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# Physio-morphological and molecular analysis for salt tolerance in chickpea (*Cicer arietinum*)

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ABSTRACT

After drought salinity is the major abiotic stress that severely affects agricultural productivity globally. Chickpea (*Cicer arietinum* L.) is the important grain legume which suffers approximately 8-10% of total global yield loss due to salinity. Screening for salt stress is difficult and traits that correlate salinity tolerance are least understood. The present study was carried out at ICAR-IARI, New Delhi 2017-18, deals with the important morphological and physiological traits like RWC (Relative water content), EL (Electrolyte Leakage), Na/K (sodium and potassium ratio) to characterize the salt tolerant genotypes under hydroponic condition which is a quick and easy method to screen large number of chickpea genotypes at initial stage under salt stress condition. Genotypes showing high RWC, low EL and Na/K ratio were tolerant like ICCV 10, JG 11, JG 62 and CSG-8962 whereas genotypes like ICC4958 and Pusa362 fall under moderately tolerant genotypes and DCP 93-3, Pusa 256, Phule G5 and SBD 377 were classified as susceptible genotypes. This study also attempts to understand the candidate genes responsible for salt-stress related pathways in chickpea genotypes based on sequence similarity approach exploiting known salt-stress responsive genes from model crops or other crop species.

**Key words**: Chickpea, Hydroponics, Salinity, Seedling Screening

Chickpea (*Cicer arietinum* L.) is the second most important grain legume and serves as a rich source of proteins (20–25%) and essential amino acids. It is also known for its unique ability to fix atmospheric nitrogen resulting in soil fertility enhancement. The global annual total production of chickpea is over 14.79 million tonnes, of which India alone contributes more than 70% (FAOSTAT 2017). Although the chickpea production potential is high, it has not been fully realized owing to several abiotic stresses, including drought and salinity stress (Jha *et al.* 2014, Kashiwagi *et al.* 2015).The yield loss in chickpea due to salinity has been estimated to be approximately 8-10% of total global production (Flowers *et al.* 2010). Chickpea is known to be sensitive to salinity at both the vegetative and reproductive stages (Samineni *et al.* 2011), which affects the productivity of the crop across the chickpea growing areas (Rengasamy 2006). Salinity stresses are among the major reasons that attenuate its production. Chickpea is highly sensitive to salinity, even a tolerant cultivar dies within 75 days when exposed to 40 mM sodium chloride (Samineni *et al.* 2011). Screening for salt stress is complex because of variation in sensitivity at various stages in the life cycle of chickpea. Seedling screening becomes the important part before going for adult plant screening which can give perfect idea of salinity tolerance in plants at early growth stage (3 to 4 week old seedlings) which is more convenient than at flowering as it is quick, take up less space and efficient in terms of time and costs. The present study deals with the important morphological and physiological parameters to characterize the salt tolerant genotypes, which may be used directly in chickpea breeding programmes for salt-stress tolerance. This study also attempts to understand the candidate genes responsible for salt-stress related pathways in chickpea genotypes based on sequence similarity approach exploiting known salt-stress responsive genes from model crops or other plant species.

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## MATERIALS AND METHODS

*Plant material*: The materials included 10 chickpea genotypes, which were procured from ICAR-Indian Agricultural Research Institute, New Delhi. These genotypes included popular released varieties of chickpea along with their pedigree in India (Table 1).

*Hydroponics experiment and salt stress imposition*: The hydroponic experiment was conducted at National Phytotron

Table 1 List of chickpea genotypes used in study along with their seedling morphological traits and physiological parameters under salt stress (EL: electrolyte leakage, RWC: relative water content, Na/K: sodium potassium ratio)

| Genotype | Pedigree | Root length (cm) | | Shoot length (cm) | | Root weight (g) | | Shoot weight (g) | | EL | RWC | Na/K |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | S | N | S | N | S | N | S | S | S | S |
| ICCV 10 | P1231 × P1265 | 15.17 | 13.53 | 17.00 | 15.00 | 0.48 | 0.39 | 0.76 | 0.49 | 21.21 | 89.22 | 0.71 |
| JG 11 | {(9Phule G5 × Narshinghpur bold) × ICCC37} | 15.00 | 12.33 | 16.67 | 15.00 | 0.50 | 0.41 | 0.72 | 0.47 | 23.36 | 88.81 | 0.81 |
| JG 62 | Local selection from west Nimar (M.P.) | 14.67 | 11.33 | 18.00 | 16.00 | 0.45 | 0.34 | 0.77 | 0.53 | 34.98 | 77.13 | 0.89 |
| CSG 8964 | Selection from GPF 7035 | 16.33 | 15.00 | 17.33 | 15.00 | 0.48 | 0.40 | 0.73 | 0.55 | 20.21 | 86.44 | 0.68 |
| ICC 4958 | Genetic stock Screened from germplasm | 11.67 | 10.00 | 14.00 | 13.00 | 0.28 | 0.22 | 0.49 | 0.37 | 32.99 | 62.97 | 1.34 |
| DCP 92-3 | Selection from local germplasm | 10.00 | 8.67 | 10.33 | 10.33 | 0.26 | 0.20 | 0.43 | 0.35 | 44.24 | 64.4 | 2 |
| Pusa 256 | (JG 62 × 850-3/27) × (L 550 × H 208) | 9.67 | 6.00 | 10.00 | 9.00 | 0.21 | 0.17 | 0.34 | 0.26 | 43.77 | 53.68 | 2.24 |
| Pusa 362 | (BG 203 × P 179) × BC 203 | 11.00 | 9.67 | 14.33 | 13.33 | 0.31 | 0.25 | 0.54 | 0.39 | 24.15 | 77.49 | 1.14 |
| Phule G-5 | Selection from local material | 8.33 | 7.33 | 11.00 | 9.67 | 0.20 | 0.14 | 0.34 | 0.27 | 31.03 | 69.75 | 1.7 |
| SBD 377 | (ICCV 88109 × PRR 1) × ICC 4958 | 9.00 | 7.00 | 8.33 | 7.67 | 0.13 | 0.11 | 0.34 | 0.24 | 47.11 | 47.45 | 2.06 |
| | Mean | 12.08 | 10.09 | 13.70 | 12.40 | 0.33 | 0.26 | 0.54 | 0.39 | 32.31 | 71.73 | 1.36 |

Facility, ICAR-Indian Agricultural Research Institute, New Delhi, India during 2017-18. The air temperature was maintained as 22/18°C (± 2°C) day/night temperature; 10/14 h light/dark photoperiod; 45% relative humidity and 450 µmol m/s/1 light intensity. Hydroponic experiment for screening was carried out by the newly develop protocol in which seed surface was sterilized with 2% sodium hypochlorite for 45 min and were soaked in Petri plate for 2 days. Soaked and sprouted seeds were transferred to sterilized germination paper on reverse osmosis (RO) water. Pre germinated seeds were transplanted on hydroponic tray and grown on Normal RO water for four days. On fifth day crates were filled with 0.5 × modified Hoagland's nutrient medium. The medium was aerated using aquarium pumps per crate. The nutrient medium was subsequently replaced with 1.0 × Hoagland's solution (*p*H 6.5) after seven days. At 18$^{th}$ day, the nutrient medium was replaced with 1.0 × modified Hoagland's with 150 mM Sodium Chloride (NaCl) (*p*H 6.5) which was to impose salinity stress. The control plants were grown in replaced 1.0 × modified Hoagland's solution (*p*H 6.5). The tissues from stressed and control plants were collected for various physiological analysis.

*Morphological traits recorded during salt stress*: Seedling response was studied by phenotyping seedling parameters like shoot length (SL); root length (RL); fresh shoot weight (FSW) and fresh root weight (FRW).

*Physiological traits recorded*: Electrolyte leakage was recorded using 10 mg fresh leaf sample, taken in test tube and immersed in 10 ml of distilled water. This test tube was kept in water bath at 45°C for 30 min. It was allowed to cool at room temperature and then water conductivity of sample ($C_1$) was measured using Electrical Conductivity Meter. Again, the test tube was kept in water bath at 100°C for 10 min. and subsequently cooled to room temperature and the final conductivity reading of the sample ($C_2$) was measured. The Electrolyte leakage was calculated as:

$$EL = [C1/ C_2] \times 100$$

Relative water content (RWC) was worked out as per protocol of Barrs and Weatherley (1962). Sodium and potassium measurement were estimated from shoot samples according to Bhargava and Raghupathi (1993). Homogenate tissue was dried at 80°C for 24 h. finely ground dried shoot samples (0.1 g) were digested in 10 ml of digestion mixture ($HNO_3$ and $HClO_4$, 9:4). The digested solution was cooled and washed in a 50 ml volumetric flask. The solution was again filtered with Whatman filter paper no. 42 and analyzed for Na and K using flame photometer (Systronics Flame photometer, microcontroller based with compressor, Type 128).

*Molecular validation of candidate genes for salt-stress tolerance in chickpea genotypes*: Genomic DNA extraction: CTAB method of DNA isolation Murray and Thomson (1980) modified by Tapan *et al*. (2014) was utilized to isolate chickpea genomic DNA.

DNA quantification: Quantification of DNA was done by analyzing the purified DNA on 0.8% agarose gel with Hind III-cut λ DNA as standard. The concentration of DNA

in individual sample was determined based on the intensity of the bands in the λ DNA ladder.

*Amplification and detection candidate genes involved in salinity tolerance:* Eight gene specific primers based on their prior information about involvement in abiotic stress especially salinity tolerance were used for validation of 10 genotypes. Primers were synthesized as per the sequences of Manish *et al.* (2013) from Bioneer, South Korea. BIORAD thermal cycler (Icycler), USA was used to carry out amplifications in 10 µl volume reaction mixture. This mixture contained 1 µl of 20 ng plant genomic DNA, 1.6 µl of 10 x Tris buffer (15 mM $MgCl_2$ and Gelatine), 1 µl of 10 mM dNTP mix, 1.0 µl each of forward and reverse primer and 0.3 µl of 3 U/µl Taq polymerase (Life Technology, India). PCR amplification profile was programmed for 35 cycles, consisting of denaturation at 94°C for 1 min, annealing at 50-60°C (depending on the primer) for 1 min and extension at 72°C for 2 min. An initial denaturation at 94°C for 5 min and a final synthesis of 10 min at 72°C were also included.

The amplification products were resolved on 3% agarose gels (Cambrex, USA) depending upon the size, stained with ethidium bromide and analyzed using the gel documentation system (AlphaImager 2200, Alpha Innotech Corp., USA).

## RESULTS AND DISCUSSION

*Phenotyping of seedling morphological traits under salt stress*: Screening for salt tolerance is more challenging because of its complex character and precise requirement of screening environment. Seedling screening analysis gives us about preliminary understanding of variation for salt tolerance in different genotypes. The analysis of variance shows that the differences among the genotypes were significant. The mean sum of square were highly significant for all the characters, viz. root length, shoot length, root weight and shoot weight indicating presence of significant variability in 10 genotypes selected for study. Therefore root and shoot length are important traits under salinity stress (Jamil and Rha 2004), similar results also seen in studies of Werner and Finkelstein (1995) and Neumann (1995). Chickpea is considered as moderately salt tolerant crop but it shows variation between genotypes in response to saline environment. The deleterious effects of salt stress on seedling growth hamper its normal development. Parameters affecting seedling growth were recorded in both stress and normal conditions and best performing genotypes were selected (Table 1).

*Characterization of physiological traits*: Physiological variables like electrolyte leakage (EL) varied from 20.21 to 47.11% with mean of 32.31%. The tolerant genotypes showed considerable lesser EL as compared to susceptible one. Similarly RWC value varied from 45.45-89.22% with average value of 71.73%, more the RWC value more is the tolerance. But Na/K ratio is inverse to the tolerance behavior; more the ratio the genotype is more susceptible to salt stress (Table 1). EL, RWC, Na/K ratio and morphological traits for biomass of plant were the parameters to access the salinity tolerance in 10 genotypes. Many workers have reported that the salinity caused yield reductions due to the effect of sodium ion (Tester and Davenport 2003, Rains and Epstein 1967, Warne *et al.* 1996, Tyerman and Skerrett 1999, Kader and Lindberg 2005). The graphical approach was followed for identification of genotypes having superior physiological parameters and morphological stress score (MSS) and these were classified as tolerant and moderately tolerant and susceptible (Fig 1). Under salt stress condition the genotypes showing high RWC, MSS, low EL and Na/K ratio were considered as tolerant like

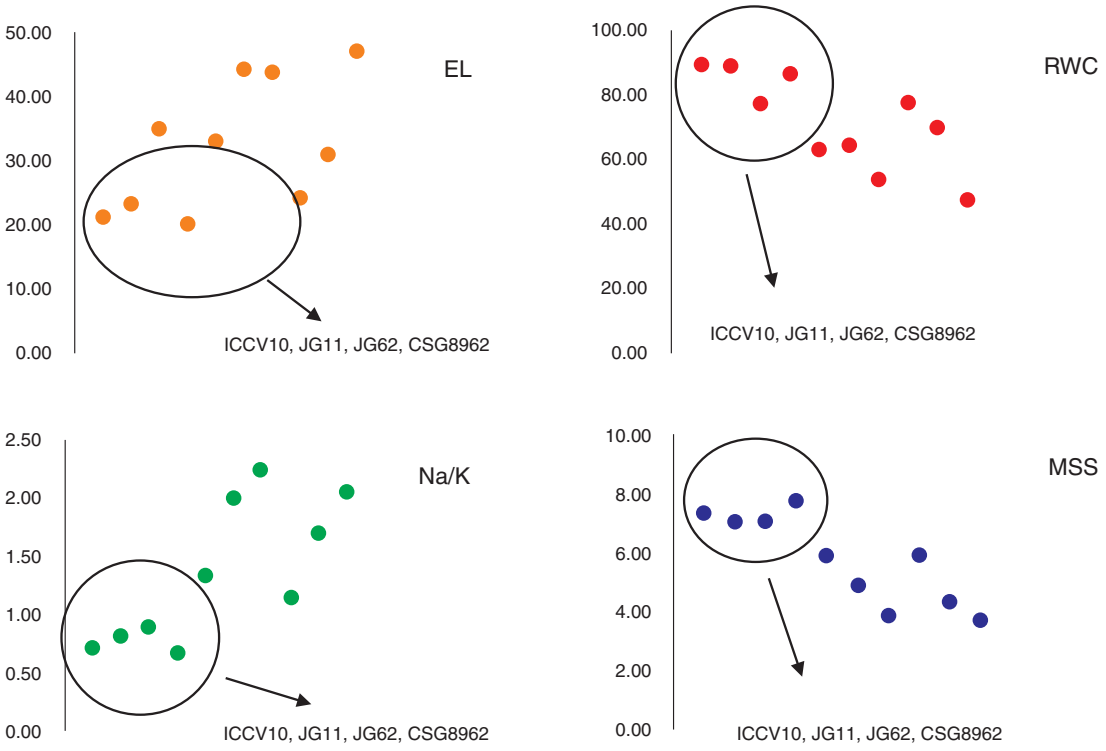


Fig 1 Graphical classification of genotypes on the basis of morpho-physiological parameters for salinity tolerance (EL: electrolyte leakage, RWC: relative water content, Na/K: sodium potassium ratio; MSS: morphological stress score).

Table 2 Correlation analysis between different traits under salt stress condition

| Correlations | EL | RWC | Na/K | RL | SL | RW | SW |
|---|---|---|---|---|---|---|---|
| EL | 1 | -.913** | .889** | -.808** | -.779** | -.779** | -.716* |
| RWC | -.913** | 1 | -.919** | .875** | .877** | .909** | .859** |
| Na/K | .889** | -.919** | 1 | -.933** | -.952** | -.914** | -.922** |
| RL | -.808** | .875** | -.933** | 1 | .889** | .937** | .944** |
| SL | -.779** | .877** | -.952** | .889** | 1 | .925** | .958** |
| RW | -.779** | .909** | -.914** | .937** | .925** | 1 | .947** |
| SW | -.716* | .859** | -.922** | .944** | .958** | .947** | 1 |

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed).

ICCV 10, JG 11, JG 62 and CSG-8962 whereas genotypes like ICC 4958 and Pusa 362 fall under moderately tolerant genotypes and DCP 93-3, Pusa 256, Phule G5 and SBD 377 were classified as susceptible genotypes on the basis of above mentioned parameters.

*Correlation analysis of morphological and physiological traits*: Correlation analysis indicates the magnitude of association between pairs of character and forms the basis of selection index. The estimates of phenotypic correlation coefficients between seven characters of different chickpea genotypes are given in Table 2. Under saline stress condition morphological traits exhibited highly significant positive correlation with RWC and significantly negative correlation with EL and Na/k ratio. The physiological parameters like Na/K ratio and EL showed significantly positive correlation (0.889), whereas RWC is significantly negative correlated with Na/K ratio (-0.919) and EL (-0.913). It was observed that plant showing more RWC and less EL and Na/K ratio were more tolerant in salt stress conditions than others. Similar results were observed by Sivasankaramoorthy (2013) indicating negative correlation for Na/K ratio content in stem with the plant yield under salt stress indicates that those genotypes are salt tolerant.

*Validation of previously reported candidate genes for salt stress tolerance*: Through this study an attempt has been made to understand the candidate genes responsible for salt stress related pathways amplifying in chickpea genotypes on the basis of Sequence similarity approach for the identification of salt stress responsive genes. Eight known candidate genes were selected on the basis of prior information about their involvement in salt tolerance mechanism in other crop species and their coding sequences available in NCBI database for abiotic stresses was utilized for similarity search against known candidate genes for salt stress tolerance (Manish *et al.* 2012) unigene sequences showing significant match with a candidate gene were selected and used for primer designing using primer3 software. Only three salt specific genes generated amplicons in the 10 genotypes studied and were CAD, DREB and DHN. Cinnamyl alcohol dehydrogenase (*CAD*) gene homologue was amplified at ~ 225bp in all chickpea genotypes using primers designed for contig showing match with cinnamyl alcohol dehydrogenase *(CAD)* gene of *Arabidopsis thaliana*. *CAD* is known to play a key role in plant defence against various abiotic stresses including salt stress. Dehydrin homologue was amplified using primer pair designed for known Dehydrin gene using chickpea unigene. Approximate amplicon size of Dehydrin (*DHN*) gene was ~300 bp. *DHNs* are one of several proteins that have been specifically associated with qualitative and quantitative changes in abiotic stresses. *DREB* (Dehydration response element binding) homologue in chickpea was also amplified using primer pairs designed using unigene showing match against *DREB* gene. Approximate amplicon size of the *DREB* gene was ~1200 bp. *DREB* is known for its role in salt tolerance in several crops. Similar results have also been established by (Manish *et al.* 2012). Further PCR amplicons can be directly used for DNA sequencing after purification using gene specific primers. Good quality sequences were then can used for confirmation of these genes in chickpea using sequence similarity approach based on sequence information of these genes from related crop species or model plants. Therefore, present study provides basic information about some salt stress responsive genes in chickpea that can be exploited to overcome salinity stress related problems limiting chickpea production.

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## REFERENCES

Allagulova C R, Gimalov F R, Shakirova F M and Vakhitov VA. 2003. The plant dehydrins: structure and putative functions. *Biochemistry* **68**(9): 945–51.

Arraouadi S, Badri M, Abdelly C, Huguet T and Aouani M E. 2012. QTL mapping of physiological traits associated with salt tolerance in *Medicago truncatula* recombinant inbred line. *Genomics* **99**: 118–25.

Barrs H D and Weatherley P E. 1962. A re-examination of the relative turgidity technique for estimating water deficits in leaves. *Australian Journal of Biological Sciences* **15**(3): 413–28.

Bhargava B S and Raghupathi H B. 1993. Analysis of plant materials for macro and micronutrients. *Methods of Analysis of Soil, Plant, Water and Fertilizers*, pp 49–82. Fertilizers Development and Consultation Organization, New Delhi, India.

Dure L, Crouch M, Harada J, Ho T HD, Mundy J, Quatrano R and Sung Z R. 1989. Common amino acid sequence domains among the LEA proteins of higher plants. *Plant Molecular Biology* **12**(5): 475–86.

FAOSTAT database. Available at http://faostat3.fao.org/faostatgateway/go/to/download/Q/QC/E (2017). Accessed 28 Jan 2019.

Flowers T J, Gaur P M, Gowda C L, Krishnamurthy L, Samineni S, Siddique K H, Turner N C, Vadez V, Varshney R K and Colmer T D. Salt sensitivity in chickpea. *Plant Cell and Environment* **33**: 490–509.

Jha U C, Chaturvedi S K, Bohra A, Basu P S, Khan M S and Barh D. 2104. Abiotic stresses, constraints and improvement strategies in chickpea. *Plant breeding* **133**(2): 163–78.

Jamil M and Rha E S. 2004. The effect of salinity (NaCl) on the germination and seedling of sugar beet (*Beta vulgaris* L.) and cabbage (*Brassica oleracea* L.). *Plant Resources* **7**(3): 226–32.

Kader M A and Lindberg S. 2005. Uptake of sodium in protoplasts of salt-sensitive and salt-tolerant cultivars of rice, Oryza sativa L. determined by the fluorescent dye SBFI. *Journal of Experimental Botany* **56**(422): 3149–58

Kashiwagi J, Krishnamurthy L, Purushothaman R, Upadhyaya H D, Gaur P M, Gowda C L, Ito O and Varshney R K. 2015. Scope for improvement of yield under drought through the root traits in chickpea (*Cicer arietinum* L.). *Field Crops Research* **170**: 47–54.

Manish R and Sharma P C. 2012. Sequence similarity base identification of abiotic stress responsive genes in chickpea. *Bioinformation* **8**(2): 092–7.

Murray M G and Thompson W F.1980. Rapid isolation of high molecular weight plant. *DNA Nucleic Acids Research* **8**(19): 4321–6.

Neumann P M. 1995. Inhibition of root growth by salinity stress: Toxicity or an adaptive biophysical response. *Structure and Function of Roots*, pp 299–304. Springer Dordrecht.

Park J M, Park C J, Lee S B, Ham B K, Shin R and Paek K H. 2001. Overexpression of the tobacco Tsi1 gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. *Plant Cell* **13**(5): 1035–46.

Purcell P C, Smith A M and Halford N G. 1998. Antisense expression of a sucrose non-fermenting-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase in tubers and loss of sucrose-inducibility of sucrose synthase transcripts in leaves. *Plant Journal* **14**(2): 195–202.

Raes J, Rohde A, Christensen J H, Van de Peer Y and Boerjan W. 2003. Genome-wide characterization of the lignification toolbox in Arabidopsis. *Plant Physiology* **133**(3): 1051–71.

Rains D W and Epstein E. 1967. Sodium absorption by barley roots: its mediation by mechanism 2 of alkali cation transport. *Plant Physiology* **42**(3): 319–23.

Rengasamy P. 2006. World salinization with emphasis on Australia. *Journal of Experimental Botany* **57**: 1017–23.

Rudrabhatla P and Rajasekharan R. 2002. Developmentally regulated dual–specificity kinase from peanut that is induced by abiotic stresses. *Plant Physiology* **130**(1): 380–90.

Samineni S, Siddique K H M, Gaur P M and Colmer T D. 2011. Salt sensitivity of the vegetative and reproductive stages in chickpea (*Cicer arietinum* L.) podding is a particularly sensitive stage. *Environmental and Experimental Botany* **71**: 260–8.

Sivasankarmoorthy S. 2013. Effect of salinity on sodium, potassium and proline content of chickpea seedlings. *International Research Journal of Pharmacy* **4**(7): 147–50.

Shukla R K, Raha S, Tripathi V and Chattopadhyay D. 2006. Expression of CAP2, an APETALA2-family transcription factor from chickpea, enhances growth and tolerance to dehydration and salt stress in transgenic tobacco. *Plant Physiology* **142**(1): 113–23.

Tapan K, Bharadwaj C, Tara Satyavathi C and Jain P K. 2014. A high throughput, improved rapid and reliable genomic DNA extraction protocol from chickpea (*Cicer arietinum* L.). *Society of Plant Research* **26**(2): 185–90.

Tester M and Davenport R. 2003. Na+ tolerance and Na+ transport in higher plants. *Annals of Botany* **91**(5): 503–27

Tyerman S D, Skerrett M, Garrill A, Findlay G P and Leigh R A. 1997. Pathways for the permeation of Na and Cl into protoplasts derived from the cortex of wheat roots. *Journal of Experimental Botany* **48**: 459–80

Warne T R, Hickok L G, Kinraide T B and Vogelien D L. 1996. High salinity tolerance in the stl2 mutation of *Ceratopteris richardii* is associated with enhanced K+ influx and loss. *Plant Cell and Environment* **19**(1): 24–32.

Werner J E and Finkelstein R R. 1995. Arabidopsis mutants with reduced response to NaCl and osmotic stress. *Physiology Plant* **93**: 659–66.

Wood A J, Saneoka H, Rhodes D, Joly R J and Goldsbrough P B. 1996. Betaine aldehyde dehydrogenase in sorghum (molecular cloning and expression of two related genes). *Plant Physiology* **110**(4): 1301–08.