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Proline Accumulation in *Vigna angularis* Seedlings Under Salt Stress

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Changes in the proline accumulation of ten-day-old seedlings of *Vigna angularis* in response to NaCl treatment were monitored. The proline content increased gradually both with an increase in the exposure time to salt stress and in a concentration-dependent manner. The increased proline accumulation was stronger in the shoots than in the roots. The salt stress by itself resulted in a significant inhibition of the chlorophyll content. Pre-treatment with proline before salinization lasting 48 h did not significantly affect the endogenous proline level in the roots, in contrast, a considerable increase of proline was observed in the shoots. The application of exogenous proline to the seedlings increased the endogenous proline content and improved the root and shoot growth under saline conditions. Detached leaves also exhibited an increased proline level in response to the applied NaCl, however, at a lower magnitude than in the intact seedlings. The proline alleviated the inhibitory effect of the NaCl in a concentration- dependent manner, thereby suggesting that salinity is a strong inducer of proline accumulation. In addition, abscisic acid eliminated the inhibitory effect of the salt salinity, thereby indicating a protective role on salinity stress and a regulatory role in proline synthesis. Accordingly, it would appear that proline may be involved in salt tolerance.

Key words : abscisic acid, chlorophyll content, proline accumulation, salt stress, *Vigna angularis*

1. Introduction

Salinity inhibits growth, disturbs pigment composition, and inhibits photosynthesis in various plants. Many plant species have evolved a variety of mechanisms to deal with salt stress. However, the mechanisms responsible for tolerance to high levels of NaCl have not as yet been well clarified, although much data has been accumulated from studies of glycophytic and halophytic plants(Wang *et al.*, 1997). The response of plants to salt stress depends on the plant growth regulators involved in the tolerance mechanisms or the susceptibility of the particular plant species. Under salinity the quantity of abscisic acid(ABA) in a plant increases and this ABA then plays a central role in regulating the osmotic adaptation of the plant, as well as in the synthesis of proline(Downton and Loveys, 198 1 ; Stewart and Voetberg, 1985). Exogenous ABA induces the accumulation of proline and eliminates the inhibitory effect of NaCl.

Plants respond to a variety of stresses by accumulating certain specific meatabolites, generally, the most conspicuous amino acids, and in particular, proline(Bassi and Sharma, 1993). Proline has been shown to accumulate in the tissue/ organs of plants subjected to water deficit, high salinity, chilling, low and high temperature stress, heat, and heavy metal exposure(Bassi and Sharma, 1993 ; Sharma *et al*, 1998). Proline plays a major role in osmoregulation and osmotolerance(Renard and Guerrier, 1997). It has been previously reported that the role of proline is related to survival rather than to growth maintenance(Greenway and Munns, 1980). Compatible solutes are also known to have an adaptive value as well as a stabilizing effect on enzyme activity(Shomer-Illan and Waisel, 1986). The accumulation of proline in a wide

variety of both halophytes and nonhalophytes after exposure to various stresses may be involved in adaptive responses(Aspinall and Paleg, 1981). Bhaskaran *et al.*(1985) found no correlation between the proline level and stress tolerance in cultured cells exposed to low water potentials and concluded that the increase of proline in their system was an incidental consequence of stress rather than an adaptive response. The proline accumulated in soybean leaves only after stress was severe enough to induce injury : whereas proline accumulation was not a sensitive indicator of salt stress in soybean plants(Moftah and Michel, 1987). However, whether or not proline exerts a protective role, as stated for the halophytic species, remains a matter of debate.

Although salinity-induced proline accumulation during germination is well-documented, it is unknown whether proline protects against the effect of toxic salts during seedling growth and, if so, what the underlying mechanism is. Accordingly, this study monitored the changes in the proline content in seedlings under saline conditions and studied the role of proline in response to salinity stress.

2. Materials and Methods

2.1. Plant growth conditions

Seeds of the azuki bean(*Vigna angularis*), selected for uniformity, were surface sterilized with a 1% sodium hypochlorite solution for 1 min and allowed to germinate in Petri dishes linked with wet filter papers kept in a growth chamber under white fluorescent lamps(35 W · m^{-2}) with a 12 h photoperiod. The day/night temperatures were 27/20℃. Five-day-old seedlings of approximately equal size were then utilized for the experimentation. These seedlings were grown on a Hoagland solution diluted 1 : 20 with the test solutions in glass beakers maintained under the above conditions. The seedlings grown on the Hoagland medium without salt were constituted as the control. All the seedlings were subjected to chlorophyll and proline determination after five days of incubation.

2.2. Pre-treatment with proline

The azuki bean seeds were germinated for 3 days in the dark. The seedlings were then transferred to glass pots containing distilled water. After 7 days, 100mM NaCl was added for 48 h both to both the proline-treated and untreated seedlings. The control plants were kept in distilled water. During the 10-day experimental period the seedlings grew in a growth chamber under the above conditions.

2.3. Proline and chlorophyll determinations

The proline was determined according to the method of Bates *et al.*(1973). Approximately 0.5 g of the plant material was homogenized in 10ml of 3% aqueous sulphosalicyclic acid and the homogenate then centrifuged. Two ml of the extract was treated with 2ml of acid-ninhydrin and 2ml of glacial acetic acid in a test tube for 1 h at 100℃ and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 ml and mixed vigorously for 15-20 sec. The chromphore containing toluene was separated and the absorbance read at 520 nm. The proline concentration was then calculated on a fresh weight basis.

The chlorophyll was determined based on the method of Arnon(1949). One gram of freshly harvested leaves was cut into small pieces with a scissors and then homogenized in 5 ml of distilled water using a mortar and pestle. The final volume was made up to 10 ml. All the experiments were done in triplicate and repeated at least twice.

3. Results

The proline accumulation in the azuki bean seedlings as a function of the salinity concentration, was within a range of 0-200mM, as shown in Fig. 1. The proline content increased progressively in response to the external NaCl concentrations. A rise of 2.5- and 4.6-fold in the proline content were observed five days after treatment with 100 and 200 mM NaCl, respectively.

The pattern of proline accumulation in the roots and shoots was different. The increase in the proline level in the shoots appeared to be a function of the length of exposure to NaCl. In contrast, the proline accumulation in the roots exhibited a small fluctuating pattern during the saline conditions(Fig. 2). As a result, no marked increase in proline content was recorded at the end of the experiment. In

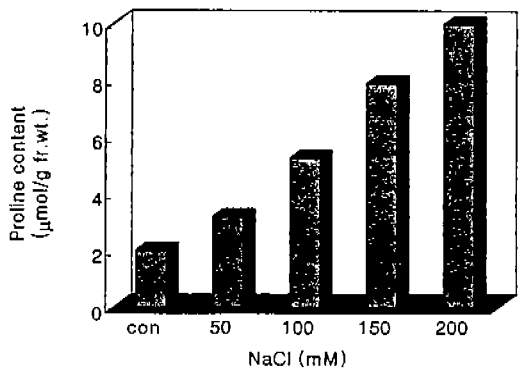

Fig. 1. Proline content in *Vigna angularis* seedlings after five days of exposure to different concentrations of NaCl.

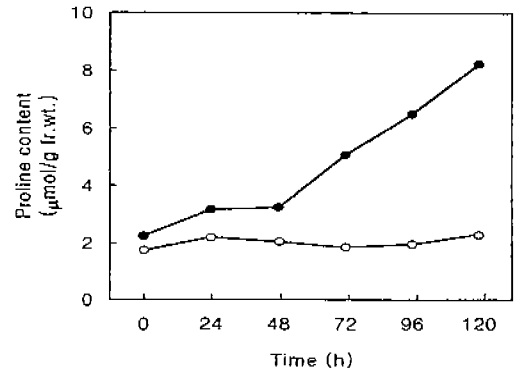

Fig. 2. Time course pattern of proline changes in *Vigna angularis* seedlings treated with 100mM NaCl. ●, shoot ; ○, root.

response to the NaCl treatment, the proline level in the roots initially increased and then declined to values close to the 0 day ones. The proline level of the roots and shoots in the untreated seedlings did not alter much during the time period used in this study(data not shown). The chlorophyll content in the seedlings as a function of salinity and proline in 100 and 200 mM NaCl is in shown Table 1. The chlorophyll content declined in response to the NaCl treatment, and the decline increased progressively with an increase of NaCl. After 5 days of treatment, 100 and 200 mM NaCl induced an inhibition of 36 and 54 %, respectively. The proline treatment under saline conditions showed an ameliorating effect on the chlorophyll accumulation and protected against the adverse effects of the salinity. In contrast, the proline content in the seedlings increased progressively with an increase in the NaCl concentration. Therefore, salt was proved again as a strong inducer of proline accumulation, plus the proline content responded differentially to NaCl and proline treatments. The external application of proline increased the proline accumulation under saline conditions, yet no significant increase was observed.

Table 1. Chlorophyll and proline content in ten-day-old seedlings treated with NaCl and proline

Treatment	Chl content (mg/g fr.wt.)	Proline content (μmol/g fr.wt.)
Control	8.02	2.32
0.1mM proline	6.43	2.86
1.0mM proline	6.55	3.05
100mM NaCl	3.75	6.02
100mM NaCl+0.1mM proline	4.80	4.45
100mM NaCl+1.0mM proline	4.62	4.98
200mM NaCl	2.74	10.87
200mM NaCl+0.1mM proline	3.38	7.25
200mM NaCl+1.0mM proline	3.17	8.04

When the seedlings were pre-treated with proline for 24 h prior to the imposition to NaCl stress, the chlorophyll content was not affected by treatment with 0.1 and 1.0 mM proline for 24 h, whereas there was a light increase of both chl a and chl b after 7 days(Table 2). Exposure to 100 mM NaCl for 2 days resulted in a 48% decrease in the chl content and the decline increased to 54% after 7 days. Treatment of the seedlings with proline for 24 h before salinization alleviated the inhibitory effects of NaCl. The application of exogenous proline to the seedlings for 7 days increased the shoot and root elongation(Table 3). Proline supplied through the roots for 24 h did not significantly affect the endogenous proline level in the roots(Table 4). In the shoots, a considerable increase of free proline was observed. 100 mM NaCl induced an increase in the proline content in both the roots and the shoots. After a 48 h treatment of NaCl in the roots and shoots, the level of free proline increased to about 49 and 94%,

respectively. Those plants exogenously supplied with proline before salinization accumulated less proline than the plants supplied with 100 mM NaCl.

Table 2. Chlorophyll content in *Vigna angularis* seedlings treated with proline for 24 h before salinization with 100mM NaCl

Treatment	Chl content (mg/g fr. wt.)		
	a	b	(a+b)
Control	482	216	698
0.1 mM proline	392	179	571
1.0 mM proline	407	187	594
Control after 2 days	559	237	796
100mM NaCl	215	197	412
100mM NaCl+0.1mM proline	326	241	567
100mM NaCl+1.0mM proline	309	231	540
0.1mM proline	425	194	619
1.0mM proline	411	187	598
Control after 7 days	575	240	815
100mM NaCl	250	118	368
100mM NaCl+0.1mM proline	319	146	465
100mM NaCl+1.0mM proline	287	135	422
0.1mM proline	560	238	798
1.0mM proline	422	203	645

Table 3. Root and shoot lengths of *Vigna angularis* seedlings treated with proline for 24h before salinization with 100 mM NaCl

Treatment	Length(cm)	
	Root	Shoot
Control	13.8	9.4
0.1mM proline	16.4	11.5
1.0mM proline	15.2	10.2
Control after 2 days	15.8	11.5
100mM NaCl	13.6	9.3
100mM NaCl+0.1mM proline	14.5	10.2
100mM NaCl+1.0mM proline	14.2	9.7
0.1mM proline	19.4	13.2
1.0mM proline	18.3	12.5
Control after 7 days	20.5	13.5
100mM NaCl	14.0	9.5
100mM NaCl+0.1mM proline	15.4	10.5
100mM NaCl+1.0mM proline	14.8	10.2
0.1mM proline	24.1	16.4
1.0mM proline	22.2	15.0

Experiments performed to check whether NaCl and abscisic acid(ABA) can induce proline accumulation in detached leaves revealed that proline accumulation due to saline and ABA treatment also occurred in detached leaves, however, the magnitude was slightly lower than that in the intact seedlings(Table 5). ABA at a concentration as high as 10-4 M had no significant effect on the proline content.

Table 4. Proline content in *Vigna angularis* seedlings treated with proline for 24 h before salinization with 100mM NaCl

Treatment	Proline content (μmol/g fr.wt.)	
	Root	Shoot
Control	1.85	2.31
0.1mM proline	1.89	2.57
1.0mM proline	2.52	2.60
Control after 2 days	1.49	2.09
100mM NaCl	2.22	4.05
100mM NaCl+0.1mM proline	1.95	3.08
100mM NaCl+1.0mM proline	1.96	3.24
0.1mM proline	1.76	2.65
1.0mM proline	2.21	2.87
Control after 7 days	1.35	1.65
100mM NaCl	2.54	16.27
100mM NaCl+0.1mM proline	1.87	5.65
100 mM NaCl+1.0mM proline	2.03	6.08
0.1mM proline	1.61	1.89
1.0mM proline	1.78	2.62

Table 5. Proline content in intact seedlings and detached leaves of *Vigna angularis* treated with NaCl, proline, and ABA

Treatment	Proline content (μmol/g fr.wt.)	
	Intact seedlings	Detached leaves
Control	2.35	2.37
0.1mM proline	2.84	2.79
100mM NaCl	5.96	5.53
100mM NaCl+0.1mM proline	4.45	4.24
0.1mM ABA	3.82	3.24
100mM NaCl+0.1mM ABA	4.03	3.78

4. Discussion

The proline content increased during germination in the presence of NaCl, thereby suggesting a possible role of proline during germination. In addition, NaCl stimulated proline accumulation in germinated seeds, therefore, proline may be considered as a compatible solute(Poljakoff-Mayber *et al.*, 1994). The free proline level in the seedlings increased with the duration of the salinity treatment (Fig. 1). Similar results were reported for *Pisum sativum*(Fedina *et al.*, 1993). The larger accumulation of proline could have been due to an increased synthesis and decreased metabolism of some of the effects related to the uptake of proline. Therefore, the tolerance of azuki bean plants to salinity stress could have been at least partially due to the large amount of free proline present in the roots.

The chlorophyll content inhibition was also concentration-dependent under salt stress(Table 1). A remarkable decrease in in the chl content was observed after a 5-day incubation in salinity conditions. The inhibition of the chl content due to NaCl treatment was partially overcome by the addition of proline(Table 2). The proline accumulation due to NaCl treatment also occurred in detached leaves, however, the magnitude was slightly lower than that in the intact seedlings (Table 5). The proline accumulation in the *Vigna angularis* seedlings in response to NaCl treatment was comparable to that exhibited by plants in response to water deficit, salinity(Hanson *et al.*, 1979), and high and low temperature stress (Aspinall and Paleg, 1981). The pattern and magnitudes, however, were different. The pattern of proline accumulation in the shoots due to salinity resembled that occurring during water deficit stress, in that it was generally a function of the length of exposure to salinity. The pattern showed by the roots was different(Fig. 2). A noticeable characteristic of salinity-induced proline accumulation was its rapidity, as a marked rise was evident as early as 24 h after treatment. Concerning the magnitude of change in the proline level due to different stresses, a much higher rise is known to occur in response to water deficit and temperature-stress when compared with the present values(Bassi and Sharma, 1993). Under moderate water stress, the increase is several-fold. Magnitude-wise, proline accumulation due to Zn and Cu occurs due to salinity stress(8.0-fold) in wheat(Bassi and Sharma, 1993). There seems to be a parallel between saline toxity(in terms of chlorophyll inhibition) and the extent of proline accumulation. No definite inference from such a correlation, however, can be drawn. The chlorophyll loss due to NaCl treatment, as observed in this data, was comparable to several other findings obtained with heavy metals(Aria and Saradhi, 1991 ; Bassi and Sharma, 1993).

Proline accumulation has been linked with stress resistance and has been ascribed to a variety of functions. Whether proline can be of any adaptive significance against saline toxicity is not clear from the present data. It is difficult to speculate on the mechanism for the proline level rise in the salinity-treated seedlings. Since proline was accumulated in response to the NaCl treatment, it is possible that proline is involved in the salinity tolerance mechanism(similar to drought resistance). A common mechanism has been suggested for proline accumulation with other types of stress(Aspinall and Paleg, 1981), where it is believed that proline accumulation occurs largely as a consequence of a disturbance in the water balance caused by salinity or high and low extremes of temperature. The application of proline improved the soil tolerance of barley and peas(Lone *et al.*, 1987) and the protection ascribed to the exclusion of Na^+ and Cl^- ions from the seedling shoots.

Proline has been reported to play an important role in osmoregulation protecting enzyme denaturation, acting as a reservoir of carbon and nitrogen sources, stabilizing the machinery of protein synthesis, regulating the cytosolic activity, and/or scavenging hydroxyl radicals(Laliberte and Hellebust, 1989 ; Paleg *et al.*, 1985 ; Smirnoff and Cumbar, 1989). However, according to Moftah and Michel(1987), proline accumulation is associated with severe damage/injury and does not have any protective value. Yet, no definite and convincing experimental evidence exists to support any of these theories, plus the influence of environmental factors on proline accumulation is still a matter of controversy.

The level of ABA, another stress metabolite, has actually been observed to increase in saline-

treated pea seedlings(Fedina *et al.*, 1994). Recent research indicates that ABA modulates the response to salt stress and is a regulator in osmotic adaptation and the synthesis of proline.

ABA eliminated the inhibitory effect of NaCl and the values of the observed parameters were similar to those of the control(Fedina *et al.*, 1994). It is possible that ABA induces the synthesis of some other solutes such as betaine, saccharose, and amino acids that maintain the osmotic balance of the cell, thereby relieving the negative effect of NaCl. Hasson and Poljakoff-Mayer(1983) showed that ABA induces proline accumulation in nonstressed plants. In the current experiments, 10^{-4} M ABA in intact seedlings and detached leaves enhanced the proline accumulation by about 62 and 36%, respectively(Table 5). However, the protective effect of ABA against salinity may not have been due to proline because, in barley, its content increased following ABA treatment(Fedina *et al.*, 1994).

The data presented here suggest that the ABA treatment of plants before salinity increases their tolerance to high concentrations of NaCl. The plants treated with ABA survived under high salinity. Yet, the protective effect of ABA against salt stress does not depend only on the lowed accumulation the of Na+ and Cl- in the shoots and may be due to some other reasons. ABA may also stimulate the synthesis of some of the solutes resulting from the induced shock protein synthesis. However, rigorous research is needed to establish the suggested involvement.

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