline (by spraying the foliage) onto the Lasura seedlings, considered to be as one of the important scientific means which participate in increasing the efficiency of the plants to tolerate the salinity and decreasing the harms to improve the growth processes in seedlings making the later more tolerate to the higher levels of salinity. So and depending upon the results of this study the researchers recommend to use the proline as one of the methods to solve the problem of salinity nowadays on other plant species in other areas.

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