CHAPTER 4

DIFFERENCES IN SALINITY STRESS TOLERANCE IN TERMS OF GROWTH AND WATER USE EFFICIENCY AMONG FOUR AMARANTH GENOTYPES

4.1 ABSTRACT

Amaranth is a promising C_4 crop for semi-arid regions due to its high nutritive value and the ability to adapt to diverse environments. Such areas are also prone to soil salinity. Crop production can be limited by saline irrigation water. Data on differential tolerance of amaranth genotypes to salinity stress is lacking. In this study the response of four amaranth genotypes, viz. *A. tricolor,* Accession '83, *A. hypochondriacus* and *A. cruentus* to saline water with different NaCl concentrations were analyzed in terms of growth, gas exchange, water use and leaf anatomical changes. The study was conducted in a greenhouse. The treatments consisted of saline water at 0, 25, 50, 100 and 200 mM NaCl, equivalent to electrical conductivities of 1.2, 4.1, 7.0, 12.8 and 24 dS. m^{-1} respectively. Plant growth, photosynthetic rate and stomatal conductance were significantly reduced at all salinity levels. *A. tricolor* and Accession '83 did not survive in the 200 mM NaCl treatment. At 50 and 100 mM NaCl the reduction in shoot growth was greater in *A. tricolor* and Accession '83 than that in *A. hypochondriacus* and *A. cruentus.* Water use efficiency increased with increasing salinity and ranged from 3.9 in *A. tricolor* to 6.7 g DM kg^{-1} H_2O in *A. cruentus* when plants were salinized with 100 mM NaCl. Specific leaf area (SLA) was decreased by salinity and differed between genotypes. A negative relationship between SLA and WUE was observed in the four amaranth genotypes. *A. tricolor* and Accession '83 had thinner leaves, more stomates per leaf area and larger stomatal apertures than *A. hypochondriacus* and *A. cruentus.*

Keywords: *Amaranthus*; gas exchange; growth; salinity tolerance; water use efficiency

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4.2 INTRODUCTION

Amaranthus spp. is used for its grain and as a vegetable. The leaves are high in protein, vitamins and minerals (Allemann *et al*., 1996). Its ability to adapt to diverse growing conditions such as low nutrient soils and a wide range of temperature and irradiation, as well as its tolerance to drought stress, emphasize the possible use of this species as a nutritious green crop in semi-arid regions (Myers, 1996). However, soils in such areas are often characterized by an excess of inorganic salts due to high evaporative water losses that exceed precipitation (Mengel and Kirkby, 1982). Hence, to enable amaranth cultivation in salt-prone regions, a better understanding of the strategies employed by amaranth genotypes in adaptation to salinity stress is required.

Salinity can affect growth, dry matter accumulation and yield (Sultana *et al*., 1999; Asch *et al*., 2000). It is well known that dry mass of plants is reduced in proportion to the increase in salinity (Pardossi *et al*., 1999; Romero-Aranda *et al*., 2001). The reduction in growth of salinized plants may be related to salt-induced disturbance of the plant water balance, and in the extreme to a loss of leaf turgor which can reduce leaf expansion and therefore, photosynthetic leaf area (Erdei and Taleisnik, 1993; Huang and Redmann, 1995b). Other causes of growth reduction under salinity stress include ionic imbalances, changes in nutrient and phytohormonal status, physiological processes, biochemical reactions, or a combination of such factors (Volkmar *et al*., 1998; Hasegawa *et al*., 2000;

Kashem *et al*., 2000a, b), accompanied by a reduction in photosynthesis (Sultana *et al*., 1999).

Although the factors that limit photosynthesis in salt stressed plants have been investigated for a number of species, the mechanistic nature of inhibition is unclear (Steduto *et al*., 2000). Salt may affect growth indirectly by decreasing the rate of photosynthesis. Photosynthesis may decrease due to stomatal closure or by a direct effect of salt on the photosynthetic apparatus. However, conflicting results on stomatal and non-stomatal limitation of photosynthesis are reported. For instance, in bean (a salt sensitive species) and cotton (a salt-tolerant species) the reduction in assimilation was found to be mostly due to stomatal limitation (Brugnoli and Lauteri, 1991), whereas other authors ascribed the reduction in photosynthesis to non-stomatal limitation (Dunn and Neales, 1993).

Among several strategies devised to overcome the problem of salinity stress, the selection of crop species or cultivars with salinity tolerance traits has been considered an economical and efficient strategy. Hence, the challenges for using salty water profitably will depend on greater knowledge of salt tolerance (Shannon and Grieve, 1999). Various workers have tried to identify physiological and biochemical differences between salt tolerant and sensitive plants in an effort to develop rapid screening methods for salt tolerance (Alian *et al*., 2000). It is well established that salt tolerance ability depends on genetic and biochemical characteristics of the species and sufficient genetic variability in relation to salinity exist in many agricultural crops (Alian *et al*., 2000; Bayuelo-Jiménez *et al*., 2003; Misra and Dwivedi, 2004).

Water use efficiency (WUE) may be one trait that can contribute to productivity when water resources are limited (Wright *et al*., 1994). Specific leaf area (SLA), an indicator of leaf thickness, is reduced under saline conditions (Bayuelo-Jiménez *et al*., 2003). Reduction of SLA is assumed to be a way to improve WUE (Wright *et al*., 1994; Craufurd *et al*., 1999). According to Liu and Stützel (2004) this is because thicker leaves usually have a higher density of chlorophyll and proteins per unit leaf area, hence, have a

greater photosynthetic capacity than thinner leaves. Nageswara Rao *et al*. (1995) recommended leaf thickness as a selection criterion for enhancing WUE in groundnut.

Several studies have shown that water uptake, and hence water use and transpiration, declined as the salt concentration in the irrigation water increased (Soria and Cuartero, 1997; Bayuelo-Jiménez *et al*., 2003). Reduction of water uptake with salinity could be related to reductions in morphological and/or physiological parameters like leaf area, stomatal density, and stomatal closure (stomatal conductance and transpiration). Since response to saline water varies greatly with species or cultivar (Bayuelo-Jiménez *et al*., 2003; Misra and Dwivedi, 2004), there is need for assessment of salinity tolerance among different species. Information on differences in salinity tolerance, and especially the relationship between WUE and stomatal conductance, photosynthesis and growth of amaranth genotypes is lacking. The objectives of this study were to (i) investigate differences in salinity stress tolerance in terms of WUE and growth among amaranth genotypes (ii) identify characteristics contributing to differences in water use and (iii) evaluate the significance of changes in these features for plant performance in a saline environment.

4.3 MATERIALS AND METHODS

4.3.1 Plant culture

This research was conducted in a greenhouse at the Experimental Farm, University of Pretoria between April and June 2002. The temperature ranged from 20^{o}C to 30^{o}C and relative humidity mainly between 60 to 70%. Seeds of four amaranth genotypes (*A. tricolor*, Accession '83, *A. hypochondriacus* & *A. cruentus*) were sown in separate seed trays. One month after sowing, one seedling per pot was transplanted into 5-liter plastic pots containing a sand/vermiculite mixture (3:1, v/v). The seedlings were irrigated daily with nutrient solution for 10 days after transplanting before commencement of the treatments. The nutrient solution used was the same as that specified in Chapter 3.

4.3.2 Salinity treatments

The nutrient solution for plants exposed to salt stress was identical to that of the control except for the addition of NaCl. The treatments consisted of a control, plus four salinity levels that were obtained by adding 25, 50, 100 and 200 mM NaCl to the basic nutrient solution. The different solutions had electrical conductivities (EC) equivalent to 1.2, 4.1, 7.0, 12.8 and 24 dS. m^{-1} respectively. In order to avoid osmotic shock, NaCl salinization initiated 10 days after transplanting of the seedlings was stepped up in daily increments of 25 mM until the final concentration was reached. The seedlings were watered daily until the solution drained freely.

A randomized complete block design with a split plot arrangement of treatments was used with the five NaCl levels as the main plots. Genotypes were allocated to the subplots and were randomized within each main plot. There were three replications.

4.3.3 Gas exchange measurements

Photosynthetic rate (P_n), stomatal conductance (g_s) and transpiration (E) were measured 28 days after initiation of the salt treatments on the second and third youngest fully expanded leaves. Measurements were made with a LI-COR, 6400 portable photosynthetic system (LI-COR, Lincoln, NE) following the same procedure as in Chapter 3.

4.3.4 Water use

Plant water use was measured gravimetrically. Everyday between 7:00h and 9:00h the plants were irrigated and pots left to drain to a constant weight before they were weighed. The pots were again weighed on the following day before irrigation in order to determine water loss per day. This procedure was carried out until termination of the experiment. Aluminium foil was placed on the surface of each pot to limit evaporation. Blank pots were prepared, one at each salinity level, to correct for water loss from plots in the absence of plants. Differences in water loss among the blank pots were small and their mean value was used as the estimate of evaporation.

Water loss through evapotranspiration was estimated during the experiment by measuring the daily mass loss of each pot. The daily increment of plant mass was small in comparison to water loss and was not taken into account in the estimate. Transpiration was estimated by subtracting the water loss from the blank pots from water loss from planted pots. Water use efficiency was calculated by dividing dry mass of shoots by the amount of water transpired (Glen and Brown, 1998).

4.3.5 Plant growth measurements

At the end of the experiment (eight weeks from the start of treatments), plant height was measured. The plants were then separated into leaves, stems and roots, and the number of leaves recorded. The roots were separated from the growth medium by washing in running water. Total leaf area per plant was measured with a LI-3100 leaf area meter (LI-COR. Inc., Lincoln, NE, USA). Fresh mass of shoots and roots were determined, and dry mass was obtained after oven drying the samples at 75^{o}C until constant weight. Relative shoot and root growth (percentage of growth of salinized vs unsalinized treatments) were determined. Root, stem and leaf mass ratio was determined as root, stem or leaf dry mass divided by plant total dry mass. Specific leaf area (SLA), the ratio of leaf area to leaf dry mass, was calculated.

4.3.6 Leaf anatomy

At the end of the experiment small leaf pieces of *A. tricolor* and *A. cruentus* from the control and 100 mM NaCl treated plants were fixed for two hours in 5% glutaraldehyde buffered with 0.075 M sodium phosphate (pH 7.4). Post-fixation followed for 2 hours in 2½% osmium tetroxide similarly buffered. The leaf segments were then dehydrated in alcohol series and specimens subsequently dried in liquid carbon dioxide (CO_2). Specimens for scanning electron microscopy (SEM) observation were mounted on aluminium stubs, coated with gold and viewed with a JEOL JSM-840 scanning electron microscope (JEOL, Tokyo). Stomatal density and dimensions were determined on three fields taken at random from each sample. Specimens for light microscopy (LM)

observation were embedded in Quetol 651 resin. Thin cross-sections of leaves (1 µm thick) were obtained with a Reichert Om U_2 ultramicrotome, stained with 1% toluidine blue O in borax, and examined with a Nikon light microscope. Total leaf thickness and mesophyll thickness were measured from three specimens taken at random.

4.3.7 Statistical analysis

All data were subjected to standard analyses of variance using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS, 1996) to determine the effect of main factors and the interaction between them. Differences at the $P \leq 0.05$ level were used as a test of significance and means were separated using Tukey's t-test.

4.4 RESULTS

4.4.1 Plant growth

Salinity stress had significant effects on all the growth parameters, and differences among genotypes for all characteristics were highly significant (Table 4.1). The interactions between genotype and salinity stress levels were also significant. All growth parameters decreased with increasing NaCl concentrations. However, their sensitivity to salinity stress varied with the level of stress and genotype. *A. tricolor* and Accession '83 did not survive in the 200 mM NaCl solution. Plants died about three weeks after the start of the treatments hence there was no data available for plant growth measurements at the end of the experiment. Although *A. hypochondriacus* and *A. cruentus* survived in the 200 mM NaCl treatment, plant growth was significantly reduced. Figure 4.1a and 4.1b illustrate the general reaction of *A. tricolor* and *A. cruentus* plants exposed to different concentrations of NaCl. Similar responses were observed with Accession '83 and *A. hypochondriacus* respectively.

a

Figure 4.1a ***Amaranthus tricolor*** **plants salinized with (A) 0 mM, (B) 25 mM, (C) 50 mM and (D) 100 mM NaCl solution.**

b

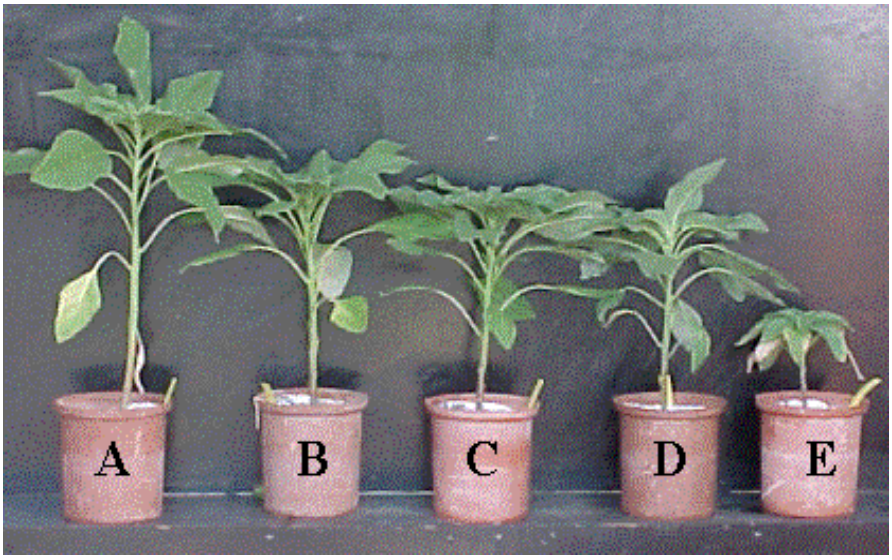

Figure 4.1b ***Amaranthus cruentus*** **plants salinized with (A) 0 mM, (B) 25 mM, (C) 50 mM, (D) 100 mM and (E) 200 mM NaCl solution.**

The most sensitive parameter was leaf area where 75% and 69% reduction was noted in *A. hypochondriacus* and *A. cruentus* respectively (Table 4.1). The least sensitive parameter at this concentration was leaf number with 54% reduction in *A. hypochondriacus*, and 49% in *A. cruentus*. Necrosis was observed at the leaf margins at 100 mM and 200 mM NaCl in *A. tricolor* and *A. cruentus* respectively (Figure 4.1).

Salinization with 25 mM NaCl did not have any significant effect on plant height in *A. tricolor*, Accession '83 and *A. cruentus*. In *A. hypochondriacus* plant height was reduced by 10%. Differences among genotypes were noted with application of higher NaCl concentrations. The reduction in plant height was more pronounced at 100 mM NaCl and especially in *A. tricolor* and Accession '83 than in *A. hypochondriacus* and *A. cruentus*. The reduction in *A. tricolor,* for instance, was 37% compared to 25% in *A. cruentus* (Table 4.1).

The effect of salt stress on the number of leaves was similar to that on plant height. Salinization with 25 mM NaCl did not have any effect on leaf number in *A. hypochondriacus* and *A. cruentus*. The number of leaves decreased with increasing NaCl concentration. *A. tricolor* and Accession '83 were more sensitive to salinity. They showed reductions in the number of leaves compared to *A. hypochondriacus* and *A. cruentus* at all concentrations of NaCl. The reduction in leaf area was similar in all the genotypes when salinized with 25 or 50 mM NaCl. At 100 mM NaCl, the reduction in leaf area in *A. tricolor* and Accession '83 was significantly higher than that in *A. hypochondriacus* and *A. cruentus*. For example, leaf area was reduced by 58% in *A. tricolor* and 49% in *A. cruentus* (Table 4.1).

Specific leaf area (SLA), an indicator of leaf thickness decreased with increasing salinity stress and varied among genotypes and salinity stress levels (Table 4.1). *A. tricolor* and Accession '83 had higher SLA values than *A. hypochondriacus* and *A. cruentus* for unstressed plants and at all concentrations of NaCl. Specific leaf area decreased with increasing NaCl, however, the reductions were higher in *A. hypochondriacus* and *A.*

cruentus than in *A. tricolor* and Accession '83. At 100 mM NaCl, for instance, the reduction in SLA was 14% in *A. hypochondriacus* compared to 10% in Accession '83.

Table 4.1 Effect of NaCl concentrations in the nutrient solution on plant height, leaf number, leaf area and specific leaf area of four amaranth genotypes

Genotype/NaCl concentration (mM)	Plant height (cm)	Leaf number	Leaf area (cm^2/plant)	Specific leaf area (cm^2 g^{-1})
A. tricolor				
Control	26gh	70a	2176b	374a
25	24gh	58b	1828f	369b
50	21hij	53c	1389j	358c
100	17j	42d	912o	333e
200	0k	0l	0r	0n
Accession '83				
Control	27g	67a	2199a	365b
25	25gh	56b	1869e	354c
50	22hi	53c	1408i	349d
100	19ij	41d	998m	327f
200	0k	0l	0r	0n
A. hypochondriacus				
Control	62a	33fg	1998d	254g
25	56bc	30gh	1690h	248h
50	50de	28hi	1314k	230j
100	44f	25i	935n	219k
200	23gh	15k	501q	196l
A. cruentus				
Control	62a	37e	2014c	246h
25	58ab	34ef	1752g	239i
50	53cd	31fgh	1324k	228j
100	46ef	29h	1030l	215k
200	25gh	19j	633p	187m
SEM	**0.78**	**0.62**	**2.8**	**0.9**

SEM: Standard error of the mean

Mean separation by Tukey's t- test. Means within each column followed by the same letter are not significantly different at P = 0.05.

Shoot and root dry masses expressed as a percentage of the control for each genotype was significantly reduced by salinity stress, and genotypic differences were observed. When plants were salinized with 25 mM NaCl the reductions in shoot dry mass ranged between 15 to 16% and no significant differences were observed among genotypes. With increasing salinity, shoot dry mass in *A. hypochondriacus* and *A. cruentus* was reduced to a lesser extend than in *A. tricolor* and Accession '83 (Figure 4.2). Root growth of *A. tricolor* and Accession '83 was less sensitive to salinization with low NaCl concentration since dry mass was reduced by only 17% when plants were salinized with 25 mM. At high NaCl concentration (100 mM) root dry mass was reduced by 57% in both genotypes. *A. hypochondriacus* and *A. cruentus* were highly sensitive to salinity stress since reductions in root dry masses of 43 and 32% were recorded even at the lowest NaCl level. Root dry mass was reduced by 71 and 68% respectively when plants were salinized with 100 mM NaCl (Figure 4.2).

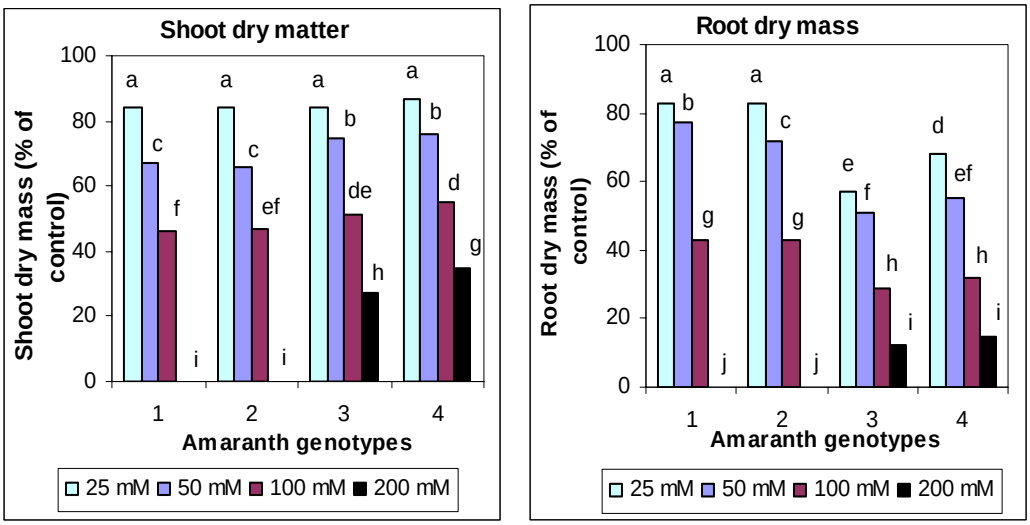

Figure 4.2 Effect of NaCl concentrations in the nutrient solution on shoot and root dry mass of amaranth genotypes: (1) *A. tricolor*, (2) Accession '83, (3) *A. hypochondriacus* and (4) *A. cruentus*. Mean separation by Tukey's t- test. For each parameter bars followed by the same letter are not significantly different at P = 0.05.

Dry matter partitioning into roots, stems and leaves expressed as a ratio of plant total dry mass for each genotype is presented in Figure 4.3. Under control conditions, all four amaranth genotypes had a similar root dry mass ratio (Figure 4.3). Salinity stress did not affect root dry mass ratio in *A. tricolor*, but it had significant effect on that of the other three genotypes. In Accession '83 salinization with 25 mM NaCl increased root dry mass ratio from 0.3 to 0.4. No further increase was noted when plants were salinized with 50 mM NaCl. At 100 mM NaCl root dry mass ratio was again reduced and was similar to that of the control. In *A. hypochondriacus* and *A. cruentus*, salinity stress decreased root dry mass ratio with the greatest reduction (from 0.3 to 0.1) obtained when plants were salinized with 200 mM NaCl.

Stem dry mass ratio was not affected by salinity stress in any of the genotypes (Figure 4.3). *A. tricolor* and Accession '83 had a higher leaf dry mass ratio than *A. hypochondriacus* and *A. cruentus* under control conditions. Salinity stress did not affect leaf dry mass ratio in *A. tricolor*, but significantly increased it in *A. hypochondriacus* and *A. cruentus*. In Accession '83 leaf dry mass ratio decreased with salinity stress at 25 and 50 mM NaCl and increased to the control value at 100 mM NaCl.

It is worthwhile to note that in the vegetable type amaranth (*A. tricolor* and Accession '83) the ratio of stem dry mass was generally lower than that of the leaf dry mass at all NaCl concentrations, while the opposite was observed for the grain types (*A. hypochondriacus* and *A. cruentus*). Figure 4.3 shows that salinity stress affected leaves, stems and roots of *A. tricolor* to the same extent, but for *A. hypochondriacus* and *A. cruentus* root growth was more affected by increasing salinity with the results that an increasing fraction of the limited assimilates were utilized for leaf growth.

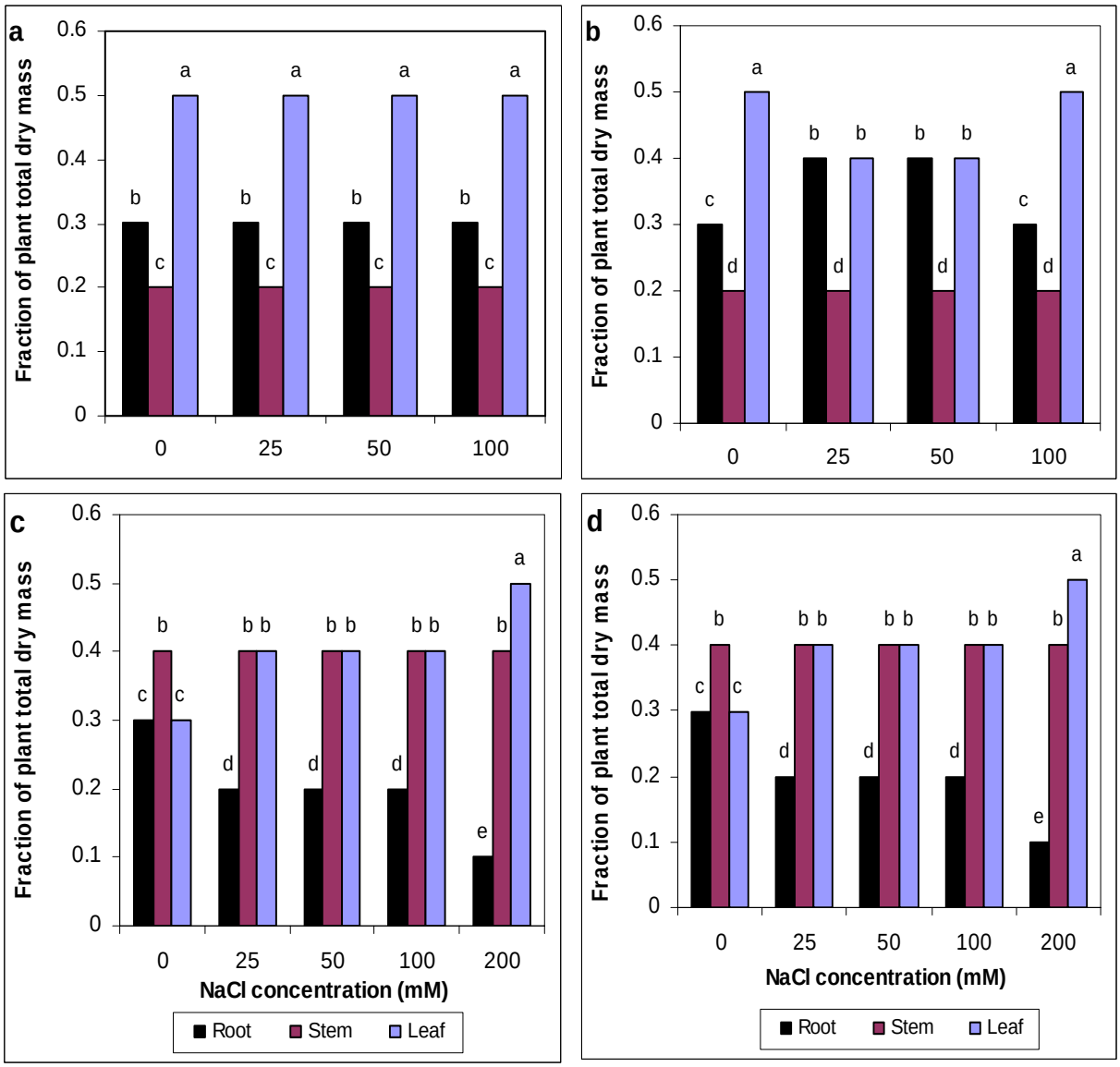

Figure 4.3 Effect of NaCl concentrations in nutrient solution on dry matter partitioning in root, stem and leaves of (a) *A. tricolor*, (b) Accession '83, (c) *A. hypochondriacus* and (d) *A. cruentus* grown under different NaCl concentrations. Mean separation by Tukey's t- test. For each genotype bars followed by the same letter are not significantly different at P = 0.05.

4.4.2 Gas exchange

Salinity significantly affected stomatal conductance (g_s) and photosynthetic rate (P_n). The interaction between salinity and genotype was also highly significant, indicating that genotypes differed in response to salinity stress. Stomatal conductances of *A. tricolor* and Accession '83 were higher than for *A. hypochondriacus* and *A. cruentus* at all salinity treatments (Figure 4.4a). Salinity stress significantly reduced stomatal conductance. The average reduction in all genotypes was 28% when 25 mM NaCl was applied. However, at 100 mM NaCl, the reduction in g_s was greater in *A. hypochondriacus* and *A. cruentus* (53%) in comparison to that of *A. tricolor* and Accession '83 (47%) (Figure 4.4a). The relative reduction of stomatal conductance exceeded that of CO_2 assimilation. A significant correlation between CO_2 assimilation rate and stomatal conductance ($r = 0.94$) indicated that the response of CO_2 assimilation to salinity was strongly associated with stomatal conductance.

The response of P_n to salinity stress was similar to that of g_s. Increasing salinity progressively reduced CO_2 assimilation rate. At the lowest NaCl concentration (25 mM) the reduction in photosynthetic rate ranged from 14% in Accession '83 to 21% in *A. cruentus* (Figure 4.4b). With increasing NaCl concentrations, the reduction in P_n was much greater in *A. hypochondriacus* and *A. cruentus* than in *A. tricolor* and Accession '83. For instance, at 50 mM NaCl, P_n was reduced by 19% in *A. tricolor* compared to 28% in *A. cruentus*.

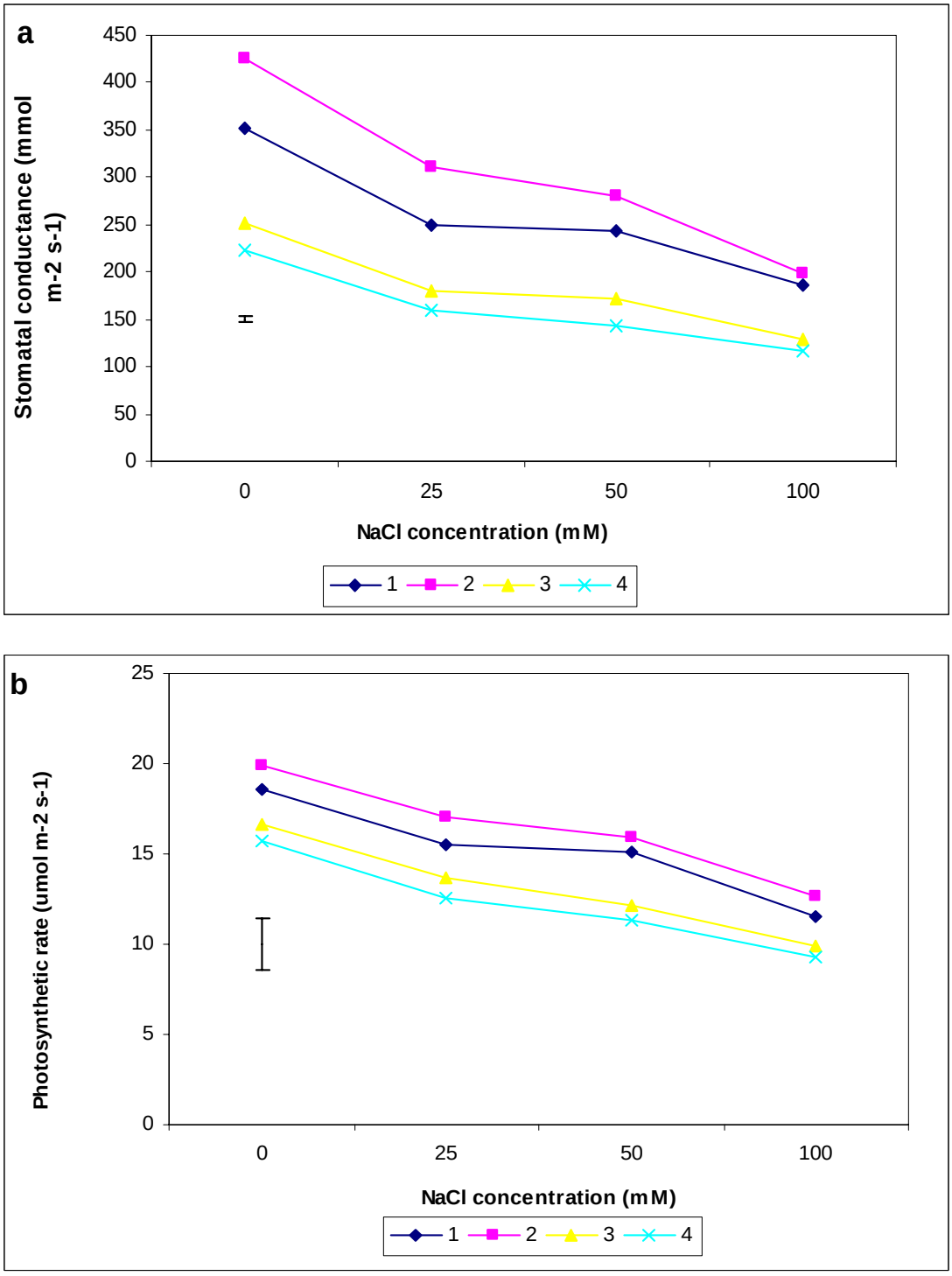

Figure 4.4 Effect of NaCl concentrations in the growth medium on (a) stomatal conductance and (b) photosynthetic rate of four amaranth genotypes (1) *A. tricolor*, (2) Accession ‘’83, (3) *A. hypochondriacus* and (4) *A. cruentus*. Mean separation by Tukey’s t- test. Verttical bars indicate least significant differences at P = 0.05.

4.4.3 Transpiration and water use

Total water loss by transpiration as well as transpiration rates (E) were significantly reduced by increasing NaCl concentrations (Table 4.2). The reduction in total water loss by transpiration was similar in all the genotypes when 25 mM or 100 mM NaCl was applied. However, when 50 mM was applied, the reduction was lower in *A. hypochondriacus* and *A. cruentus* (38%) compared to that in *A. tricolor* and Accession '83 (44%). In *A. hypochondriacus* and *A. cruentus* the rate of transpiration was reduced to a greater extent than in *A. tricolor* and Accession '83. Salinization with 50 mM NaCl, for instance resulted in E being reduced by 35% in Accession '83 and 47% in *A. cruentus*.

Water use efficiency (WUE) increased with increasing salinity, and differences among genotypes occurred. Photosynthetic water use efficiency (WUE^a) derived from instantaneous gas exchange parameters (P_n/E) increased with increasing salinity since salinity reduced leaf transpiration rate more than that of CO_2 assimilation (Table 4.2). Water use efficiency (WUE^b) determined as the amount of dry matter produced per unit of water transpired also increased with salinity. Among genotypes, *A. cruentus* followed by *A. hypochondriacus* were the most efficient in water use, while *A. tricolor* was the least efficient genotype. The increases in WUE^b between 0 and 100 mM NaCl ranged from 21% in *A. tricolor* to 34% in *A. cruentus* (Table 4.2).

Table 4.2 Effect of NaCl concentrations in the nutrient solution on total water loss by transpiration, transpiration rate (E), and water use efficiency (WUE) of different amaranth genotypes

Genotype/NaCl concentration (mM)	Total transpiration (kg/plant)	Transpiration rate (E) (mmol m^{-2} s^{-1})	WUE[a]	WUE[b] g/kg
A. tricolor				
Control	2.5c	5.5ab	3.4de	3.3l
25	1.9f	3.9d	3.9c	3.6k
50	1.4g	3.6de	4.2bc	3.8j
100	0.9h	2.6fg	4.4ab	3.9j
200	0.0j	0.0i	0.0f	0.0m
Accession '83				
Control	2.6c	5.8a	3.4de	3.5k
25	2.0ef	4.5c	3.8cd	3.9j
50	1.5g	3.8d	4.2bc	4.1i
100	1.0h	2.8f	4.5ab	4.3h
200	0.0j	0.0i	0.0f	0.0m
A. hypochondriacus				
Control	3.5a	5.3b	3.1e	4.9g
25	2.6c	3.7de	3.7cd	5.5f
50	2.1de	2.9f	4.1bc	5.9e
100	1.4g	2.2g	4.5ab	6.3c
200	0.7i	1.6h	4.7a	6.9b
A. cruentus				
Control	3.5a	5.1b	3.1e	5.0g
25	2.8b	3.3e	3.8cd	5.6f
50	2.2d	2.7f	4.2bc	6.1d
100	1.5g	2.0g	4.6ab	6.7b
200	0.9h	1.5h	4.7a	7.3a
SEM	**0.51**	**0.92**	**0.10**	**0.04**

[a] - Expressed as the ratio P_n/E
[b] - Expressed as dry matter per kg of water used
SEM: Standard error of the mean
Mean separation by Tukey's t- test. Means within each column followed by the same letter are not significantly different at P = 0.05.

4.4.4 Effect of salinity stress on leaf cell ultrastructure

4.4.4.1 *Stomatal density and pore size*

In both genotypes, increasing NaCl concentration resulted in a decrease in the number of stomata (Table 4.3). *A. tricolor* had a higher number of stomata than *A. cruentus.* Salinity stress reduced the number of stomata on the upper and lower leaf surface by 47 and 45% in *A. cruentus* compared to 20 and 19% in *A. tricolor*. The length of stomatal aperture on the upper leaf surface was not affected by salinity in *A. cruentus,* while that of *A. tricolor* was reduced by 45%. On the lower leaf surface, the length of stomatal aperture was reduced by 38% in *A. cruentus* and 42% in *A. tricolor* (Table 4.3). Differences between the effect of salinity stress on stomatal density and closure are shown in the micrographs (Figure 4.5). The stomata of plants under salt treatment were either completely or partially closed when compared to plants in control (Figure 4.5). The effect of salinity stress on epidermal cell size is also clear in the micrographs. Epidermal cells were reduced in size with increasing NaCl concentration in both genotypes (Figure 4.5).

Table 4.3 Effect of NaCl concentrations on the number of stomata and stomatal aperture on the upper and lower leaf surfaces of two amaranth genotypes

Genotype/NaCl concentration	Upper leaf surface		Lower leaf surface	
	Stomatal number per mm^2	Length of stomatal aperture (µm)	Stomatal number per mm^2	Length of stomatal aperture (µm)
A. tricolor				
0 (control)	267a	9a	272a	12a
100 mM	213b	5b	221b	7bc
A. cruentus				
0 (control)	152c	6b	163c	8b
100 mM	80d	4b	89d	5c
SEM	**0.57**	**0.54**	**0.61**	**0.56**

SEM: Standard error of the mean
Mean separation by Tukey's t- test. Means within each column followed by the same letter are not significantly different at P = 0.05.

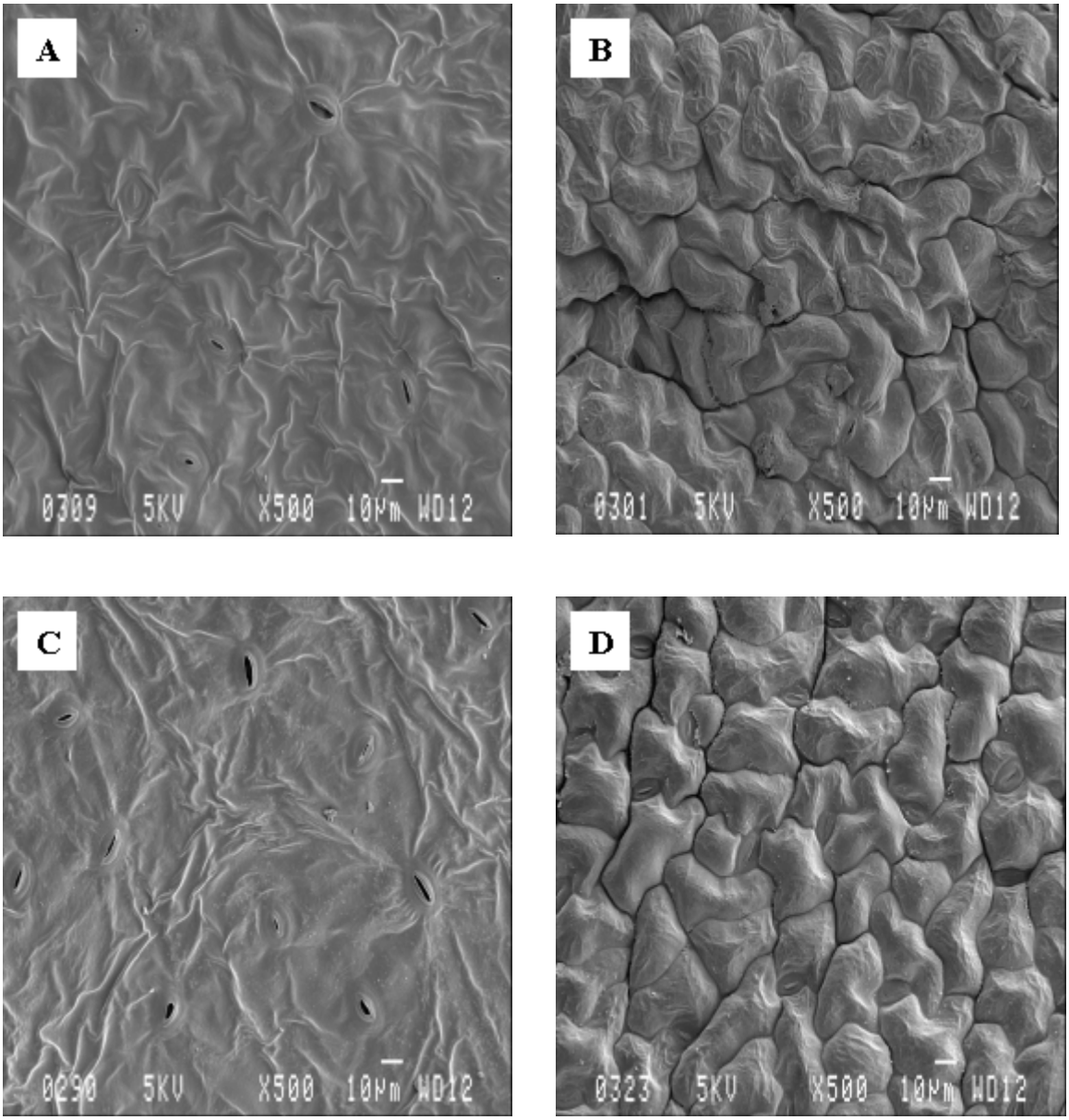

Figure 4.5 Scanning electron micrographs showing stomates and cell size on the upper leaf surface of two amaranth genotypes. A, B (*A. cruentus* at control and salinized with 100 mM NaCl); C, D (*A. tricolor* at control and salinized with 100 mM NaCl). Bars = 10 µm.

4.4.4.2 Mesophyll thickness

Salinity stress induced changes in leaf anatomical characteristics. In particular, it resulted in an increase in the size of almost all the cells of the mesophyll, as well as the entire lamina thickness (Table 4.4; Figure 4.6). The main treatment effects (genotype and salt level) were significant but the interaction between these factors was not significant indicating similar reactions in the two genotypes. The palisade layer and total leaf thickness of *A. cruentus* were greater than in *A. tricolor*. However, when other leaf histological components were considered no genotypic differences were found. Salinity stress induced an increase in the thickness of all the leaf components compared to the control (Table 4.4).

Table 4.4 Main effects of genotype and salinity stress on leaf tissue thickness of amaranth

Main effect	Tissue thickness (µm)				
	Upper epidermis	Palisade	Spongy	Lower epidermis	Total thickness
Genotype					
A. tricolor	17a	29.0b	30.5a	13.0a	90.0b
A. cruentus	18a	43.5a	30.0a	12.5a	104.0a
SEM	**0.73**	**0.64**	**0.64**	**0.64**	**0.54**
NaCl level (mM)					
0	15.5b	31.0b	29.0b	11.0b	86.5b
100	20.0a	41.5a	31.5a	14.5a	107.5a
SEM	**0.73**	**0.64**	**0.64**	**0.64**	**0.54**

SEM: Standard error of the mean
Mean separation by Tukey's t- test. Means within each column followed by the same letter are not significantly different at P = 0.05.

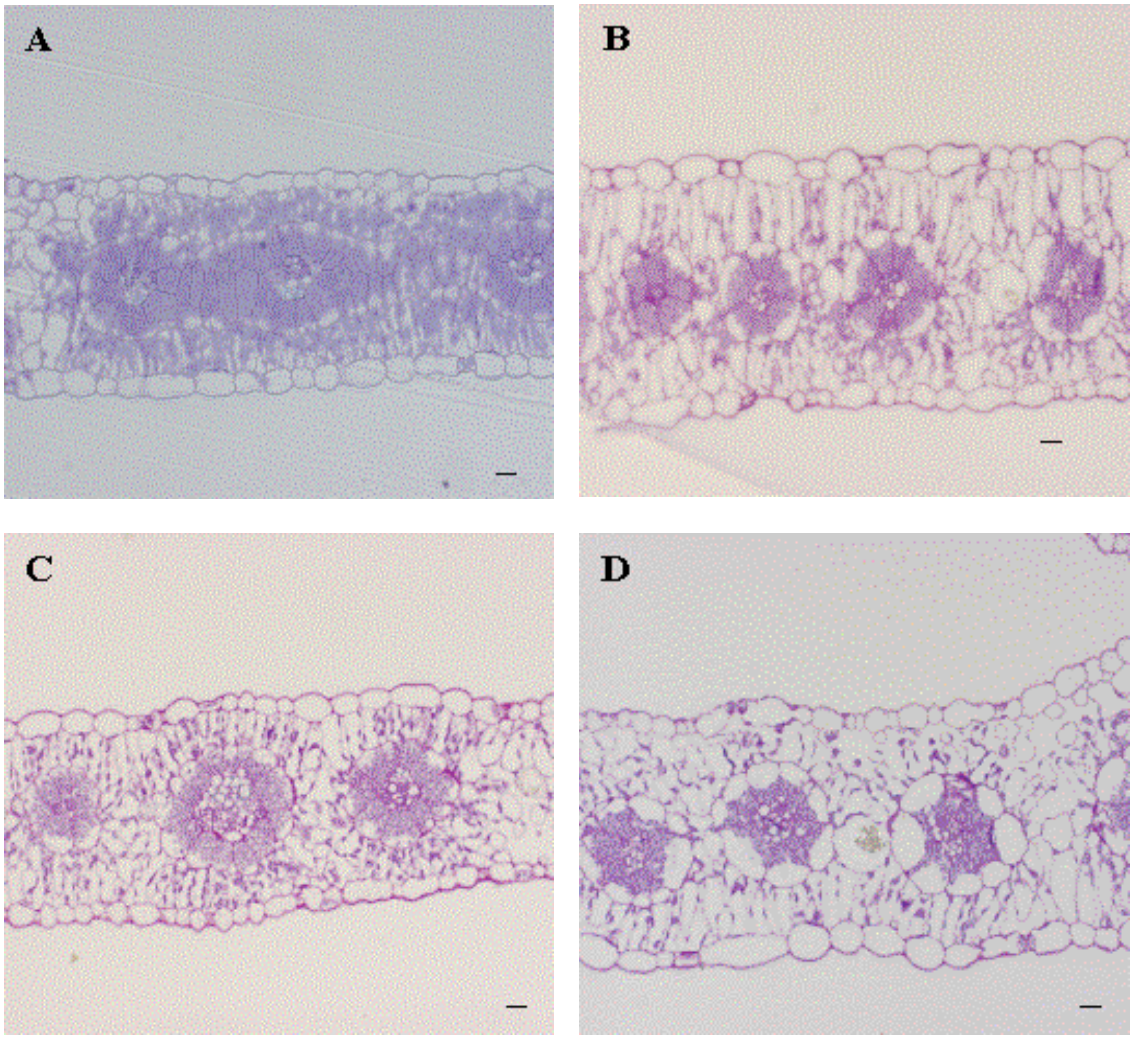

Figure 4.6 Leaf cross sections of *A. cruentus* (A – control, B – salt stressed) and *A. tricolor* (C – control, D – salt stressed) showing different tissue thickness. Bars = 10 µm

4.5 DISCUSSION

4.5.1 Plant growth

Salinity stress caused reductions in plant height, leaf area shoot and root dry mass in all amaranth genotypes, although the relative effects varied and the classification of the genotype for its salt tolerance would vary according to the parameter used. For example, on the basis of leaf area, plant height and shoot dry mass *A. hypochondriacus* and *A. cruentus* were more tolerant than *A. tricolor* and Accession '83 at 100 mM NaCl, while the reverse was true for root dry mass. Differences in sensitivity among genotypes could not be distinguished when 25 mM NaCl was applied. At the highest NaCl concentration (200 mM), *A. hypochondriacus* and *A. cruentus* were more tolerant. The effect of salinity on leaf area was greater than on shoot length (Table 4.1). It is well known that salinity reduces plant growth and that there are differences in tolerance to salinity among species and among cultivars (Cruz *et al*., 1990; Bolarin *et al.,* 1991; Romero-Aranda *et al*., 2001). Ashraf (2001) recorded a significant reduction in mean fresh and dry mass of shoots of *Brassica* species with increase in salt concentration and observed that the response to salinity stress differed with species and the measured growth variable.

Leaf area was significantly correlated to leaf number (r = 0.88), suggesting a lower leaf production as one of the reasons for reduced leaf area development under salinity stress. The size of individual leaves also decreased as salinity increased (Figure 4.1). The reduction in leaf area implies that the area available for transpiration and assimilate production would be reduced, thereby reducing the growth of plants. Thus, assessment of the ability of a genotype to maintain a large leaf area, during salinity stress would help in characterizing the genotypes as salinity stress tolerant or susceptible. At 100 mM NaCl, the reduction in leaf area in *A. tricolor* and Accession '83 was greater than that in *A. hypochondriacus* and *A. cruentus*. Hence, the latter two amaranth genotypes may be considered more salt tolerant than the former genotypes (Table 4.1).

The response of root growth to salinity stress in relation to shoot growth differed with the level of NaCl used and the genotype. Genotypic differences in dry matter production and partitioning under stress can be used as indicators of tolerance to salinity stress. Similar sensitivity in terms of root and shoot growth was noted in *A. tricolor* and Accession '83 when plants were salinized with either 25 or 100 mM NaCl. However, at 50 mM NaCl root biomass was higher than shoot biomass (Figure 4.2). Sobrado and Turner (1986) found similar root to shoot ratios in well-watered and water-stressed plants of *Helianthus petiolaris* Nutt. and *Helianthus annuus* L. and suggested that it might have been due to a similar degree in osmotic adjustment in root and leaf cells. A high root dry mass could indicate an increased capacity of water uptake, thereby maintaining the shoot in a well-hydrated condition (Blum, 1996). In *A. hypochondriacus* and *A. cruentus* shoot biomass was higher than root biomass at all levels of NaCl. Sustaining shoot growth during salinity stress is particularly important for genotypes cultivated as leafy vegetable crops. A reduction in root growth with increasing root zone salinity was also observed by Bassil and Kaffka (2002) with safflower. According to Bassil and Kaffka (2001) earlier physiological maturity could have accounted for some of the observed reduction in root growth of safflower grown in saline plots.

The results from this study contrast with observations from most investigators who found that roots were less affected by salinity than shoots (Brugnoli and Bjorkman, 1992; Chartzoulakis *et al*., 1995; Perez-Alfocea *et al*., 1996). Fisarakis *et al*. (2001) reported that at 50 mM and especially at 100 mM NaCl, root growth of sultana vines was less affected than that of shoots resulting in high root/shoot ratios. Dalton *et al*. (1997; 2001) have discussed in great detail functional drawbacks of such a response in saline versus drought environments. More recently, Munns (2002) pointed out that under certain conditions high root/shoot ratios may actually enhance the accumulation of toxic ions into the shoot. De Pascale *et al*. (2003a) proposed that the smaller root/shoot ratio observed in salinized vs. drought affected plants may be functionally associated with the need of salt stressed plants to restrict the uptake of toxic ions to the shoot while still maintaining high turgor and a positive growth rate. According to Gunes *et al*. (1996); Hayashi *et al*. (1997); Shen *et al*. (1997) and Maggio *et al*. (2001) this may be

accomplished by simultaneously reducing root vs. shoot development and activating specific metabolic pathways (i.e., osmolyte biosynthesis), both of which occur in saline environments.

At the highest NaCl concentration (200 mM), the leaf dry mass was higher than stem dry mass in *A. hypochondriacus* and *A. cruentus*. Similar results were obtained by Sifola and Postiglione (2002) who found that increasing salt concentration in the irrigation water increased assimilate partitioning towards the leaf and decreased that towards the stem.

4.5.2 Transpiration and water use

In the four amaranth genotypes, total transpiration as well as transpiration rate (E) decreased with increasing NaCl concentration. Similar decreases in transpiration rate with increasing salinity were recorded by Ashraf (2001) with *Brassica* species. Bassil and Kaffka (2002) observed that consumptive water use and biomass declined at high EC and that safflower's evaporative demand was correlated with reduced height and leaf area in saline plots. According to Pang and Letey (1998) increasing soil or water salinity reduces transpiration and increases drainage for a given irrigation volume.

The reduction in transpiration with salinity should be related to the reduced g_s and the lower stomatal density of leaves developed under saline conditions as indicated by the close correlation found between these parameters. Amaranth growth reduction resulting into reduced leaf number and leaf area could probably be the main origin of the observed reduction in water uptake and transpiration. Reduction in water uptake has also been related to reduction in hydraulic conductance of the root system (Rodriguez *et al*., 1997). This may explain the reduction in water absorption rate and may contribute to a similar reduction in nutrient uptake, resulting in retarded plant growth and decreased dry-matter yield under salt stress conditions.

Water use efficiency in the four amaranth genotypes increased with increasing NaCl concentration. This increase may be due to the large decrease in transpiration rate

compared to photosynthetic rate. Ashraf (2001) recorded increasing water use efficiency of the salt tolerant *Brassica* species with increasing external salt concentration and attributed this increase to relatively higher assimilation rates and lower stomatal conductance in these species. Studies have shown that WUE of a crop is related to the morphological characteristics of leaves. Wright *et al*. (1994) proposed that under field conditions at moderate temperatures, there is a close negative relationship between WUE and SLA. Salinity stress reduced SLA and increased WUE in amaranth. This is probably part of an adaptive mechanism to reduce leaf area and transpiration (Craufurd *et al*., 1999). According to Thumma *et al*. (2001) the relationship between WUE and SLA may be due to the fact that plants with low SLA (thicker leaves) have more mesophyll cells per unit area, leading to higher rates of CO_2 assimilation, and consequently, higher biomass production. However, CO_2 assimilation in amaranth was also reduced with increasing salinity. Bayuelo-Jiménez *et al*. (2003) argued that the lower SLA of salt stressed plants probably reflects an overloading of the leaves with inorganic and organic solutes, which allows osmotic adjustment but reduces the efficiency for gaining carbon.

4.5.3 Gas exchange

Salinity significantly reduced P_n and g_s of all the genotypes and the reduction was proportional to the increase in NaCl level. Similar results were reported by Ashraf (2001) with *Brassica* species where both photosynthetic rate and stomatal conductance showed significant decreasing trends with increase in salt concentration in the rooting medium. Gas exchange response of tobacco to saline treatments was markedly decreased even at the lowest level of salinity (2.5 dS m^{-1}) (Sifola and Postiglione, 2002). Similarly, Bayuelo-Jiménez *et al*. (2003) reported a reduction in photosynthetic carbon assimilation in *Phaseolus* species and attributed this decrease to reduced stomatal conductance.

The reduction in net carbon dioxide assimilation by increased salinity could be due to a limitation of CO_2 supply as a result of stomatal closure (Perera *et al*., 1994; Steduto *et al*., 2000); to non-stomatal factors related to the toxic effect of salts in the activity of the photosynthetic mesophyll thus depressing specific metabolic processes in carbon uptake

(Seemann and Critchly, 1985; Sultana *et al*., 1999; Chen *et al*., 1999); inhibition in photochemical capacity or a combination of these factors (Everard *et al.*, 1994; Dubey, 1997). Although the role of stomatal vs non-stomatal responses to NaCl salinity was not distinguished in amaranth, the results showed a close relationship between P_n, g_s and stomatal density making it clear that the reduction in net CO_2 assimilation with salinity could be explained by the reduction in g_s and stomatal density. Xu *et al* (1994) and Romero-Aranda *et al.* (2001) demonstrated for tomato exposed to high electrical conductivity in the root medium, that net CO_2 assimilation is more affected by the limitation of CO_2 supply than by biochemical processes in the leaf mesophyll. Meloni *et al*. (2003) also reported that the stomatal closure limited leaf photosynthetic capacity in the NaCl-treated cotton plants.

Higher stomatal conductance in plants is known to increase CO_2 diffusion into leaves thereby favoring higher photosynthetic rates. Higher net assimilation rates could in turn result in a higher biomass and higher crop yields (Taiz and Zeiger, 1998). However, this may not always be the case as was observed by Ashraf (2001). In his study with six *Brassica* species he found no significant relationship between photosynthetic rate and stomatal conductance although these two variables declined consistently with increase in salt concentration of the growth media. It was similarly shown by Melesse and Caesar (1992) with *Vicia faba* that stomatal conductance bore little relationship with photosynthetic rate.

It is noteworthy that higher CO_2 assimilation rates and stomatal conductance occurred in *A. tricolor* and Accession '83 than in *A. hypochondriacus* and *A. cruentus*. The characteristic of higher stomatal conductance and CO_2 assimilation can be regarded as an adaptive mechanism to salinity. According to Plaut *et al*. (1990) a higher stomatal conductance could be associated with a higher stomatal density. A higher stomatal density was observed in *A. tricolor* compared to *A. cruentus* (Table 4.3). This trait may allow maintenance of CO_2 exchange (Lynch *et al*., 1992). Stomatal density is a character of special interest in terms of its potential utility in genetic improvement since it may be related to leaf water use and hence, water use efficiency. Further studies to determine the

extent of variability in physiological traits related to leaf photosynthesis for salinity tolerance are greatly needed.

4.5.4 Leaf anatomy

In amaranth, salt-stressed leaves were thicker than in control plants (Table 4.4, Figure 4.6). The dense arrangement of palisade and spongy cells may result in reduction of the diffusion conductance in salt-stressed amaranth leaves. According to Evans *et al*. (1994) and Syvertsen *et al*. (1995) changes in leaf anatomy are likely to affect the conductance to CO_2 diffusion. Reduction of mesophyll conductance was related to mesophyll thickening in olive leaves (Bongi and Loreto, 1989). Salt-induced increase of mesophyll thickness may have contributed to reduced mesophyll conductance and photosynthesis in cotton (Brugnoli and Bjorkman, 1992). However, it is possible that the observed reduction in mesophyll conductance in salt-stressed leaves is caused in part by ultrastructural features related to the stress such as reduced chloroplast adherence to the cell wall (Sharkey *et al.*, 1991). Contrary to the results obtained with amaranth, low salt accumulation slightly decreased the thickness of spinach leaves (Delfine *et al*., 1998). However, the reduction in P_n in the salt-stressed spinach leaves was suggested to be associated with a reduction of the intercellular spaces in the mesophyll with respect to controls and may have caused a restriction of carbon flow towards the chloroplasts. These results support the idea that a direct relationship between leaf porosity and mesophyll conductance exists (Loreto *et al*., 1992; Evans *et al*., 1994; Syvertsen *et al.*, 1995).

A reduction in the number of stomata as well as size of stomatal apertures with salinity stress was noted in amaranth. A mechanism of water economy in salt-stressed plants is the reduction of transpiration by closure of stomata. On the other hand, the rate of photosynthesis is also reduced since CO_2 is prevented from entering the mesophyll. This conforms to data obtained from this study. *Amaranthus tricolor* which had a higher number of stomata and larger stomatal apertures also had a higher photosynthetic rate and transpiration rate compared to *A. cruentus* (Table 4.2, 4.3, Figure 4.4). Salinity stress also resulted in a decrease of the cell size (Figure 5). According to Oertli *et al*. (1990), the

small size contributes to a resistance against cell collapsing due to arid conditions. Small epidermal cells have been found to be at least 20 times more resistant to collapse than large ones. Cutler *et al*. (1977) and Steudle *et al*. (1977) have considered the reduction in cell size under water stress as a drought adaptation mechanism. According to Cutler *et al*. (1977) the reduction in cell size appears to be a major response of cells to water deficiency that may be caused either by drought or salinity stress.

The salinity level at which amaranth shoot yield was reduced by 50% was approximately 75 mM NaCl (10 dS m^{-1}) for *A. tricolor* and Accession'83, 100 mM (12 dS m^{-1}) for *A. hypochondriacus* and 125 mM (14.6 dS m^{-1}) for *A. cruentus.* According to the classification of salt tolerance of herbaceous crops (Maas, 1986) and vegetable crops (Shannon and Grieve, 1999), amaranth can be classified as moderately tolerant and compares well with other vegetables crops such as cowpea and Brassica.

4.6 CONCLUSIONS

In all four amaranth genotypes, salinity stress decreased plant growth. However, the sensitivity to stress differed with the level of salinity, genotype and the measured parameter. The reduction in growth due to salinity in most of the parameters was more severe for *A. tricolor* and Accession '83 than for *A. hypochondriacus* and *A. cruentus*. Salinity stress resulted in genotypic differences in dry matter partitioning. High salt tolerance of *A. hypochondriacus* and *A. cruentus* was found to be associated with their high water use efficiency, but there was little association of the tolerance of these genotypes with respect to stomatal conductance and photosynthetic rate.

The effect of salinity stress on photosynthetic rate and water use efficiency was closely related to leaf anatomical features. Salt stress induced a reduction of stomatal conductance in amaranth leaves and this reduction may have contributed to the inhibition of photosynthesis. The reduction of mesophyll conductance was associated with leaf thickness and less intercellular spaces in the mesophyll of salt-stressed leaves, which may have made the path towards the sites of CO_2 fixation more difficult.

According to the classification of salt tolerance of herbaceous crops, amaranth can be rated as moderately salt tolerant similar to other leafy vegetable crops.