^{22}Na influx is significantly lower in salt tolerant groundnut (*Arachis hypogaea*) varieties

J. A. Smitharani · M. L. Sowmyashree · K. M. Vasantha ·
M. Srivastava · V. R. Sashidhar

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Abstract Distinct varieties differing in salt tolerance were initially identified from two separate green house experiments using two systems; solution as well as soil culture. The first screening involved a diverse group of 27 cultivars. Several physiological traits; Chlorophyll Stability Index (CSI), Salt Tolerance Index (STI) and ion content were determined to screen the cultivars for differences in salt tolerance using solution culture in the first experiment. A set of six varieties (three tolerant and three susceptible) were selected from this experiment and then subjected again to salt stress adopting a natural soil system in the second experiment which involved a screening approach essentially similar to that of the first experiment. In the third experiment using two distinct cultivars differing in salt tolerance selected from experiment II, ^{22}Na influx rate was determined in the root and shoot at the end of a 24 h salt imposition in Hoagland's nutrient system containing 180 KBq of ^{22}Na . The results suggested that there were distinct differences in ^{22}Na influx rate into root and concurrently in the shoot. The salt tolerant *Spanish improved* and one of the moderately tolerant Trombay variety *TAG 24*, showed good regulation of ^{22}Na influx resulting in low ^{22}Na concentration. The salt susceptible variety *JSP39* had nearly 7–8 fold higher root ^{22}Na content as compared to the tolerant and moderately tolerant cultivars. The results have highlighted the importance of Na exclusion as an important determinant of salt tolerance in groundnut.

Keywords ^{22}Na uptake · Salt susceptible · Salt tolerant cultivars · Groundnut

Abbreviations

CMS	Cell membrane stability
CSI	Chlorophyll stability index
MI	Membrane integrity
NOA	Net osmotic adjustment
OA	Osmotic adjustment
OD	Optical density
RWC	Relative water content
STI	Salt tolerance index
TTC	2,3,5 Triphenyl tetrazolium chloride

Introduction

It is well known that there are two specific effects of salt stress (Munns et al. 1995). In this model, the first phase is represented by the osmotic component, while the second phase describes the salt specific or ion excess effects of salinity.

This model implies that there is very little evidence of genetic differences in the osmotic component of salt stress. Much of the work between 1995 and 2007 greatly emphasized the second component as the main pathway to improved salt tolerance (Tester and Davenport 2003; Munns and Tester 2008; Almansouri et al. 1999). The concept of salt exclusion has recently come in for scrutiny and re-assessment based on the work of (Davenport et al. 1997), who found no correlation between the ability to exclude Na^+ and salt tolerance among genotypes of bread wheat. The results of the study were in direct contradiction to the earlier study of (Munns and James 2003), where a high correlation was observed between leaf 3 Na^+ concentrations and salt tolerance in durum wheat. The

J. A. Smitharani · M. L. Sowmyashree · K. M. Vasantha ·
V. R. Sashidhar (✉)

Department of Crop Physiology, Stress Physiology Lab, University
of Agricultural Sciences, GKVK, Bangalore 560 065, India
e-mail: vrsashi51@gmail.com

M. Srivastava
NABTD, BARC, Trombay, Mumbai 400085, India

fact that genetic differences in the first phase i.e., osmotic effects also cannot be overlooked has gained importance from the recent re-evaluation of tolerance amongst varieties of durum wheat (James et al. 2008).

With this background, we have attempted to determine differences in Na^+ flux rates into the root using a radiometric approach as a reflection of exclusion ability among contrasting cultivars of Rice. In this approach, the ^{22}Na isotope is invariably used as it has a longer half life (2.6 years) as compared to ^{24}Na which has an extremely short half life of less than 15 h. ^{22}Na uptake differences were assessed in two wheat cultivars differing in salt tolerance (Davenport et al. 1997). A similar approach was taken to determine variation in ^{22}Na uptake among 232 accessions of tetraploid wheat (Nevo et al. 1992). The results of both these studies were surprisingly contradictory. While the former study (Davenport et al. 1997) found no significant differences in ^{22}Na uptake in contrasting wheat varieties differing in salt tolerance, the latter study (Nevo et al. 1992), on the other hand, found significantly low ^{22}Na uptake in accessions adapted to salt affected sites. In our study, selection for contrasting cultivars with significant differences in salt tolerance was based on a preliminary screening and included ion content Na^+ K content, K/Na selectivity and a few other physiological traits. The results suggested that differences in ^{22}Na flux rates was significantly lower in the salt tolerant varieties of groundnut.

Materials and methods

Two experiments were conducted in the green house of Department of Crop physiology, University of Agricultural Sciences, Bangalore.

Experiment 1

Twenty-seven varieties of groundnut representing a diverse collection from different agro-climatic zone were screened for salt tolerance using a Soilrite-Hoagland system. Included in this was three potentially salt tolerant varieties developed by mutation breeding at the Bhabha Atomic Research Centre (BARC) Trombay, Mumbai. 1. TPG-41, 2. TAG-24 and 3. TG-37-A. Three drought tolerant varieties developed by National Research Centre on Ground nut (NRCG) were also included; Junagadh 11, (T-11), Gangapuri and GG-2. The following traits were used for the screening. Ion content (Na^+ and K^+ content) of the root and shoot in response to a salt stress of 200 mM NaCl.

Raising the plant material

The Soilrite/Hoagland's system for imposition of salt stress

The method developed by USDA (Peel et al. 2004) was followed with minor modifications. Twenty cultivars

representing the entire range of diversity from different agro climatic zones of India were raised in thermocole cups (10 cm long and 5" diameter). In order to facilitate the easy entry of roots through the cup, five small holes were made at the bottom of the cup. These cups were filled with Soilrite and placed on the sand bed to facilitate easy entry of roots into the sand, and also to enable the removal of cups easily. Around five to eight seeds were sown in each cup. After 10 days of sowing, thinning was done to maintain two seedlings per cup. The seedlings were grown for a period of 25 days and the roots were allowed to grow into the sand bed. Later they were taken out of the sand bed to free the emerged roots. There were six replications of each genotype in each treatment.

These cups along with the free hanging roots were transferred to the plastic trays containing quarter strength Hoagland's solution. The cups were placed in such a way that the free hanging roots were completely immersed in the solution, while care was taken not to damage roots. This facilitates quick absorption of nutrients from the Hoagland's solution through the roots. Six such cups (six replications) were placed per tray. The seedlings were allowed to acclimatize for the new environment for a period of 24 h induction with 50 mM 5:1 ratio of (NaCl : CaCl_2). This setup was maintained under rainout shelter to protect from rain. After 24 h of induction period, Hoagland's solution was decanted from the tray and two treatments were imposed. a) T1-control or non stress b) T2-250 mM NaCl stress. A continuous stream of air bubbled through the solution in the lower tray ensured that the seedlings were not subjected to anaerobic conditions.

Screening for salt tolerance: physiological traits

- a) **Chlorophyll Stability Index:** Chlorophyll Stability to salt stress was assessed according to the method of Murthy and Majumdar 1962, and Chlorophyll Stability Index (CSI) was modified as the percentage of chlorophyll of the salt stressed sample relative to its content in the fresh sample as follows

$$\text{CSI} = \frac{\text{Content of chlorophyll in salt stressed sample}}{\text{Content of chlorophyll in control sample}} \times 100$$

Total chlorophyll content was calculated using the method of Hiscox and Israel Stam 1979 involving the use of acetone: DMSO. The advantage of this method over other methods involving acetone alone is that the leaf tissue need not be macerated.

- b) **Salt tolerance index (STI):** The procedure of Seydi et al. 2007 was adopted. Salt tolerance was determined using the formula,

$$\text{STI} = \frac{\text{Total dry weight of salt stressed plant}}{\text{Total dry weight of control plant}} \times 100$$

Ion content Na content, K content in root and shoot.

Sample preparation Samples were ground separately in pestle and mortar to make a fine powder. 5 mL of concentrated nitric acid was added to 200 mg of powdered sample and incubated in a digestion hood overnight. The next day 5 mL of di-acid mixture (10:4::nitric acid: perchloric acid) was added to it and placed on a sand bath till all the white fumes evaporated and thick white residue was left out in flask. It was allowed to cool and volume was made up to 25 mL using triple distilled water and further dilutions were made if the concentration of the solution was too high. These diluted solutions were used for sodium and potassium estimation (Mohammed et al. 2010).

Estimation of sodium and potassium Sodium and potassium were estimated in the root and shoot samples using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Thermo Fisher).

Standard preparation Using the 1000 ppm Na^+ and K^+ standard solution, 100 ppm standards of Na^+ and K^+ solution were prepared and suitable dilutions were made to get standard solution ranging from 0 to 10 ppm. A standard curve was obtained and the ion content of the standards was determined from this. The sample readings were compared with the standard.

$$\text{Na}^+/\text{K}^+ = \frac{\text{Average ppm} \times \text{Dilution factor}}{\text{Weight of the sample}}$$

Sodium and potassium content was expressed in mmols/g dry weight (Munns and James 2003)

$$\text{K}^+/\text{Na}^+ \text{ ratio} = \frac{\text{Potassium (mmols/g dry weight)}}{\text{Sodium content (mmols/g dry weight)}}$$

Statistical analysis

Data in experiment 1 and experiment 2 was subjected to statistical analysis using methods described in Snedecor and Cochran (1989). Data in Tables 1 and 3 has Critical Difference (CD) calculated from Standard Error of Means (SEM). Data in Figs. 2 and 3 has standard errors determined by methods described by Snedecor and Cochran (1989).

Experiment 2

Three salt tolerant and three susceptible variety along with three moderately tolerant Trombay varieties from experiment 1, using the Soilrite-Hoagland system were again screened for salt tolerance in the green house in containers holding up to 15 kg soil by a method developed at the Central Soil Science Research Institute (CSSRI) Karnal, India. The method involves equilibrating the soil with a mixture of salts (NaCl , CaCl_2 , MgCl_2 and Na_2SO_4) to simulate natural salt stress in the field. Salt stress of 8 dS/m was imposed when the plants were 30 days old. Screening for salt tolerance was done using a physiological approach based on three parameters. Only those cultivars which were either tolerant or susceptible with respect to all three traits were included for uptake studies in the third experiment.

Cell Membrane Stability (CMS) CMS was determined adopting the procedure of (Tripathy et al. 2000).

$$\text{CMS}(\%) = [1 - \text{T1}/\text{T2}] [1 - \text{C1}/\text{C2}] \times 100$$

Where T1 and T2 are treatment conductivities before and after autoclaving and C1 and C2 are the respective control conductivities.

Relative Water Content (RWC)

The RWC was determined using the fresh weight and turgid weight of leaves (Annick et al. 2004).

$$\text{RWC}\% = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

Osmotic adjustment

Leaf samples were frozen in liquid nitrogen, thawed, and centrifuged for 5 min at 20000 rpm. The Ψ_s of the extracted sap was measured by VAPRO vapour pressure osmometer (Wescor Inc., Logan, UT, USA).

$$\Psi_{S100} = (\Psi_s \times \text{RWC})/100$$

Net osmotic adjustment (OA) was determined using the method of (Ludlow et al. 1983).

$$\text{Net Osmotic adjustment (OA)} = A - B$$

Where A denotes Ψ_{s100} of plants from salt stress treatment and B denotes Ψ_{s100} of plants from non stressed treatment.

Table 1 Range and mean of a few physiological traits including root and shoot Na content, K/Na ratio in 27 varieties, subjected to salt stress (250 mM NaCl) in the soilrite Hoagland system (Experiment 1)

Parameters		Range	Mean	CD $P \leq 0.05$
Root Na (mmol/g DW)	Control	0.59–0.96	0.75	0.13
	Salt stress	1.01–2.86	1.64	
Shoot Na (mmol/g DW)	Control	1.09–1.33	1.18	0.19
	Salt stress	1.70–5.17	3.07	
Root K/Na ratio	Control	0.45–1.58	0.86	1.68
	Salt stress	0.11–0.94	0.48	
Shoot K/Na ratio	Control	0.23–1.11	0.44	0.68
	Salt stress	0.16–0.50	0.3	
Chlorophyll Stability Index (CSI)	Control	18.24–89.30	57.95	25.76
	Salt stress	8.12–68.89	29.55	
Relative Water Content (RWC)	Control	47.18–81.87	58.67	11.29
	Salt stress	38.79–69.72	48.32	
Net Osmotic Adjustment (NOA)	Control	0.02–0.34	0.18	0.229
	Salt stress	0.14–0.33	0.25	
Triphenyl Tetrazolium Chloride (TTC) Test	Control	0.32–0.90	0.56	0.304
	Salt stress	0.23–0.73	0.42	
Cell Membrane Stability (CMS)	Control	17.70–57.47	34.68	13.98
	Salt stress	24.40–72.81	50.18	
Salt Tolerance Index (STI)	Salt stress	41.13–89.36	68.34	28.06

Experiment 3

Selection of varieties for ^{22}Na studies

Two contrasting varieties were selected from experiment II for ^{22}Na influx studies. They were a) *Spanish improved* (tolerant) b) *JSP-39* (susceptible). In addition, three moderately tolerant Trombay cultivars *TPG-41*, *TAG-24* and *TG-37-A*, were also selected for ^{22}Na influx studies (Fig. 1a–c)

The time interval for influx rate was standardized in initial experiment as 1, 6 and 24 h. The seedlings were incubated for 24 h in a solution containing 180 kBq of ^{22}Na . At the end of incubation, root and shoot were excised and flux rates were calculated in shoot and root (Fig. 1d).

Results

Experiment 1: Preliminary screening for salt tolerance in 27 diverse cultivars, based on growth and physiological traits

In this experiment, a soilrite Hoagland system was used to screen 27 diverse genotypes ranging from three potentially salt tolerant genotypes developed by mutation breeding to two drought tolerant genotypes developed by selection. A set of six physiological traits which included ion content in root and shoot, K/Na selectivity, water relations. Data on NOA, CSI, and CMS was utilized to screen the cultivars. The data on the range and mean (Table 1) values of these traits for the 27 cultivars showed highly significant genotypic variability for all the traits. The variability

Fig. 1 Experimental set up for ^{22}Na flux rate in groundnut. **a**, **b**, **c** & **d** indicates the different stages of seeds sowing to imposing the stress. **a** Sowing of selected groundnut varieties in the tubes, **b** Growth of the

seedlings in the tube after 30 days, **c** Experimental set up for radioisotope treatment and **d** After treatment with radioisotope ^{22}Na showing the root and shoot

for root and shoot Na content in salt stress treatment was 3–4 folds, suggesting wide variability in exclusion of Na.

Experiment 2

From the first preliminary screening in experiment-I only nine cultivars, three moderately tolerant, three tolerant, and three susceptible were selected (Table 2). These were raised in containers in a soil system and a salt stress of 8 dS/m was imposed. The data are presented in Table 3.

Cell membrane stability Among the three tolerant varieties, variety *Spanish improved* had significantly high CMS, suggesting that it was most consistently tolerant in both experiments, with lesser membrane injury. The mean CMS (%) of the three tolerant varieties was 28.38 as compared to the mean of the salt susceptible varieties of 22.74 suggesting a significantly higher CMS. Among these the CMS of susceptible varieties was nearly similar, and also similar to the moderately tolerant cultivar.

Net osmotic adjustment The data presented in Table 3 showed enhanced osmotic adjustment, in concert with that of external stress, Genotype *Spanish Improved* showed highest OA among the tolerant cultivars. OA of salt susceptible genotypes was on par. The mean OA was 0.55 MPa in tolerant cultivars as compared 0.27 MPa in salt susceptible varieties selected from the earlier experiment in Soilrite Hoagland system. The mean OA of the moderately tolerant lines was significantly higher than the mean of the susceptible varieties, but lower than mean of tolerant varieties.

Table 2 Groundnut varieties selected from experiment 1 for the second screening in experiment 2 using natural soil system

Sl. no.	Class	Variety name
1.	Moderately tolerant ^a	TPG-41
2.		TAG-24
3.		TG-37-A
4.	Tolerant ^a	R-9227
5.		M-28-2
6.		<i>Spanish improved</i>
7.	Susceptible ^b	Jsp-39
8.		R-2001-3
9.		J-11

^a Moderately tolerant and tolerant varieties had values of root and shoot Na significantly lower than mean, and a K/Na ratio significantly higher than mean both in root and shoot. RWC, NOA and CMS and STI were also significantly higher than the mean

^b Susceptible varieties had values of root and shoot Na significantly higher than mean. Similarly values of K/Na ratio in shoot and root, RWC, NOA, CMS and STI were correspondingly lower

Salt tolerance index The mean STI of the salt tolerant genotypes (Table 3) was 79.42 while it was 55.30 for the salt susceptible genotypes. The mean STI of three moderately tolerant Trombay varieties was higher than the mean of susceptible varieties. Cultivar *Spanish improved* was superior among three tolerant genotypes selected from experiment No.1, while JSP-39 had the lowest STI among the three susceptible cultivars, although differences were non-significant. Among three moderately tolerant cultivars, the differences in STI between TAG-24 and TG-37-A was statistically non-significant. The STI values although on par was significantly higher than TPG-41.

Experiment 3

Data on ²²Na influx rate in root (Fig. 2) showed significant differences in influx rate with the susceptible cultivar JSP 39, having a seven fold higher ²²Na influx rate as compared to the tolerant cultivar *Spanish improved*. The mean of the moderately tolerant cultivars was 0.12 Bq/DW, which was comparable to that of the tolerant cultivars but significantly lower than the susceptible cultivars. ²² Na concentration in shoot was low in the salt tolerant variety and high in salt susceptible varieties (Fig. 3).

Discussion

Salinity is a complex abiotic stress that simultaneously presents an osmotic and an ionic component (Munns et al. 1995). Many studies have tried to determine whether the major influence of high salinity is primarily osmotic or due to a specific ion toxicity. More recently (Almansouri et al. 1999), a study has shown that expression of this two-phase response, initially suggested (Munns et al. 1995), as a simplistic explanation for genetic differences in salt tolerance of wheat cultivars, was even influenced by how the salt stress was imposed; whether sudden or progressive. More recently, it has been observed (Tavakkoli et al. 2010) that the expression of differences in these two components amongst barley cultivars is also influenced by what kind of system was used, whether a hydroponic system or a soil system. The concept of exclusion ability of genotypes being the primary determinant of salt tolerance has itself come in for re-evaluation. This has been primarily due to the fact that several studies have shown a lack of correlation between the ability to exclude Na and the overall tolerance of a genotype (Genc et al. 2007). It is evident that a combination of different mechanisms (exclusion, extrusion, compartmentation and cellular tolerance) are responsible, and not solely ion exclusion or ion extrusion (Tavakkoli et al. 2010).

With this background, we have chosen not only exclusion ability (as reflected by ion content, K/Na selectivity) as

Table 3 Effects of salt stress (8 dS/m) on the Net Osmotic Adjustment (OA)* (MPa), Salt Tolerance Index (STI) and Cell Membrane Stability (CMS) in selected contrasting varieties differing in salt tolerance in solution culture

STI*=(Total dry matter produced by stress plants/Total dry matter of control plants)*100	4.	Tolerant	R-9227	0.46	73.03	28.00
	5.		M-28-2	0.59	79.73	22.81
	6.		Spanish improved	0.60	85.50	34.34
	Mean			0.55	79.42	28.38
OA* - Determined by the method of Ludlow et al. (1983), Ludlow and Muchow (1990). The osmotic or solute potential (Ψ _{s100}) at 100 % RWC is calculated for both non stressed and stressed pants	7.	Susceptible	Jsp-39	0.26	42.54	24.23
	8.		R-2001-3	0.24	67.12	22.10
	9.		J-11	0.32	56.26	21.90
	Mean			0.27	55.30	22.74
Ψ _{s100} =(Ψ _s *RWC)/100	Min–Max value			0.32–0.60	41.13–89.36	17.40–34.34
	CD Genotypes (<i>P</i> ≤0.05)			0.35	39.9	2.13
	CD Stress (<i>P</i> ≤0.05)			0.25	32.76	2.69
	CD Genotypes v Stress (<i>P</i> ≤0.05)			0.23	28.06	1.87
Net OA (MPa)=Ψ _{s100} of stressed plants-Ψ _{s100} of control plants		CV (%)		13.52	11.86	14.75

STI*=(Total dry matter produced by stress plants/Total dry matter of control plants)*100

OA* - Determined by the method of Ludlow et al. (1983), Ludlow and Muchow (1990). The osmotic or solute potential (Ψ_{s100}) at 100 % RWC is calculated for both non stressed and stressed plants

$\Psi_{s100}=(\Psi_s \cdot \text{RWC})/100$

Net OA (MPa)= Ψ_{s100} of stressed plants- Ψ_{s100} of control plants

parameters to screen diverse cultivars of groundnut for salt tolerance, but also a range of other well accepted physiological parameters (Chlorophyll Stability Index). Such an approach has been widely followed (Almansouri et al. 1999) by others. Four salt tolerant and three salt susceptible cultivars were identified in both solution and soil culture (experiment I and II respectively) based on set of well established physiological traits; used extensively in salt stress screening (Almansouri et al. 1999; Tavakkoli et al. 2010). Based on the results of experiment I and II only two distinct contrasting varieties differing in salt tolerance was taken for ^{22}Na uptake

studies (experiment III). Differences in Na uptake were highly significant and the salt tolerant variety had low Na concentration both in root and shoot, a result in confirmation with that obtained by Nevo et al. 1992 in Wild emmer wheat accessions. The results have confirmed the importance of Na exclusion as an important criterion for salinity.

In summary, sodium exclusion itself has two aspects (a) restricting Na flux into roots from the surrounding salt environment, so that much less gets into the root cytoplasm (b) low transport to the shoot (Munns and James 2003). What this essentially means is that two varieties could be differentially

Fig. 2 ^{22}Na influx rate (Bq/g Dw/day) in roots of selected contrasting groundnut genotypes. 30 day old of each variety seedlings were incubated for 24 h in a solution [(2.0 mol.m⁻³ Ca (NO₃)₂), 1.0 mol.m⁻³ NaCl, 100 mmol.m⁻³ KNO₃] containing 180 kBq of ^{22}Na . At the end of incubation, roots were excised, dried in an oven at 100 °C for 12 h, and ground to a fine powder. 100 mg of this was taken for quantification of radioactivity in a Liquid Scintillation Counter (LSC) Perkin–Elmer

Fig. 3 ^{22}Na influx rate (Bq/g Dw/day) in shoots of selected contrasting groundnut genotypes. 30 day old of each variety seedlings were incubated for 24 h in a solution [(2.0 mol.m⁻³ Ca (NO₃)₂), 1.0 mol.m⁻³ NaCl, 100 mmol.m⁻³ KNO₃] containing 180 kBq of ^{22}Na . At the end of incubation, shoots were excised, dried in an oven at 100 °C for 12 h, and ground to a fine powder. 100 mg of this was taken for quantification of radioactivity in a Liquid Scintillation Counter (LSC) Perkin–Elmer

tolerant or susceptible with similar root Na content simply by exercising greater control over transport to shoot. This is perhaps the reason why Genc et al. 2007 found no relationship between the level of exclusion of Na (as determined by root Na content) and salt tolerance in genotypes of bread wheat, whether based on vegetative growth or grain yield.

In addition to differential transport of Na to shoot in two varieties with similar influx rates the third factor which determines net Na content of root is the Na efflux rate. Differences in Na efflux rate could negate the importance of low Na influx rate. Evidence for this has come from the work of Naqvi and Tandon 1991 which showed that the salt tolerant Indian land race Kharchia 65 showed high Na accumulation under salt stress. What then is responsible for the high salt tolerance of Kharchia 65, is it greater compartmentation of accumulated Na into vacuoles or greater efflux? As Genc et al. (2007) emphasized, future research should focus on identifying mechanisms of, and developing robust screening methods for tissue tolerance. This study is a step in that direction.

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