EFFECTS OF OSMOTIC STRESS ON GROWTH AND RIBONUCLEASE ACTIVITY IN *Vigna unguiculata* (L.) Walp. SEEDLINGS DIFFERING IN STRESS TOLERANCE[1]

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ABSTRACT - Seeds of Vita 5 and Vita 3 cultivars of cowpea (*Vigna unguiculata* (L.) Walp.) were allowed to germinate in paper towels wetted with distilled water, mannitol or NaCl solutions of different osmotic potentials. The seedlings were harvested during the process of seedling establishment, separated into cotyledons, roots, stems (hypocotyl + epicotyl), and leaves for extracting and determining ribonuclease activity. After 7 days from sowing they were harvested for growth measurements. Growth inhibition due to water and salt stresses were more conspicuous in Vita 5 than in Vita 3 cultivar, and root/shoot ratios for the latter were higher than for the former at all stress levels shudied. These results suggest that Vita 3 is more tolerant to stress than Vita 5. The effect of salt stress on RNase activity varied within seedling parts and stages of development. It also depended upon the way the enzyme activity was expressed, that is as total or specific activity. The development of both total and specific ribonuclease activity as a function of salt stress in different seedling parts of the two cultivars differing in salt tolerance did not support the hypothesis that there is a correlation between salt tolerance and changes in ribonuclease activity, and that this enzyme could be used as a biochemical marker for salt stress.

Additional index terms: cowpea, mannitol, NaCl, RNase activity and salt tolerance.

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EFEITOS DO ESTRESSE OSMÓTICO NO CRESCIMENTO E NA ATIVIDADE RIBONUCLEÁSICA DE PLÂNTULAS DE *Vigna unguiculata* (L.) Walp. COM DIFERENTES GRAUS DE TOLERÂNCIA AO ESTRESSE

RESUMO - Sementes de *Vigna unguiculata* (L.) Walp. dos cultivares Vita 5 e Vita 3 foram postas para germinar em papel toalha umedecido com água destilada ou soluções de manitol e de NaCl de diferentes potenciais osmóticos. As plântulas foram colhidas durante o processo de estabelecimento da plântula, separadas em cotilédones, caules (hipocótilo + epicótilo), raízes e folhas para extração e determinação da atividade da ribonuclease. Após 7 dias da semeadura as plantas foram colhidas para medição do crescimento. A inibição do crescimento decorrente dos estresses hídrico e salino foi mais conspícua no cultivar Vita 5 do que no Vita 3 e a relação raíz/parte aérea do último cultivar foi maior do que a do primeiro, em todos os níveis de estresse estudados. Estes resultados sugerem que o cultivar Vita 3 é mais tolerante ao estresse do que o Vita 5. O efeito do estresse salino na atividade da ribonuclease variou com a parte da plântula e com o estágio de desenvolvimento. Ele também dependeu da forma como a atividade enzimática foi expressa, isto é, como atividade total ou específica. O desenvolvimento, tanto da atividade ribonucleásica total como da específica em fiunção do estresse salino, nas diferentes partes da plântula dos dois cultivares com diferentes graus de tolerância à sais não deu suporte a hipótesede que existe uma correlação entre tolerância à sais e mudanças em atividade ribonucleásica e de que esta enzima poderia ser usada como um marcador bioquímico para estresse salino.

Termos adicionais para indexação: feijão-de-corda, manitol, NaCl e tolerância à salinidade.

INTRODUCTION

Salt and water stresses are responsible for both inhibition or delayed seed germination and seedling establishment (Uhvits, I 946; Prisco & O'Leary,1970a; Bewley and Black,1994). Under these stress conditions there is a decrease in water uptake both during imbibition and seedling establishment, and in the case of salt stress, this can be followed by excessive uptake of ions (Uhvits, 1946; Prisco & OZeary, 1970a). This results in physiological and biochemical changes in both anabolic Prisco & O'Leary,1970b; Prisco, I 971) and catabolic organs of the seeds and seedlings (Prisco & Vieira,1976; Gomes Filho & Prisco,1978; Sheoran & Garg,1978; Prisco et al., 1981; Gomes Filho & Sodek, 1988).

Among the physiological and biochemical changes induced by salt and water stresses it has been reported inhibition of translocation of reserve hydrolysis products from cotyledons toward the embryo-axis (Prisco & Vieira,1976; Prisco et al.,1981), inhibition of the protein synthesizing capacity (Rauser & Hanson,1966; Kahane & Poljakoff Mayber,1968; Prisco & O'Leary, 1970b), delayed enzyme solubilization and activation (Prisco et al., 1981; Prisco,1987), and enhancement (Porath & Poljakoff Mayber,1968; El-Fouly & Jung, l 972; Arad & Richmond, 1973; Sheoran & Garg, I 97g; Gopal & Rao,1982) or inhibition of enzyme activity (Porath & Poljakoff Mayber,1968; Hason-porath & Poljakoff Mayber,1969; Kalir & Poljakoff Mayber, 1976; Sheoran & Garg,1978; Abdel Wahab & Zahran,1981). It has also been observed that phosphatase and ribonuclease activities increase with water stress (Vieira da Silva,1968a;1968b; 1969), and that this increase was correlated to stress sensitivity for *Gossypium* species differing in water stress sensitivity (Vieira da Silva,1970). These and other results (Yi & Todd,1979. Sheoran & Gargg, 1978; Gomes Filho et al.,1983; Lauriere, 1983; Gomes Filho & Sodek,1988) led Rouxel et al., (1989) to postulate that ribonuclease might be considered as a good marker enzyme for stress conditions.

Based on the existance of cowpea cultivars differing in water stress sensibility (Guimarães,1988), this work studied differences in salt tolerance and if there was any correlation between ribonuclease activity changes and salt tolerance that could be used as marker for selection in this species.

MATERIAL AND METHODS

Plant material, seed germination conditions, and growth measurements

Vita 3 and Vita 5 cowpea (*Vigna unguiculata* (L.) Walp.) seeds obtained from the Centro Nacional de Pesquisa do Arroz e Feijão (CNPAF/EMBRAPA), were multiplied at the Fazenda Experimental do Vale do Curu, Universidade Federal do Ceará, Pentecoste, Ceará, Brazil.

After drying seeds were stored in sealed bottles containing silica gel at 10^{o}C until required. Seeds were immersed for 10 minutes in sodium hypochlorite solution containing 1.3% of active chloride (Gomes Filho & Prisco,1978), and allowed to germinate in paper towels (Prisco & Vieira,1976) wetted in water, mannitol or NaCl solutions, at 25$\int$2^{o}C in darkness. The osmotic potentials of mannitol and NaCl solutions were -0.23, -0.46, and -0.69 MPa, prepared according to Van't Hoff equation (Lang,1967). Seedling at the 7th day from sowing were harvested, and both shoot and root length measured. Four replicates of 10 seedlings per treatment were used.

Enzime extraction and activity

After harvesting the seedlings were separated into cotyledons, stems (hypocotyl + epicotyl), roots, and leaves, and ribonuclease (EC 2.7.7.17) was extracted according to Gomes Filho & Enéas Filho (1991). Each seedling part was homogenized separately in 0.1 M potassium phosphate buffer, pH 5.7, at 4^{o}C, and centrifuged at 3000 g for 10 min. The pellet was discarded and the supernatant used for enzyme assay. Ribonuclease (RNase) activity was determined as the capacity to hydrolyze RNA according to Tuve & Anfinsen (1960), modified by Gomes Filho & Sodek (1988). It was used 0.1 M (I=0.1) sodium acetate buffer, pH 5.8, in the assays for cotyledons, stems,, and roots RNases, and 0.05 M (I=0.1) sodium phosphate buffer, pH 6.8, for leaf RNases assays (Gomes Filho & Enéas Filho,1991). One unit of RNase activity (UA) was defined as a difference in absorbance (ΔA_{260}) of 0.01 (Gomes Filho et al., 1983). Three different extracts for each plant part were prepared, and each one was assayed three times both for RNase and proteins. Protein was assayed by the method of Bradford (1976), with 2 x cristallized and lyophilized bovine serum albumin (Sigma Chemical Company, USA) as standard.

RESULTS AND DISCUSSION

Stress tolerance

The root/shoot ratio increased as a result of both salt and water stresses (Fig. 1), and it was due to a greater inhibition of shoot as compared to root growth (Fig. 2). The root/shoot ratios for Vita 3 were higher than for Vita 5, at all strress levels, and the inhiibitory effect of both NaCl and mannitol on total seedling growth was more pronounced on Vita 5 than on Vita 3 . Based on these results and on the fact that higher

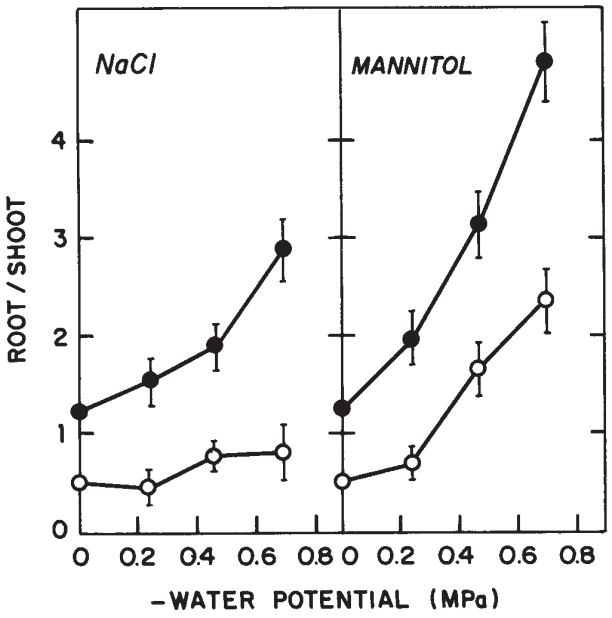

FIGURE 1- Effect of NaCl and mannitol on root/shoot length of 7 day-old seedlings of *Vigna unguiculata* cultivars diffeering in stress tolerance. Cultivar Vita 5 (❍), and cultivar Vita 3 (❁); the bars represent the standard deviation of the mean.

root/shoot ratio has been used as an index of water stress tolerance (Kramer,1983) it seems that Vita 3 showed a higher salt and water stress tolerance when compared with Vita 5. Using a different selection criteria for water stress tolerance, Guimarães (1988) also considered Vita 3 as promissing as far as water stress tolerance was concerned. When the inhibitory effects of mannitol and NaCl were compared it was evident that at isosmotic concentrations the former fiunctioned as a stronger growth inhibitor, specially of shoot growth, for both Vita 5 and Vita 3 (Fig. 2). These observations agreed with those obtained by Machado et al. (1976) with mannitol, and Kawasaki et al. (1983) with polyethyleneglicol, which induced more effective water stress than NaCl. One possible explanation for these findings is that the cell membrane reflection coeficient for NaCl is lower than for mannitol (Slatyer,1967): a result, the seedlings subjected to salt stress should osmotically adjust faster and more efficiently in NaCl than those grown in mannitol solutions.Thus the salt stressed seedlings should experience lower water stress than the ones grown in mannitol solutions.

Ribonuclease activity

Total cotyledonary activity (Fig. 3A and 3B) in the control treatment for both Vita 5 and Vita 3 cultivars increased up to day 5, decreasing thereafter until the end of the experimental period. The development of enzyme activity in the salt treatment followed a

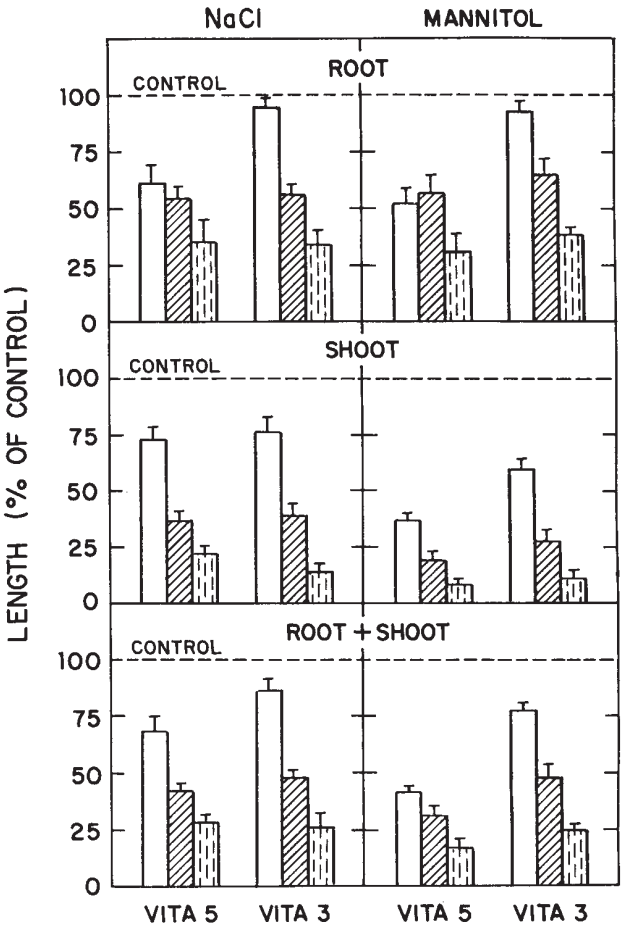

FIGURE 2- Effect of NaCl and mannitol on growth of 7 day-old of *Vigna unguiculata* cultivars differing in stress tolerance. The water potentials (Ψ) of the seedling establishment medium were 0 (Control), -0.23(□), -0.46 (▨), and -0.69 MPa (▥), respectively; the bars represent the standard deviation of the mean.

different pattern in the two cultivars. In Vita 3, enzyme activity was lower at days 3 and 5; the opposite was observed at days 7 and 9, that is, it was higher in the salt treatment than in the control (Fig. 3B). In Vita 5 the enzyme activities at days 3 and 5 were the same in both treatments, but an increase in activity due to salt stress was observed at days 7 and 9 (Fig. 3A). When the values of RNase specific activity in cotyledons were determined (Fig. 4A and 4B), it was observed a delay in the development of enzyme activity as a result of salt stress in both Vita 5 and Vita 3. A similar lag in the development of enzyme activity was also found for cotyledonary amylases (Prisco et al.,1981), RNases (Gomes Filho et al.,1983), and galactosidases (Enéas Filho et al.,1995) from Pitiuba cowpea. These results reinforce the idea that salinity may either delay solubilization/activation, or *de novo* synthesis, or even the turnover of cotyledonary hydrolytic enzymes during seedling establishment (Prisco, 1987). In stem

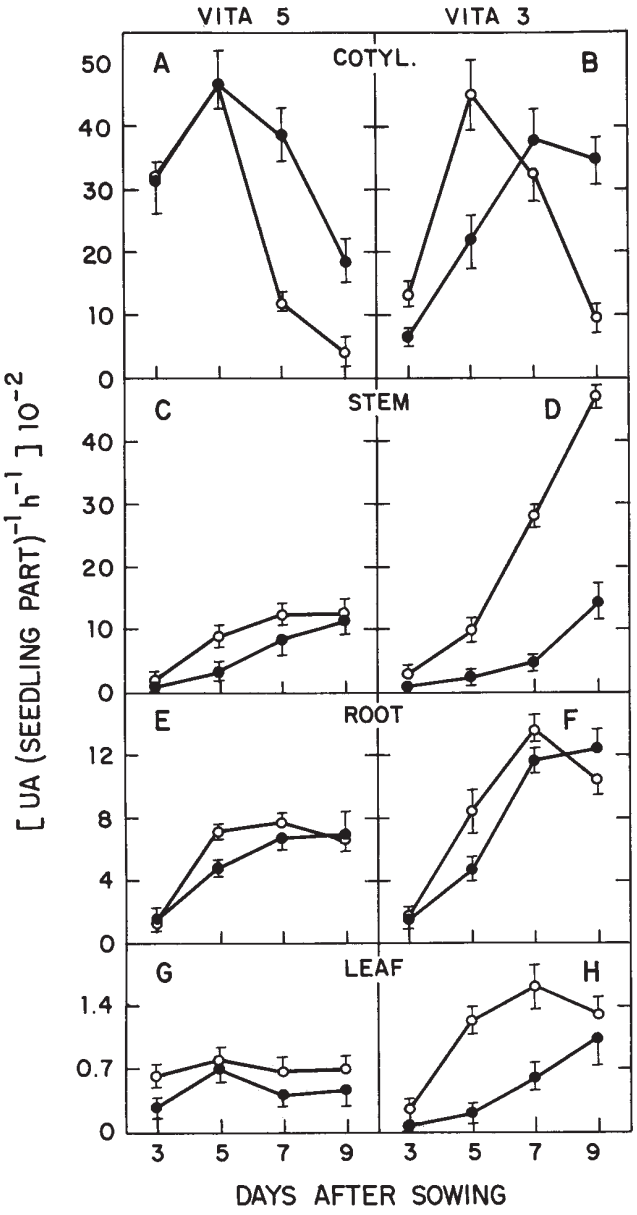

FIGURE 3- Effect of NaCl on total ribonuclease activity of different seedling parts of *Vigna unguiculata* cultivars differing in stress tolerance. Control (❍) and salt treatment (●); the water potential (Ψ) of the salt treatment was -0.46 MPa; the bars represent the standard deviation of the mean.

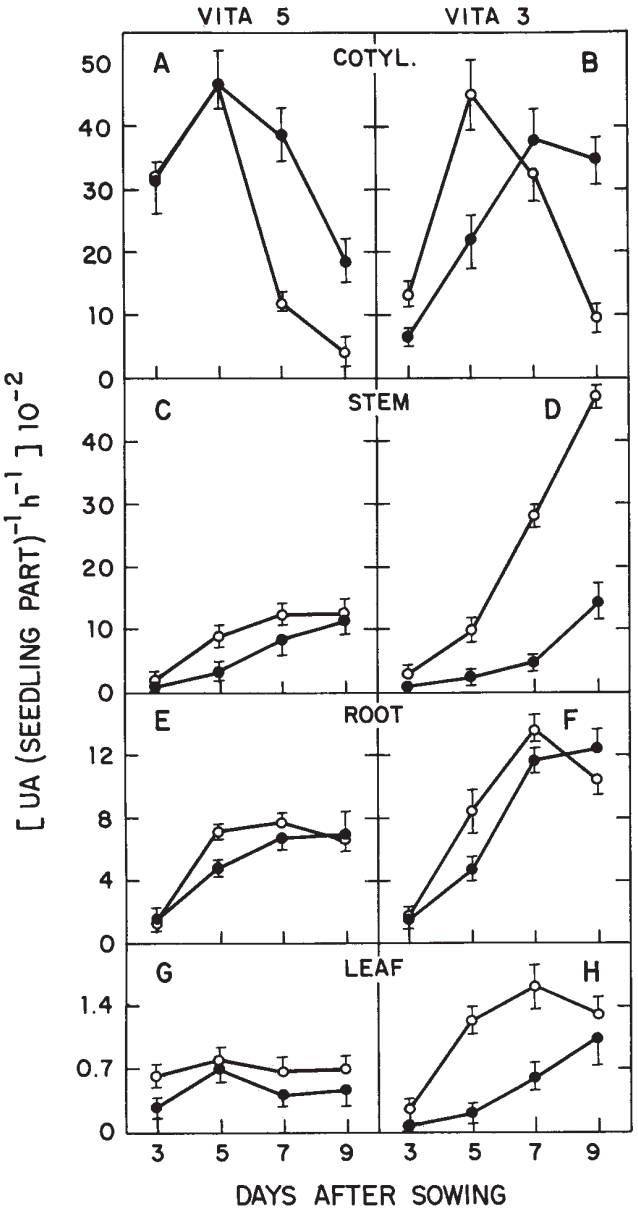

FIGURE 4- Effect of NaCl on specific ribonuclease activity of different seedling parts of Vigna unguiculata cultivars differing in stress tolerance. Control (❍) and salt treatment (●); the water potential (Ψ) of the salt treatment was -0.46 MPa; the bars represent the standard deviation of the mean.

tissues, total RNase activity increased during seedling establishment, in both treatments, for Vita 5 (Fig. 3C) and Vita 3 (Fig. 3D) cultivars. However, the activities were lower in the salt than in the control treatment.

These differences in total RNase activities were much higher in the cultivar less sensitive to stress (Vita 3) than in Vita 5. These results were exactly the opposite compared with those found in leaves of *Gossypium* species differing in water stress sensitivity (Vieira da Silva,1970), suggesting that the way this enzyme responds to stress varies among species and plant organs. In the roots (Fig. 3E and 3F) and leaves (Fig. 3G and 3H) of both cultivars, total RNase activity was much lower than the ones obtained for cotyledons and sometimes for stems as it was reported previously for Pitiuba cowpea (Gomes Filho & Enéas Filho, 1991). Root enzyme activity in the control treatment increased up to day 7, decreasing thereafter. Inhibition of enzyme activity under saline conditions was observed only at days 5 and 7, and this effect was

TABLE 1- Protein concentration (mgProt per seedling part) of different parts fromVita 5 and Vita 3 cultivars of *Vigna unguiculata* along seedling establishment. Seedlings grown in distilled water (C, Yw = 0 MPa), and in NaCl solutions (S, Ψw = -0.46MPa). The values represent the mean SD (n=3).

Cultivar and seedling part	3rd day		5th day		7th day		9th day	
	C	S	C	S	C	S	C	S
VITA 5								
Cotyl.	1.10 0.10	1.43 0.20	0.37 0.05	0.87 0.02	0.08 0.01	0.43 0.04	0.05 0.00	0.14 0.00
Stem	0.25 0.04	0.18 0.04	0.27 0.02	0.39 0.02	0.16 0.04	0.45 0.04	0.15 0.03	0.31 0.04
Root	0.08 0.01	0.05 0.01	0.12 0.00	0.05 0.0I	0.101 0.1	0.07 0.00	0.06 0.01	0.05 0.01
Leaf	0.08 0.02	0.04 0.00	0.11 0.01	0.08 0.01	0.15 0.01	0.07 0.02	0.16 0.01	0.09 0.02
VITA 3								
Cotyl.	2.90 0.19	3.59 0.62	0.93 0.05	2.08 0.06	0.31 0.05	1.04 0.04	0.07 0.01	0.58 0.14
Stem	0.32 0.04	0.13 0.03	0.73 0.07	0.43 0.04	0.55 0.01	0.83 0.03	0.36 0.03	1.14 0.09
Root	0.07 0.00	0.05 0.01	0.19 0.03	0.10 0.01	0.18 0.02	0.16 0.01	0.18 0.02	0.15 0.02
Leaf	0.07 0.01	0.03 0.00	0.38 0.04	0.06 0.01	0.54 0.04	0.23 0.01	0.40 0.05	0.34 0.03

similar to the one observed for cotyledons and stems, that is, a delay in the development of enzyme activity. However, the specific activity values (Fig. 4E and 4F) showed that there was no delay in enzyme activity due to salt stress in this case. In Vita 5, total enzyme activity in the leaves (Fig. 3G) did not change much during seedling development, being the salinity inhibition fairly small. However, in Vita 3, leaf RNase activity (Fig. 3H) in the control treatment increased up to day 7, and then started to decrease. In this cultivar, enzyme activity in the salt treatment was always lower, throughout seedling developemnt, suggesting inhibition or delayed onset of enzyme activity as it was observed for cotyledons and stems. However, the analysis ofthe specific activity values (Fig. 4G and 4H) indicates that the effect of salt stress was not a delay in enzyme activity as it was observed in the cotyledons and stems.

The development of cotyledonary and stem specific RNase activities in the control treatment varied in the two cultivars (Fig. 4). In Vita 5, enzyme activity in the cotyledons increased up to day 7 and then started to decrease (Fig. 4A) while in Vita 3 (Fig. 4B) it increased continuosly up to the end ofthe experimental period. In the stems of Vita 5, the values increased up to ay and then stayed constant (Fig. 4C), while in Vita 3 (Fig. 4D) they increased exponentially until the experimental period was completed. Similar changes in specific cotyledonary RNase activities during seed germination and seedling development have also been observed in pea (Beevers & Guernsey, 1966), and cowpeas (Gomes Filho et al., 1983; Gomes Filho & Enéas Filho, 1991) cotyledons, as well as in rice endosperms (Palmiano & Juliano,1972) of seeds sown in distilled water. Salt stress inhibited the development of this enzyme specific activity in these plant parts for both cultivars, but this inhibition was more pronounced in Vita 3 than in Vita 5. Inhibitory effects of salinity on cotyledonary specific activity have also been observed in *Phaseolus vulgaris* (Sheoran & Garg, 1978), and in *Vigna unguiculata* cv Pitiuba (Gomes Filho et al., 1983). The higher salinity inhibition on the specific activity when compared to the inhibition on total cotyledonary enzyme activity was probably due to the fact that the protein content of this tissue in the salt treatment was always higher than in the control (Table 1). This might be interpreted as resulting from a salinity-induced delay of protein reserve mobilization from the cotyledons (Prisco & Vieira,1976; Gomes Filho & Prisco,1978). The development of stem specific (Fig. 4C and 4D) and total (Fig. 3C and 3D) enzyme activities during seedling development were similar for both cultivars. However, in Vita 3 there was a larger difference in these activities, particularly at days 7 and 9. This fact might be associated with both higher total activities found for Viita 3 in the control treatment and higher protein contents in the salt treatment at these days (Table 1).

Root specific RNase activity increased during seedling establishment for both cultivars (Fig. 4E and 4F), while leaf specific activity did not change much along the same period in both Vita 5 and Vita 3 (Fig. 4G and 4H). Salt stress did not affect Vita 3 root or leaf specific RNase activity (Fig. 4F and 4H), and had a small promotive effect in both root (Fig. 4E) and leaf (Fig. 4G) specific enzyme activity of Vita 5. This

enhanced effect can be atributed to the lower protein contents of these seedling parts in the salt treatment (Table 1), resulting from the salt inhibition of translocation of materials from the cotyledons to these organs (Prisco & Vieira,1976).

Several authors (Vieira da Silva,1970; Arad & Richmond,1976; Sheoran & Grag,1978; Yi & Todd, 1979; Rouxel et al.,1989) have shown significant increases in leaf specific RNase activity under salt or water stresses. It was suggested that there was a correlation between water (Vieira da Silva,1970) and salt (Rouxel et al. ,1989) stress tolerance and increase in leaf specific RNase activity. The analysis of the effects of salt stress on cotyledons and stem specific RNAse activity (Fig. 4A, 4B, 4C, and 4D) showed that salt stress, instead of increasing, caused inhibition or delayed the development of enzyme specific activity in both tolerant (Vita 3) and susceptible (Vita 5) cultivars.

The results for root and leaf specific activities were also in desagreement with the ones cited before, that is, while the root and leaf specific RNase activities of the more tolerant cultivar (Vita 3) were not affected by salt stress (Fig. 4F and 4H), there was a slight increase in specific activity as a result of salt stress in the less tolerant Vita 5 (Fig. 4E and 4G). These results do not support the hypothesis that salt and water stresses always induces increase in RNase activity (Vieira da Silva,1970; Lauriere,1983; Rouxel et al., 1989). On the contrary, they showed that the differences in enzyme behavior in the different seedling parts of the two cultivars in response to salt stress varied depending upon the seedling part. Therefore, the use of RNase as a biochemical marker for stress conditions (Rouxel et al. ,1989) is unreliable because its behavior in response to stress will vary in time during seedling development and in the space according to the plant organ.

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