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Evaluation of rough lemon (*Citrus jambhiri* Lush.) as rootstock for salinity tolerance at seedling stage under *in vitro* conditions

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***In vitro* approach was adopted to study the effect of salinity on survival and growth of rough lemon (*Citrus jambhiri* Lush.) seeds. North-West (Punjab) part of India has been facing a major problem of soil salinity for citrus orchards. Therefore, it is logical to study the salinity tolerance of common citrus rootstock, rough lemon (*Citrus jambhiri* Lush.), grown in the region. The seeds were treated with nine different doses of sodium salt. In all the treatments, leaves of rough lemon seedlings showed severe injury symptoms of chlorosis and necrosis while the seeds cultured in control did not show any injury. There was a significant decrease in seed germination, seedling height, internodal length, and subsequently plant weight with increasing concentration of salt. In contrast to the above characteristics, the length of primary roots increased proportionally with the increase in salt concentration in the culture media. As under stress conditions, the *in vitro* grown seedlings tend to increase the root length for its survival. In comparison to the control, salt treatments showed increased level of Na^+ and Cl^- ions in the seedlings and also resulted in a decrease of K^+/Na^+ ratio. Tolerance index was found minimum (100) in control and maximum in 154mM NaCl treatments after 4 and 8 weeks.**

Key words: Citrus, salt, sensitivity, chlorosis, sodium chloride.

INTRODUCTION

Around the world, citrus is one of the major horticultural crops and is relatively salt sensitive. In India, citrus industry is a third largest fruit industry after mango and banana with an area of 0.99 million ha area with an annual production of 96.4 x 10^8 kg (CSO, 2007). In Punjab state, citrus crop covers an area of 44724 ha with an annual production of 9 x 10^8 kg (DHP, 2011). Due to the negative influence on the yields of many crops, salinity is a major problem for citrus crop. In citrus, it has an adverse effect on tree growth and causes many physiological disorders. Primarily, salt-stress lowers the net CO_2 assimilation, stomata conductance, as well as water potential of citrus tree leaves in addition to the accumulation of excessive concentration of chloride or sodium in leaves (Al-Yassin, 2004). Excessive salts in soils interfere generally in plant metabolism and causes disturbance in water relations (Xoing and Zhu, 2002). In Southwestern region of Punjab, salinity is the major

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Abbreviations: WS, Plants without symptoms; **VMC,** very mild chlorosis; **PMC,** partial mild chlorosis; **BMA,** mild chlorosis to burns on the margins and apices of the lower leaves; **SDD,** intense damage with leaf loss followed by plant death; **NG,** no germination.

abiotic stress threatening citrus industry. Salt stress inhibits plant growth and reduces plant production due to water scarcity, ionic toxicity, and nutritional imbalance resulting in major yield loss.

Along with osmotic effects of soil salinity, Na and Cl uptake and their accumulation in plant tissues result in specific ionic toxicities. Competitive interactions among toxic ions (Na and Cl) and nutrients in the soil could induce nutrient imbalance and deficiencies (Grattan and Grieve, 1998). Numerous studies have shown that chloride tends to accumulate in citrus leaves. However, this accumulation may also be function of the age, position, and genotype of the shoots (Chapman, 1968; Walker et al., 1982; Banuls et al., 1997) which implicates the factors other than the root system in salt tolerance. Thus, Cl^- resistance was affected not only by the root system, but also by shoot properties. Increased concentration of NaCl in the nutrition solution reduced growth proportionally and altered leaf and root mineral concentrations of all citrus rootstocks. The shoot height, leaf number, fresh weights of the seedlings, relative chlorophyll contents, chlorophyll fluorescence yields (Fv/Fm), net photosynthetic, and respiration rates in the leaves decreased with the increase in salinity level in the irrigation water (Zekri and Parsons, 1992; Anjum, 2008). Assessment of nutritional behavior of the new citrus rootstocks, *Forner-Alcaide no.5* (FA-5) and *Forner-Alcaide no.13* (FA-13), under saline conditions revealed that the accumulation of saline ions inhibits growth and nutrient uptake by citrus plants as elevated salt levels in the growth medium reduced the absorption of the mineral elements in all scion-rootstock combinations (Ginner et al., 2011). This problem can be prevented by soil and water management and/or using tolerant genotypes (Ashraf and Ahmad, 2000). In some glycophytes, the osmotic adjustment is related to the accumulation of proline, glycine-betaine, soluble sugars, and salt stress proteins (Ashraf and Harris, 2004), which results in adaptation by the plant to the saline environment (Munns et al., 2006). Apart from soil and water management, *in vitro* approaches have been adopted as one of the options to overcome salinity problems, under the laboratory conditions. In one such study, the plantlets were regenerated from the selected salt tolerant cell line of shamouti orange (*Citrus sinensis* L. Osbeck) and therefore, salt tolerance on the whole plant level was achieved (Ben-Hayyim and Goffer, 1989). Salt tolerant somaclonal variants in rice and *Poncirus trifoliate* through *in vitro* selection have been developed successfully (Beloualy and Bouharmont, 1992; Dang and Nuguyen, 2003)

Thus, keeping in view the salinity problems being faced in citrus plantations, the present investigation was conducted in rough lemon (*Citrus jambhiri* Lush), a major citrus rootstock in India and around the world, to find out a threshold value of salt tolerance and its influence on the growth and survival of *in vitro* grown seedlings.

MATERIALS AND METHODS

Growing media and treatments

The study was conducted in the tissue culture laboratory of Fruit Science Department, Punjab Agricultural University, Ludhiana. Seeds of rough lemon (*Citrus jambhiri* Lush.) extracted from healthy fruits, collected from the orchard of Punjab Agricultural University, Ludhiana, were surface sterilized with 0.1% mercuric chloride (w/v) for 3 min followed by washing with sterile distilled water twice. The sterilized seeds (20 for each treatment and 80 in total) were cultured on Murashige Skoog basal medium (Murashige and Skoog, 1962) consisting of MS salt solution supplemented with 30 g/l of sucrose, 7.5 g/l of agar and different concentrations of salt (NaCl) ranging from 0.1 to 0.9% and a control where no salt was added. One seed per test tube was cultured for every treatment. The pH of the medium was adjusted to 5.8 by adding 1N NaOH or 1N HCl solution drop wise before autoclaving for 20 min at 121°C. The cultures were maintained at 16 h photoperiod at 26°C temperature at a relative humidity of 70%. Uniformity of seeds were maintained by passing the seeds through standardized sieve. Each treatment was replicated four times, the LD50 dose was determined from number of seedlings that emerged or survived up to 56 days after sowing, and percent germination was calculated.

Growth measurements

Seedlings height, internodal length, and length of primary roots was measured using a measuring scale while number of leaves was determined on visual observation basis. To study the comparative qualitative foliar symptoms, the severity of the plants condition was classified as follows: Plants without symptoms (WS), very mild chlorosis (VMC), partial mild chlorosis (PMC), mild chlorosis to burns on the margins and apices of the lower leaves (BMA), intense damage with leaf loss followed by plant death (SDD) and no germination (NG) that is, completely dead.

Tolerance index (TI) as given by La Rosa et al. (1989) was used to summarize the general effect of different concentrations of NaCl on rough lemon on the basis of reaction to salt treatment. The dry weight (DW) of the seeds/seedlings cultured on various concentrations of NaCl was measured after drying the samples at 70°C. The control treatment is designated as S10 for this parameter.

$$TI = 100 + \Sigma^{n}[X\ (T_x/T_o)\ 100]$$

Where, n is the Number of treatments; X is the salt concentration (1-9 g/l of NaCl), T_x, is the shoot/root weight of NaCl treated seedlings (1-9 g/l of NaCl) and T_o, is the shoot/root weight of untreated cuttings (g). The concentration of ions was expressed on percent dry weight basis.

Chemical analysis

For chemical analysis, all the samples were washed with distilled water, dried at 70°C for 48 h to obtain constant weight and then ground. In the treatments beyond S5 (0.5%) where no germination took place, the swollen seeds were used for carrying out the analysis. The Na^+ and K^+ contents were determined by flame photometrically (Mapa, 1971) and Cl^- by titration with silver nitrate approach (Gilliam, 1971).

Statistical analysis

The data was analyzed with the Statistical Analysis System (SAS) V9.2 (SAS Institute, Cary, NC). LSD was used to compare the

Table 1. Rough lemon seed germination and growth parameters of *in vitro* grown rough lemon (*Citrus jambhiri* Lush) under saline conditions after 8 weeks.

Treatment	*GRM	SMT**	SH (cm)	NOL	INL (cm)	LPR (cm)	FW†	DW†	SDW (g)	RDW (g)	S/R
S1-NaCl 17 mM	89.3	WS-VMC	6.5	4.2	3.1	5.1	4.32	1.02	0.64	0.34	1.88
S2-NaCl 35 mM	88.7	VMC-PMC	6.1	3.3	3.1	5.9	3.89	0.92	0.60	0.31	1.94
S3-NaCl 51 mM	84.7	PMC-BMA	5.6	3.1	2.9	6.2	3.52	0.81	0.54	0.26	2.08
S4-NaCl 68 mM	69.7	BMA	3.8	3.0	1.1	6.6	2.12	0.62	0.42	0.19	2.21
S5-NaCl 86 mM	54.3	BMA-SDD	3.6	2.8	1.0	7.0	1.77	0.48	0.33	0.13	2.53
S6-NaCl 103 mM	0.00	NG	0.0	0.0	0.0	0.0	0.41	0.09	-	-	-
S7-NaCl 120 mM	0.00	NG	0.0	0.0	0.0	0.0	0.51	0.11	-	-	-
S8-NaCl 137 mM	0.00	NG	0.0	0.0	0.0	0.0	0.54	0.12	-	-	-
S9-NaCl 154 mM	0.00	NG	0.0	0.0	0.0	0.0	0.62	0.14			
S10-NaCl 0mMControl	100.0	WS	6.8	4.4	3.3	4.9	5.01	1.33	0.86	0.49	1.75
LSD (P= 0.05%)	2.88		0.20	0.14	0.84	0.41	0.1	0.17	0.11	0.12	0.10

GRM, Germination percentage; SMT, symptoms; SH, seedling height; NOL, number of leaves per seedling; INL, internodal length; LPR, length of primary root; FW, fresh weight; DW, dry weight; SDW, stem dry weight; RDW, root dry weight; S/R, shoot to root ratio; **WS, plants without symptoms; VMC, very mild chlorosis; PMC, partial mild chlorosis; BMA, mild chlorosis to burns on margins and apices of the lower leaves; SDD, intense damage with leaf loss followed by plant death; NG, no germination; *, after 56 days; †, fresh and dry weight of seedlings (S1-S5 and Control) and seeds (S6-S9).

treatments with each other. The P value at 0.05 was used to find the significance between the treatments. Microsoft excel 2010 was used to make the figures. The correlation coefficient (r^2) value was used to evaluate the relationship between the salt treatment and growth parameters.

RESULTS

Treatment effect

Growth and nutrient acquisition in rough lemon were studied under *in vitro* conditions where salinity was induced by incorporating different concentrations (S1 to S9, Table 1) of salt (sodium chloride) in the culture media along with a control where no salt was added (Tables 1 and 2). There was significant effect on the survival and growth dynamics of rough lemon seedlings with gradual increase in concentration (NaCl). No seedling formation took place (Table 1) beyond S5 of salt concentration. The severity of symptom of NaCl injury can be evaluated from the data given in Table 1.

Maximum germination was found in control. Beyond S5, the seeds got swollen, sprouted but no seedling formation took place. The growth of the single shoot (measured as shoot length), number of leaves, and the internodal length of *C. jambhiri* under *in vitro* conditions was found to be maximum in control (Table 1). With increase in salt concentration in MS medium, the growth of single shoot under *in vitro* conditions decreased proportionally (Table 1). In comparison to the plant height, leaf number, and internodal length, the length of primary roots increased in proportion to the increase in salt concentration in the media and found positively correlated to increase in salt concentration until S5 after which no germination took place. The data depicted that total fresh weight as well as the dry weight of seedlings was found to be maximum in control. It decreased with increase in salt concentration treatment.

The dry weights of shoot and root were found significantly more in the control than other treatments whereas, minimum shoot and root dry weight was observed in S5 of NaCl (Table 1). Maximum shoot to root ratio was recorded in the S5 of NaCl treatment and minimum was observed in the control treatment. Nutrient uptake by the rough lemon seeds and seedlings is as shown in Table 2. Data in Table 2 clear the fact that in comparison to control, salt treatments resulted in increased levels of Na^+ and Cl^- ions in seedlings including seeds, which got swollen but did not germinate. Maximum Na^+ and Cl^- ions were observed in seeds with highest level of salt treatment. Compared to the control, Na^+ content in the seeds was almost 7 times while Cl^- content was 9 times in the S9 treatment indicating that the concentration of Cl^- was higher than that of sodium (Na^+) (Akilan et al., 1997).

It is evident from the Figure 1 that degree of tolerance index did not change with treatment and time, that is, the trend during 4 and 8 weeks of study was almost similar. Tolerance index was found minimum in control (S10). After 8 weeks of seed culturing in different salt treatments, changes in tolerance Index was more apparent where it increased to 391.04 in S5 of NaCl and then decreased to 308.95 in S6 of NaCl, while increasing to S10 in 154mM of NaCl treatment. It is interesting to observe that after 8 weeks, tolerance index from S1 to S5 treatments was higher while it was slightly lower for S5 to S10 of NaCl treatments than that observed after 4 weeks. Nevertheless, the trend was similar in both periods.

Figures 2a and 2b depict that the degree of injury increased with increase in concentration of salt in the

Table 2. Nutrition uptake of *in vitro* raised rough lemon (*Citrus jambhiri* Lush) seedlings/seeds under saline conditions (after 8 weeks).

Treatment	Na (ppm)	K (ppm)	Cl (ppm)	K/Na	Na/Cl
S1-NaCl 17 mM	18.2	32.8	40.4	1.4	0.45
S2-NaCl 35 mM	21.3	33.6	53	1.23	0.4
S3-NaCl 51 mM	21.8	34.2	55.8	1.23	0.39
S4-NaCl 68 mM	22.3	35.1	60.4	1.22	0.37
S5-NaCl 86 mM	22.9	35.7	61.3	1.21	0.37
S6-NaCl 103 mM	23.1	34.6	62.5	1.17	0.37
S7-NaCl 120 mM	23.4	33.5	63.6	1.11	0.37
S8-NaCl 137 mM	23.5	34.2	64.3	1.13	0.36
S9-NaCl 154 mM	23.8	32.4	65.6	1.06	0.36
S10-NaCl 0 mMControl	3.2	6.6	7.2	1.62	0.44
LSD- (P= 0.05%)	0.164	0.169	1.84	NS	0.36

*, Fresh and dry weight of seedlings (S1-S5 & Control) and seeds (S6-S9).

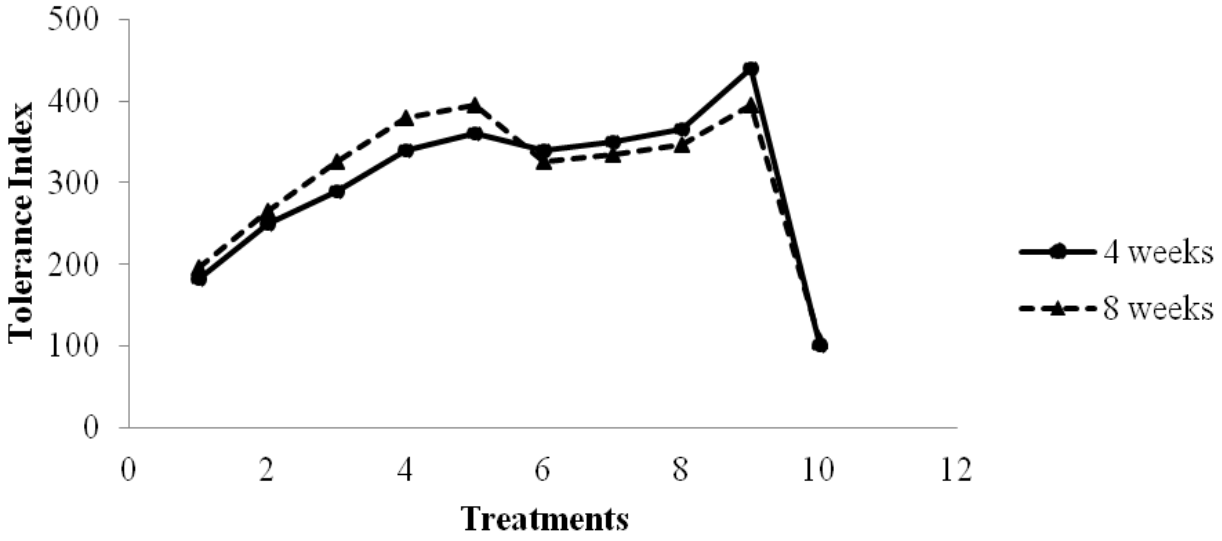

Figure 1. Tolerance index of rough lemon seeds and seedlings under salt conditions after 4 weeks and 8 weeks *in vitro* conditions.

culture media after 4 and 8 weeks, respectively. There was no injury (0 degree of injury) in control seedlings. Maximum degree of injury was observed in S9 of NaCl, might be due to high salt toxicity, where no germination took place and high concentration of sodium, potassium, and chloride found in the seeds. After 4 and 8 weeks, 3 degree level of injury was found in the S9 of NaCl treatment.

Correlation and regression analysis

Correlation and regression coefficient were used to define the strength of relationship between the salt treatment and growth parameters as well as within growth parameters. The correlation between Na^+ and Cl^- with K^+ was positive and significant (Table 3). It is evident from the Table 3 that there was strong correlation between the different growth parameters. It is being clear that with increase in Na^+ inside the plant tissue there is similar increase in K^+. The correlation between the germination, single shoot (measured as shoot length), number of leaves, and the internodal length, with salt treatment was negative and significant (Table 3). The correlation between fresh and dry weight with salt treatment was negative. High r2 value explained the strong relationship between the Na and K (Figure 3). In addition, Cl and K were found to have strong relationship with each other (Figure 4). However, negative correlation was found between the

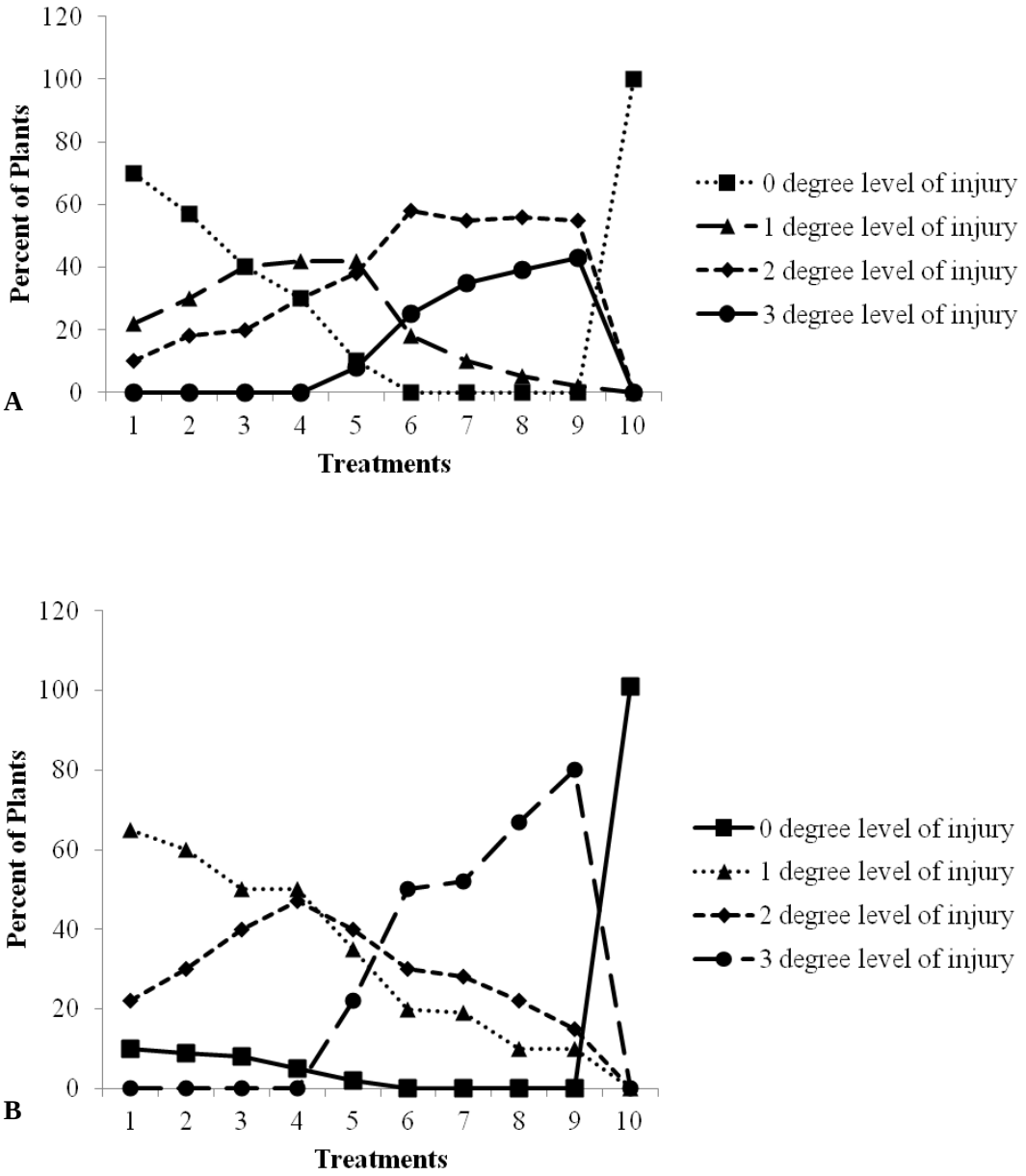

Figure 2. Degree of injuries in rough lemon seeds and seedlings after A, 4 weeks; B, 8 weeks.

salt treatments with fresh and dry weight of seeds and seedlings (Figure 5).

DISCUSSION

The present investigation demonstrates the effects of increasing salt concentration on *C. jambhiri* seeds and seedlings. In all salt treatments (17 to 154 mM) leaves of rough lemon seedlings showed severe injury symptoms of chlorosis as well as necrosis which progressed fairly rapidly in the tips and along the leaf margins and then advanced basipetaly (Al-Yassin, 2004). The leaf injury symptoms were observed mostly in mature leaves. The seeds cultured in control treatment did not show any injury while the extent of chlorosis, necrosis and death shown by plants were in proportion to amount of NaCl added in the culture media (Sykes, 1985; Bongi and Loreto, 1989; Munns, 1993). Beyond 86 mM NaCl, the seeds got swollen, sprouted but no seedling formation took place (Yokas et al., 2008; Murkute, 2004). Increasing reverse osmotic pressure and concomitant salt toxicity could be the predisposal factor to reduce the physico-chemical processes in seed germination (Maathuis and Amtmann, 1999). It is evident from the results that NaCl treatments caused inhibition in plant growth due to

Table 3. Correlation between growth parameters and salt treatments.

COR	Trt	FW	DW	Na (ppm)	K (ppm)	Cl (ppm)	K/Na	Na/Cl	SDW	RDW	S/R	GRM	SH	LPS	INL	LPR
Trt*	1.00															
FW*	-0.96	1.00														
DW*	-0.96	0.99	1.00													
Na*	0.70	-0.71	-0.76	1.00												
K*	0.51	-0.55	-0.60	0.97	1.00											
Cl*	0.79	-0.80	-0.84	0.99	0.92	1.00										
K/Na*	-0.87	0.85	0.88	-0.94	-0.82	-0.97	1.00									
Na/Cl*	-0.88	0.89	0.87	-0.75	-0.59	-0.85	0.88	1.00								
SDW*	-0.96	0.98	1.00	-0.72	-0.56	-0.80	0.86	0.84	1.00							
RDW*	-0.96	0.99	1.00	-0.79	-0.64	-0.86	0.90	0.88	0.99	1.00						
S/R*	-0.75	0.73	0.76	-0.30	-0.12	-0.38	0.52	0.47	0.81	0.73	1.00					
GRM*	-0.94	0.96	0.96	-0.58	-0.40	-0.67	0.76	0.76	0.98	0.95	0.89	1.00				
SH*	-0.95	0.98	0.97	-0.60	-0.41	-0.70	0.78	0.81	0.98	0.96	0.86	0.99	1.00			
LPS*	-0.94	0.94	0.96	-0.61	-0.42	-0.70	0.80	0.79	0.97	0.94	0.90	0.99	0.98	1.00		
INL*	-0.94	0.99	0.96	-0.62	-0.45	-0.72	0.77	0.85	0.96	0.96	0.74	0.95	0.97	0.92	1.00	
LPR*	-0.75	0.73	0.75	-0.28	-0.10	-0.36	0.50	0.45	0.81	0.73	1.00	0.89	0.86	0.89	0.74	1.00

COR, Correlation; Trt, treatment; FW, fresh weight; DW, dry weight, SDW, stem dry weight; RDW, root dry weight; S/R, shoot to root ratio; GRM, germination percentage; SH, stem height; LPS, length of primary shoot; INL, internodal length; LPR, length of primary root. *Correlation values were significant at 0.05, 0.01, and 0.001 level of significance.

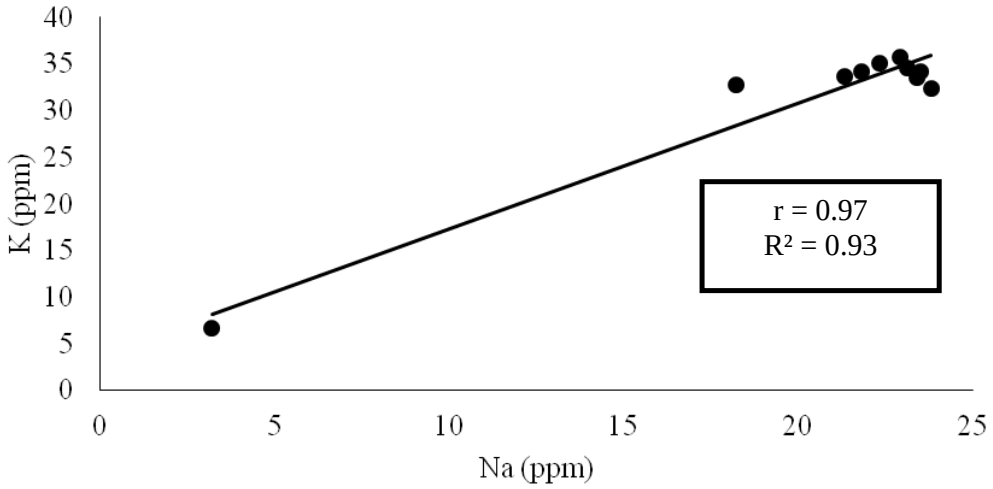

Figure 3. Regression and correlation between potassium and sodium level in rough lemon seed and seedlings.

decrease in proliferation ratio, fresh weight, shoot length, and number of leaves (Zidan et al., 1990; Pérez-Tornero et al., 2009). Since plant growth is a result of massive and irreversible expansion of young cells produced by ongoing merismetic divisions, salinization can inhibit both cell division and cell expansion in growing tissue of roots, stem and leaves thereby affecting shoot growth (Aazami et al., 2010; Forner-Giner et al., 2011). Under stress conditions, the *in vitro* grown seedlings tended to increase the root length for its survival (Abed et al., 2005) as compare to other fact. The effect of *in vitro* NaCl treatments on rough lemon supported the previous findings of *in vitro* NaCl treatments on grapevine cultivars (Downton and Millhouse, 1985).

Decrease in the number of leaves were not only due to the growth inhibiting effects of salt, but also due to the injurious effects of salt toxicity resulting in defoliation of the damaged leaves (Moya et al., 1999; Khoushbakht et al., 2010). First visual symptoms on the *in vitro* grown seedlings of rough lemon were withered shoot tips and

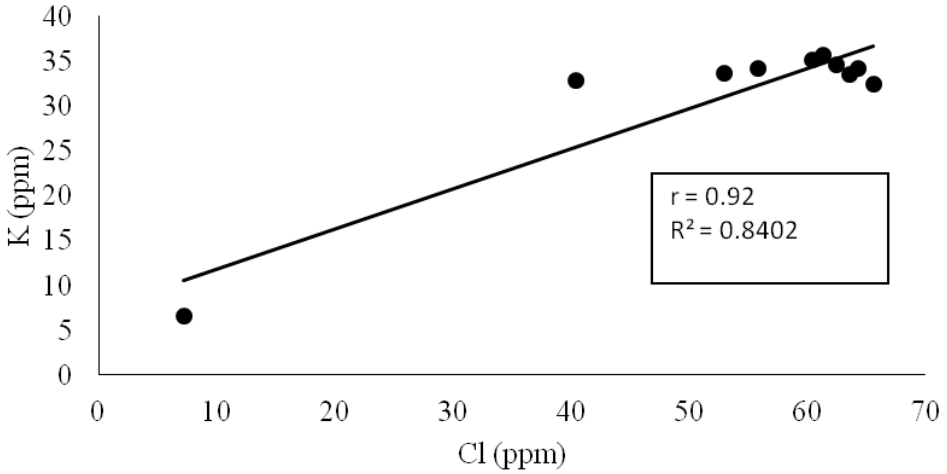

Figure 4. Regression and correlation between potassium and chloride levels in rough lemon seed and seedlings.

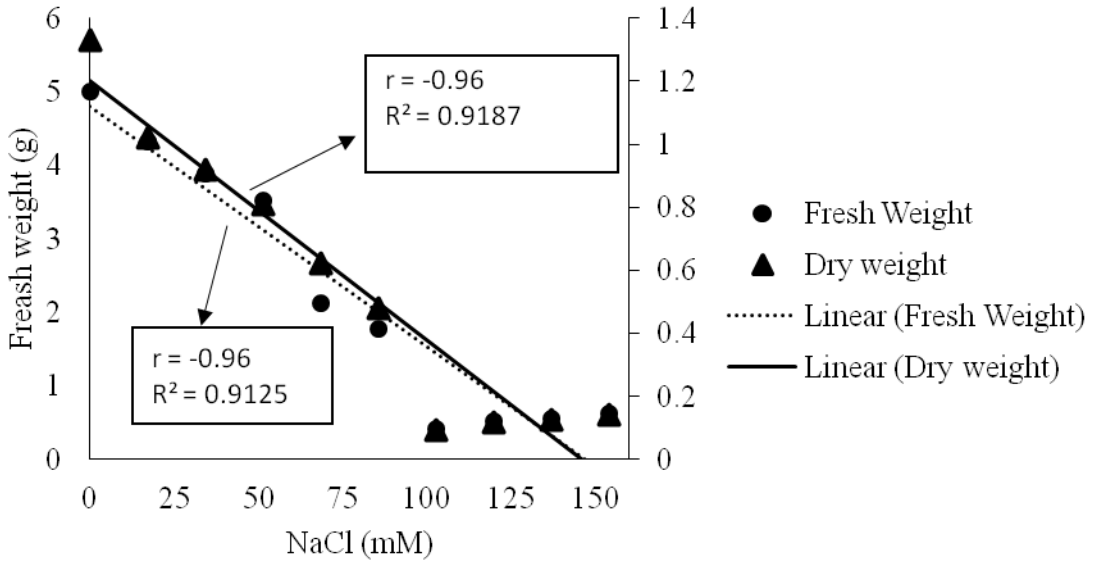

Figure 5. Regression and correlation between fresh and dry weights of rough lemon seed and seedlings with salt treatment.

leaf edges, which progressively increased inwards with increased in salinity levels and the length of treatment period (Yokas et al., 2008). These injurious effects progressed exponentially with increasing salinity levels.

In the present investigation, the reduced plant weight due to higher NaCl concentration and treatment period (8 weeks) can be attributed to the osmotic effect of salts in the soil that hinders plant growth and reduces production (Alkilan et al., 1997). As the citrus plant is not capable of excluding sodium from its system, sodium concentration builds up in the leaves thus causing leaf injury. The seeds cultured in treatments S6 to S9 (17 to 154 mM) did not germinate due to high salt concentration but got swollen and were used for mineral analysis. The growth inhibition due to salinity has been explained by suppression of nutrient ions absorption due to higher uptake of Na^+ and Cl^- ions in competition with the nutrient ions (Alkilan et al., 1997). A portion of Na^+ was probably transported from the leaves through the phloem to root, relative to Cl, which accumulated in the leaves producing burns, nutriational imbalance, and reduced transpiration (Hassan and Catlin, 1984).

Potassium ion (K^+) is one of the most important solute which plays crucial role in maintaining the osmotic poten-

tial especially in roots. The K^+/Na^+ ratio decreased with the increase in NaCl concentration (Storey, 1995). Data demonstrates that salt treatments had a comparatively greater influence on the shoots compared to the roots. This was clearly evident in shoot to root ratio in the control seedlings (1.75), the ratio was less as compared to salt treated cultured media (LaRosa et al., 1989) Tolerance index for both 8 and 4 weeks salt treatment shows similar trend with slight variation. High tolerance index was shown by 8 week treatment for lower salt concentration from 17-86 mM. However, reverse pattern have been observed for high salt concentration (103 to 154 mM) than that observed after 4 weeks.

The study indicated that the addition of sodium chloride in the culture media decreased the osmotic potential of the media thus inducing salinity stress that adversely affected the *in vitro* germination capacity of seeds of *C. jambhiri*. The seeds of *C. jambhiri* did not germinate in the culture media containing salt beyond 103 mM of salt. Thus, the LD_{50} value for seed germination of *C. jambhiri* was 0.5% NaCl in MS media.

Conclusion

An inverse relationship was observed between the salt concentration and seed germination, seedling height, leaves, internodal length, root length. Salt injury was mostly on the mature leaves, which moved from tip to margin and then towards basipetal. The amount of chlorosis, necrosis, and death shown by seeds and seedlings were in proportion to amount of NaCl added into the culture media. In addition, the injurious effects of salt were exponential with increasing salinity levels. Salt had more adverse effect on the shoots than roots. Tolerance index with both 4 and 8 weeks salt treatment showed similar trend. Total fresh weight as well as the dry weight of seedlings decreased with an increase in salt concentration. Salt uptake was also increased with an increase in salt in the growing media. Selected seedlings were further evaluated under greenhouse conditions.

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