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In vitro Cellular Salt Tolerance of *Troyer citrange*: Changes in Growth and Solutes Accumulation in Callus Tissue

HOUDA EL YACOUBI[1], KOUTOUA AYOLIÉ AND ATMANE ROCHDI
Unité Ecophysiologie & Biotechnologies Végétales, Université Ibn Tofail, Faculté des Sciences, BP 133, Kénitra, Marocco
[1]Corresponding author's e-mail: elyacoubihouda@gmail.com

ABSTRACT

Salt tolerant cell lines of *Troyer citrange* were obtained by exposing calli to increasing concentrations [0 - 8 g L^{-1}] of NaCl. At 6 and 8 g L^{-1} NaCl, the morphology and growth of the tolerant calli were similar to the control maintained in salt-free medium, but in meanwhile the sensitive wild-type calli show salt injury symptoms resulting in tissue browning and drastically inhibited growth. The selected cell lines maintain their growth after transfer to salt-free medium and after retransfer to salt-containing medium, respectively indicating the independence and the stability of the salt tolerance. The implication of accumulation of both inorganic (Na^+, K^+) and organic (proline, soluble sugars) solutes were also evaluated. K^+ content of the selected tolerant lines was close to that of the control and together were greater than that of the sensitive calli. But Na^+ content in both tolerant and sensitive calli was relatively higher than in the control. Subsequently sodium has been accumulated in two cellular levels according to the calli types: vacuolar sequestration for the tolerant calli (halophytic behavior) and cytosol invasion for the sensitive calli. Increased vacuolar Na^+ concentrations have been supported by increased accumulation in proline and soluble sugars, which are compatible solutes in the selected tolerant but not in the sensitive wild-type calli.

Key Words: *Troyer citrange;* Salinity tolerance; Tissue culture; Solutes accumulation

INTRODUCTION

Salinity tolerance is a multigenic character controlled at different levels of organisation, from the cell to the whole plant. For *Citrus*, sensitive glycophytes plants (Ben hayyim & Moore, 2007), the tolerance is associated with salt transport restriction from the root to aerial part (Syvertsen & Yelenosky, 1988). Also in forest species, the strategy to tolerate stress is done by excluding the salt ions at the point of uptake and reducing the translocation of ions to the shoot (Niknam & McComb, 2000). Indeed, growth reduction is closely related to the $Na^+$and Cl^- contents in leaf. Some rootstocks tolerate the effect of salt stress by combining the selective exclusion of Cl^- (relatively slow absorption of Cl^- ions) and the compartmentalization of excess Na^+ (Rochdi *et al.*, 2005).

Transmission of tolerance has been already demonstrated (Sykes, 1992). Conventional breeding by hybridization is often inefficient towards salinity tolerance because of difficulty using the pre-existing variability i.e., quantitative nature of resistance, high degree of heterozygoty etc. (Rains, 1982; Gorham & Wyn Jones, 2002).

These observations have guided research to use techniques of tissue culture, that contributes to improvement programs for the acquisition of salinity tolerance and to help elucidate the cellular mechanisms contributing to this tolerance. Thus *in vitro* culture was used to select cell lines tolerant to salinity (Beloualy & Bouharmont, 1992; Javed, 2002a,b,c; Haouala *et al.*, 2003). However the techniques of biotechnology have not always yielded positive results because of instability and the loss of tolerance after transfer of cultures to the environment without selective stressor.

Comparison between selected and non-selected cells with regard to the nature of salinity resistance has identified various physiological mechanisms that contribute to the adaptation of cells to salt stress. Thus Stavarek and Rains (1984) have shown that cell tolerance to NaCl is correlated with the capacity of ionic regulation. Some studies also show that tolerant cells of tobacco accumulate Na^+ and Cl^- ions (Watad *et al.*, 1983; Binzel *et al*., 1987). In contrast, it has been shown that tolerant cells of *Citrus* survive to salinity by partial avoidance to NaCl (Ben hayyim & Kochba, 1983). Other authors have shown that, in *Citrus*, tolerance of callus may be associated with the retention of K^+ (Ben hayyim *et al.*, 1985; Bouharmont *et al.*, 1993; Piqueras *et al*., 1996) and on a wide range of salt concentrations the internal potassium was linearly and positively correlated with growth (Ben hayyim *et al.*, 1987). Furthermore under saline environment, osmotic adjustment requires the accumulation of organic solutes such as amino acids, sugars and other compounds (Misra *et al.*, 1990; Das *et al*., 1990; Liu & Van Staden, 2000).

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The aim of this study was to develop *in vitro* salt tolerant cells of *Troyer citrange* (*Tristeza* resistant but salt sensitive) and in a second step, to assess the effect of salinity on growth of callus cell lines and determination of inorganic (K^+, Na^+) and organic (proline & soluble sugars) solutes that may be involved in their response to salt stress.

MATERIALS AND METHODS

Callus establishment: Polyembryonic seeds, genetically heterogeneous, from *Troyer citrange* (*Citrus sinensis [L.] x Poncirus trioiliata* [L.] Raf.), were used in this study. Callus was induced from embryos (one embryo is taken from each seed) on Murashige and Tucker (1969) medium supplemented with 50 g L^{-1} sucrose, 1 mg L^{-1} 2,4-D and 5 mg L^{-1} BAP (MB medium). The cultures were incubated at 30°C in the dark and subcultured after four weeks.

Selective salt concentration determination: In order to determine the salt concentration at which growth of normal calli was reduced by more than 50% (threshold often used for classification of tolerance degree) compared to controls, calli were exposed to different concentrations of NaCl. One hundred and twenty fragments of two month old homogeneous (yellowish colour & crumbly texture) calli were used. Their initial fresh weight (FW) was 0.2 g corresponding to 0.013 g dry weight (DW). These calli were been allowed to grow in MB medium supplemented with five concentrations (0, 4, 6, 8 & 9 g L^{-1}) of NaCl. Explants were cultured in Petri dish with four batches for each saline concentration tested.

Sixty calli were harvested after 1 month, while the others 60 remaining calli were subcultured and harvested later after two months. FW and DW of calli were measured and expressed as growth rate (GR), relatively to the initial weight [GR = (final weight–initial weight)/initial weight] and as relative growth rate (RGR), relatively to the GR of control [RGR = 100 x GR*stress*/GR*control*].

Cellular salt tolerance screening: The appropriate NaCl concentration, once determined, was used for selection of a callus line tolerant to this concentration (8 g L^{-1}) of NaCl. A total of 1400 pieces (each 0.2 g FW corresponding to 0.013 g DW) of two month old established calli, were cultured for one month at 4 g L^{-1} NaCl supplemented MB medium. All these calli were then transferred on 6 g L^{-1} NaCl for a second month and subsequently on 8 g L^{-1} NaCl for a third month as a progressive salt stress. Meanwhile another 300 pieces of calli were subcultured on the control medium (MB medium).

To determine phenotype, growth characteristics and *in vitro* screening for salinity tolerance at the cellular level were carried out to find and isolate fast growing yellowish cell clusters deriving from callus subculturing on MB medium supplemented with increasing salt concentration (0 to 8 g L^{-1} NaCl). After each month, callus phenotype (texture & colour) was noted. The callus weight (FW & DW) and the water content ((FW-DW)/FW) were measured, at the end of progressive salt stress, on 36 samples taken randomly from representative distinguished type calli (12 calli of each three distinguished type): control "calli cultured on MB salt-free medium", tolerant "yellowish crumbly calli with actively growing and a size equivalent to that of controls" and sensitive "more or less brownish calli with retarded/inhibited growth". Fresh and dry growth rate (FGR & DGR) were expressed relative to the initial weight of calli.

NaCl independence test and tolerance stability assay: After the progressive salt stress phase, remaining calli were transferred for two months on MB salt-free medium (independence test) and then retransferred for two other months on 8 g L^{-1} NaCl supplemented MB medium (stability assay). Meanwhile control calli are subcultured each month on MB medium. The callus FW and its FGR were determined after the second month of the independent test, then at one month and two months during the stability assay, in 36 samples (12 calli of each one of the three distinguished type ''control, tolerant, sensitive'').

Na^+, K^+, Proline and soluble sugars measurement: At the end of that selection process, for each three distinguished calli types (control, stable tolerant, sensitive), 12 calli were mixed in 4 representative mean replicates and were used for organic and inorganic assay. On 0.1 g fresh matter, proline content was determined by ninhydrin reagent at 528 nm (Dreir & Goring, 1974) and soluble sugars content were assayed at 625 nm using the anthrone reagent (Ashwell, 1957).

Sodium and potassium were determined from the dried calli by flame photometric method at 590 nm for Na^+ and 680 nm for K^+ (Novozamsky *et al.*, 1993).

Statistical analysis: The experimental device is a random model. Quantitative data were processed for the analysis of variance and means were compared by Duncan test.

RESULTS

Selective salt concentration determination (NaCl concentrations effect on callus growth): Determination of the salt concentration suitable for developing salt tolerant cells lines was made on a percentage reduction basis of relative growth rate. With increasing salinity concentration (0 to 9 g L^{-1} NaCl), a gradual decrease in callus growth was observed (Fig. 1). In fact at 4 and 6 g L^{-1} NaCl concentrations, salt treated calli still had higher RGR (these salt concentrations were insufficient to cause a relative reduction of 50% compared to controls). However 8 and 9 g L^{-1} NaCl concentrations had more significant retarding effect since, the stressed calli RGR were strongly decreased by 65% and 75%, respectively. In addition to slow callus growth at higher NaCl levels, salt stressed calli showed dehydration (increment of DW/FW ratio by 1.65 fold compared to control).

Table I: Variance analysis for dry weight (DW) and dry growth rate (DGR) of calli

Variable	Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value
DW	Duration (d)	1	0.00001321	0.00001321	1.53 ns
	Salinity (S)	4	0.00283446	0.00070861	82.31 ***
	(d) x (S)	4	0.00003595	0.00000899	1.04 ns
	error	110	0.00094703	0.00000861	
	Total	119	0.00383065		
DGR	Duration (d)	1	0.01343544	0.01343544	2.18 ns
	Salinity (S)	4	2.37326066	0.59331516	96.26 ***
	(d) x (S)	4	0.02623019	0.00655755	1.06 ns
	error	110	0.67801970	0.00616382	
	Total	119	3.09094598		

ns: not significant; ***: significant at p<0,001

Variance analysis showed a highly significant effect of NaCl on callus DW (Table I). Three different groups [(0), (4 & 6), (8 & 9) g L^{-1} NaCl] were distinguished for NaCl concentration. However, classification on the basis of GR revealed four distinct groups ({0} > {4 & 6} > {8} > {9} g L^{-1} NaCl).

Progressive selection of NaCl tolerant callus lines: The culture of 1400 explants for a month of stress on 4 g L^{-1} NaCl concentration generally led to slower growth with the exception of 57 calli that showed fast growth and equivalent size to that of controls. After transferring to the second medium with 6 g L^{-1} NaCl concentration, 38 other callus pieces showed a growth remarkably similar to those of the first identified calli. The other calli had much retarded growth and became smoother and slightly brownish. Moreover transfer of all explants on 8 g L^{-1} NaCl selective pressure (final stress) exacerbated salt injury symptoms in the sensitive calli. However the 95 earlier identified calli showed tolerance also to 8 g L^{-1} NaCl continuing healthy fast growth and keeping the yellowish colour (tolerant calli). The fresh and dry weights, measured at the end of the experiment, were affected by NaCl and the growth rate was more reduced in the sensitive calli (Table II). However DGR was higher for tolerant (69%) but reduced more for sensitive calli (26%). On the other hand, the water content of callus tissues was always higher and did not show large variation among the three types of calli (91% for control & 90% both for tolerant & sensitive calli).

The analysis of variances carried out for growth parameters revealed a very highly significant difference for calli type factor. Three different groups (controls > tolerant calli > sensitive calli) were highlighted (Table II). However for selected calli, the percentage of GR reduction significantly below 50% as compared to controls is an evidence of their tolerance to salt stress.

NaCl independence and tolerance stability of the selected cells/callus lines: Independence test consisted of culturing all calli types for two months in callusing salt-free medium. Growth of the selected calli was slightly lowered initially during the first week but thereafter resumed normal and showed best fresh weight equal to 101.6% of control calli (Table III), demonstrating their independence of NaCl. In contrast sensitive calli exhibited greater loss of weight (7.2% relatively to controls).

Table II: Fresh and dry weight (FW & DW) and growth rate (GR) of three distinguished type (control, tolerant, sensitive) of calli at the end of progressive stress

	Weight (g)		GR	
Type of calli	FW	DW	FGR	DGR
Control	0.86±0.11a	0.08±0.01*a*	3.30±0.56a	5.30±0.65a
Tolerant	0.57±0.08b	0.06±0.01*b*	1.87±0.42b	3.66±0.83b
Sensitive	0.29±0.01c	0.03±0.01*c*	0.43±0.03c	1.38±0.24c

For each column, the values assigned by different letters differ at p<0,05

Table III: Fresh weight (FW) and growth rate (GR) of three differentiated type (control, tolerant, sensitive) of calli after 2 months of culture on salt-free medium (independence test)

Type of calli	FW (g)	GR
Control	4.55±1.12a	4.30±1.30b
Tolerant	4.63±1.22a	7.07±2.13a
Sensitive	0.33±0.05b	0.15±0.08c

For each column, the values assigned by different letters differ at p<0,05

Table IV: Fresh weight (FW) and growth rate (GR) of three discriminated type (control, tolerant, sensitive) of calli after 1 and 2 months of culture on salt (8 g L^{-1} NaCl) medium (stability test)

	FW (g)		GR	
Type of calli	1 month	2 months	1 month	2 months
Control	6.12±1.03a	8.63±1.72a	0.34±0.15a	0.94±0.33a
Tolerants	6.11±1.06a	8.02±1.03a	0.32±0.13a	0.84±0.39a
Sensitive	0.37±0.04b	0.39±0.04b	0.13±0.06b	0.19±0.07b

For each column, the values assigned by different letters differ at p<0,05

Statistical analysis showed a highly significant effect of callus type factor and two distinct groups (tolerant & controls calli in the same group & sensitive calli in the second group) were been clustered, confirming that the growth of tolerant calli was independent of NaCl. In addition, classification based on the growth rate presented three separate groups revealing a better performance of tolerant calli (Table III).

The salt tolerance stability assay consisted of retransferring calli culture (tolerant calli & sensitive calli)

Table V: Analysis of variance of fresh weight (FW) and growth rate (GR) in the stability test

Variables	Source of variation	Degree of freedom	Sum of squares	Mean squares	F.value
FW	Duration (d)	1	0.84	0.840	75.51***
	Type of calli	3	51.52	17.173	1543.07***
	(d) × (Type)	3	0.29	0.089	8.04***
	error	88	0.98	0.011	
	Total	95	53.61		
FGR	Duration (d)	1	0.54	0.542	50.23***
	Type of calli	3	0.60	0.199	18.47***
	(d) × (Type)	3	0.12	0.040	3.73*
	error	88	0.95	0.012	
	Total	95	2.21		

*: significant at p<0,05; ***: significant at p<0,001

for two other months on salt medium containing 8 g L^{-1} NaCl. On this selective pressure under saline concentration, the tolerant calli retained their yellowish colour and grew satisfactorly *i.e.,* almost equal GR to that of the control (Table IV). Sensitive calli growth was more inhibited and some of them became ultimately necrotic resulting in death of many calli. Fresh GR of the tolerant calli was 2.5 and 4.4 fold greater than that of the sensitive calli after one and two month.

Variance analysis showed a highly significant effect both of callus type factor and culture duration (Table V). Moreover two groups were distinguished for callus type (tolerant & control > sensitive) at one and two months of callus culture on 8 g L^{-1} NaCl (Table IV), which proved that the tolerant calli growth varies slightly in the presence of NaCl.

Determination of K^+, Na^+, proline and soluble sugars contents: The K^+ content in tolerant calli remained unaltered. But, in sensitive calli, the potassium level showed a sharp decline. However Na^+ content both in tolerant and sensitive calli was much higher than in the controls calli (Table VI). The K^+/Na^+ ratio decreased in all the calli grown on NaCl-media, but the reduction was twice lower in the tolerant than in the sensitive calli (ratios were 1.79 in control, 0.18 in tolerant & 0.09 in sensitive calli).

The total amount of free proline and soluble sugars (Table VI) greatly increased in tolerant calli; 2.7 fold for proline and 1.5 fold for sugars, compared to control. However these amounts remained low in the sensitive calli and didn't show any difference with control. Statistical analysis showed a highly significant effect of the callus type for the four variables (K^+, Na^+, proline & soluble sugars) (Table VII). Two groups have been distinguished for K^+ as well as for proline and sugars ({control & tolerant calli} > {sensitive calli}). But concerning Na^+, classification rather showed three groups (control < tolerant calli < sensitive calli).

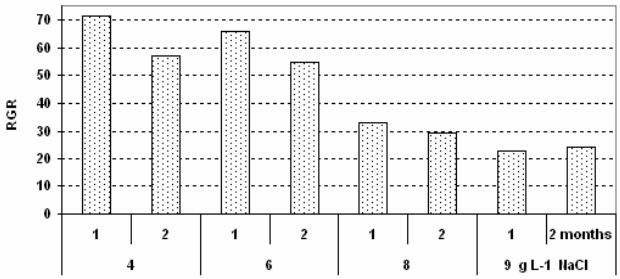
Correlation analysis (Table VIII) revealed that in tolerant calli, the growth weight was strongly correlated with both mineral (K^+ & Na^+) and organic (proline & soluble sugars) solute contents. Otherwise for this callus type, all of these parameters were positively correlated with each other. However sensitive calli showed no correlation except a negative one between K^+ and Na^+ contents.

Table VI: K^+, Na^+, soluble sugars and proline contents in the three separated type (control, tolerant & sensitive) of calli

Variables	Controls	Tolerant	Sensitive
K^+ (µéq/g DW)	157.79±4.02a	150.6±2.56a	78.59±3.46b
Na+ (µéq/g DW)	87.94±5.89c	822.5±58.58b	912.6±45.54a
Sugars (µmoles/g FW)	16.96±1.19b	25.93±1.86a	16.16±0.82b
Proline (µmoles/g FW)	21.45±2.43b	56.73±7.11a	21.55±0.82b

For each column, the values assigned by different letters differ at p<0.05

Fig. 1: Relative growth rate (RGR) of callus dry matter depending on the NaCl concentration and duration of culture



DISCUSSION

Overall salt stress led to reduced growth, browning and necrosis of the cell clusters. The percentage of reduction of the dry weight, generally considered as a sensitivity index, showed that concentrations 4 and 6 g L^{-1} NaCl are not sufficient to cause a relative reduction near to 50% compared to control (threshold widely used for the ranking of the plants tolerance). However the concentrations 8 and 9 g L^{-1} NaCl can be used for screening tolerant cell lines, as they lead to more pronounced reductions. These results are used to set the salt dose selective 8 g L^{-1} NaCl at which the tolerant cells should survive.

In fact, during the application of progressive salt stress, some calli showed NaCl-tolerance (lack of browning & maintenance of a continuous growth). These tolerant calli isolated under selective conditions (high level of NaCl), represent 6.8% of 1400 original homogenous fragments. This relatively high percentage may be due to the *in vitro*

Table VII: Analysis of variance for variables Na^+, K^+, soluble sugars and proline

Variable	Source of variation	Degree of freedom	Sum of squares	Mean squares	F value.
	callus type	2	1.195	0.597	623.92***
K^+	Error	9	0.009	0.001	
	Total	11	1.203		
	callus type	2	13.856	6.929	1737.31***
Na^+	Error	9	0.036	0.004	
	Total	11	13.894		
	callus type	2	0.491	0.245	65.85***
Sucres	Error	9	0.034	0.004	
	Total	11	0.524		
	callus type	2	2.354	1.177	131.04***
Proline	Error	9	0.081	0.009	
	Total	11	2.434		

***: significant at p<0,001

Table VIII: Correlations for parameters studied in *Troyer citrange* calli

Type of calli	Variables	K^+	Na^+	Proline	Sugars
	Weight of matter	0.994	0.994	0.996	0.992
		0.006	0.006	0.004	0.008
	K^+	1	0.989	0.996	0.974
Tolerant		0	0.011	0.004	0.026
	Na^+	0.989	1	0.982	0.982
		0.010	0	0.018	0.018
	Proline	0.996	0.982	1	0.982
		0.004	0.018	0	0.018
	K^+	1	-0.933	0.591	0.205
Sensitive		0	0.067	0.409	0.795
	Na^+	-0.933	1	0.399	0.149
		0.067	0	0.601	0.851

variation of cells resulting from the use for a long time of growth regulators at high concentrations (1 mg L^{-1} 2,4-D & 5 mg L^{-1} BAP) but also to the existence of the tolerance character in some initial embryonic explants (genetic heterogeneity). Indeed the seeds of the hybrid rootstock *Troyer citrange* are highly polyembryonic and therefore behaved as zygotic embryos and several nucellar embryos. This intrinsic genetic heterogeneity (uniparental & biparental inheritance) of the embryos (Batygina & Vinogradova, 2007) necessarily predicted the involvement of this genetic component in the *in vitro* response of initial excised explants (El Yacoubi *et al*., 2004).

Growth maintenance in salt-free medium (independence test), demonstrated that the selected calli was independent from NaCl. In addition, preservation of this tolerance (stability test) later at 8 g L^{-1} NaCl, indicates an assured stability in the character of tolerance/resistance and confirms the selection of salinity tolerant variants. In addition, growth reduction and salt damage appear to be associated with ions toxicity (Rochdi *et al*., 2003 & 2005). Indeed mineral analyses showed firstly that the calli culture on NaCl medium caused a significant accumulation of Na^+ with a toxic effect only in non-selected calli resulting in growth reduction and necrosis of the sensitive cells. On the other hand, K^+ in the tolerant selected calli did not differ significantly from that of controls. However the decrease in K^+ content in the non-selected calli is consistent with literature and reflects the sensitivity of glycophytes submitted to salt stress (Ben hayyim *et al*., 1985). In addition, Rochdi *et al.* (2003) have already reported lower levels of K^+ in salt-sensitive calli of *Troyer citrange*. These authors consider the decline in potassium content as a criterion for sensitivity to NaCl. Cramer *et al*. (1985), noted that high concentrations of Na^+ caused the displacement of Ca^{2+} from plasmalemma and resulting in a disruption of membrane permeability and K^+ efflux from the cytosol. However, maintaining sufficient concentration of K^+ is essential for growth (Marshner, 1995).

Cells tolerance to NaCl can be correlated with changes in transport properties and the capacity of ionic regulation (Sun *et al*., 2009). Our results also supported this close relationship between callus growth and their content of K^+ and that the tolerance of selected calli is associated with the retention of K^+. Other studies have also shown that the amount of biomass produced is related to the potassium efficiency absorption and that the Citrus tolerance at callus level is related to the retention of K^+ (Bouharmont *et al.*, 1993; Piqueras *et al.*, 1996). However Ben-Hayyim and Kochba (1983) showed that in *Citrus sinensis*, tolerant calli may have lower K^+ content than in sensitive calli.

The relative low level of K^+/Na^+ ratio in sensitive calli, suggests that tolerant calli have a better selectivity and higher capacity to absorb K^+. We have also noted a significant decrease in this ratio from other sensitive citrus (Rochdi *et al*., 2003). However K^+/Na^+ ratio remains lower than that of control grown in salt-free medium, because the Na^+ content (the denominator) has increased among sensitive and tolerant calli that (tolerant) kept a good growth. Other studies have noted that the accumulation of sodium in saline conditions may be an adaptive value (Erdei & Kuiper, 1979). Our results also showed that the growth of tolerant calli is strongly correlated with their sodium content. This evidence, now is clear that Na^+ was accumulated at two levels, in the vacuolar space for selected tolerant cells (compartmentalization of excess Na^+) and in the cytosol (toxicity effect) for sensitive calli. The vacuolar sequestration of sodium ions blurs cancels the toxic effect of Na^+, while increasing the cytoplasmic K^+/Na^+ ratio and maintaining the water balance of selected calli through

increasing osmotic pressure of tolerant cells. Some researchers have also noted an accumulation of salt ions in cells tolerant (Watad *et al*., 1983) and other works have shown ion compartmentation as a component of salt adaptation of glycophyte cells (Binzel *et al.*, 1988). However Maathuis and Amtmann (1999) indicated that the key element in salinity tolerance is the ability to sustain a high cytosolic K^+/Na^+ ratio.

The accumulation of sodium is also strongly associated with increased proline and soluble sugar contents of *Troyer citrange* tolerant calli. Other studies have also registered the similar results under salt stress (Das *et al.*, 1990; Misra *et al.*, 1990; Patnaik & Debata, 1997; Liu & Van Staden, 2000; Elavumoottil *et al.*, 2003). Otherwise when the organic solutes accumulates in the cytosol it provides an osmotic adjustment, where the toxic salt ions are sequestered in the vacuole. The accumulation of organic solutes is therefore a metabolic character, which could be an indicator of salt stress tolerance of *Troyer citrange* calli. In contrast sensitive calli showed no significant change in these solutes content (Das *et al.*, 1990; Misra *et al*., 1990; Rochdi *et al*., 2003). Concurrently absence of increment in organic solutes levels, associated with high sodium content, registered in the non-selected calli of this rootstock due to an inability to restrict the absorption of these ions (together with browning, growth reduction & loss of potassium), confirmed that these sensitive calli were unable to sequester salt ions in vacuoles, which lead to intoxication by salt.

Significant correlation noted between proline and sugars in our study among tolerant calli of *Troyer citrange* would be explained by Stewart hypothesis (Stewart, 1981) suggesting that high soluble sugars levels can induce the accumulation of proline by inhibiting its oxidation (Das *et al*., 1990; 1992). Indeed, in many species under water stress, the accumulation of proline occurs only when there is sufficient carbohydrate concentration in the tissues (Hsiao, 1973; Misra *et al*., 2002).

CONCLUSION

Maintaining an active growth both during their transfer to salt-free medium and during their retransfer in medium with 8 g L^{-1} NaCl, respectively demonstrated their independence to NaCl and stability of their salinity tolerance. The accumulation of organic solutes allowed the osmotic adjustment between the vacuolar compartment and the cytosol of the selected tolerant cells. Thus the mechanism of salt tolerance at callus/cellular level is found to be associated with changes in transport properties and the ability to ionic regulation and compartmentation.

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