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NaCl stress-induced growth, water and ions contents changes on *in vitro* selection of salt tolerant and salt sensitive callus of wheat (*Triticum durum* Desf.)

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Abstract

Callus cultures tolerant to NaCl were developed from eight wheat genotypes using *in vitro* selection techniques. The accumulation of inorganic (Na^+, Cl^- and K^+) solutes, water content and relative fresh weight were determined in selected (tolerant and sensitive) calli after a NaCl shock in order to evaluate their implication in salt tolerance of the selected lines. No growth reduction was observed in salt-tolerant calli compared to control while a significant ($P<0.05$) decrease about 46.54% was observed in salt sensitive ones when both were cultivated under NaCl stress. Water content is significantly ($P<0.05$) high in salt-sensitive calli than salt-tolerant ones. Selected calli accumulate less K^+ as compared with control. However, K^+ content of salt-tolerant calli is greater than that of salt-sensitive. Accumulation of Na^+ and Cl^- were more important in salt-sensitive calli in comparison with salt-tolerant ones while K^+ content was lower in salt-sensitive than in salt-tolerant calli when both were exposed to salt. The results indicated Na^+ and Cl^- exclusion combined to less K^+ accumulation may play a key role in *in vitro* salt-tolerance in wheat calli lines obtained by *in vitro* selection and they could contribute mainly to counteract the negative effects of salt stress in wheat tolerant calli. Comparison of K^+/Na^+ ratio permitted to classify Sebou, Anouar and Tarek which are as most salt-tolerant wheat genotypes and on contrary, Marzak, Ourgh, Massa and Amjad as salt-sensitive wheat genotypes. K^+/Na^+ ratio can be use as a criterion of wheat genotypes classification.

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Introduction

Wheat production in the arid areas is confronted with several kinds of stresses. Salinity of soil and/or irrigation water is among them. Salinity is a major factor limiting the crop productivity in arid and semi-arid areas of the world (Sairam *et al*., 2002; Poustini and Siosemardeh, 2004; Zaman *et al*., 2005). It has been estimated that salts affected nearly 950 million ha of land in the world (Munns *et al*., 1999; Flowers, 2004). The understanding of the mechanisms that enable plants to adapt to salt stress is necessary for exploiting saline soils. Salt-tolerance in higher plants is the result of numerous physiological and biochemical processes. Many techniques have so far been adapted to alleviate this problem. Of these, one is the selection of salt tolerant genotypes. This technique has successfully been used by many researchers for the last many years. They reported that changes in salinized plants growth appear to be associated with accumulation of toxic elements and/or osmotic adjustment and turgor maintenance against these elements (Morgan 1984; Noaman *et al*., 2004). Plants under saline environment accumulated organic solutes such as sugars, amino acids, proteins and/or other compounds against the deleterious effects of Na^+ and Cl^- (Yancey *et al*., 1982; Rochdi *et al*., 2003). Accumulation of these compounds in response to stress is a metabolic adaptation found in a number of stress-tolerant plants. Many authors reported that salt tolerance in wheat is related to its low uptake of Na^+ and Cl^- (Wyn and Gorham, 1986; Ashraf and O'Leary, 1996). In the last decade, this conventional technique was supplemented with in vitro technique. Plant tissue culture techniques have been used to produce salt-tolerant cell lines in several plants (Benavides *et al*., 2000; Alvarez *et al*., 2003). This suggests that tissue culture selection is an adequate model to select tolerant clone from overall non-tolerant populations and to research the adaptative mechanisms of plants living in saline environment. Several researchers also reported that in vitro selection of plants cell lines that exposed to saline environment can be selected for enhancement of tolerance to salinity (Kirti *et al*., 1991; Lutts *et al*., 1999). Moreover, studies at cellular level provide better knowledge to understand the mechanism of salt tolerance, since they require relatively little space and lower time for the selection, as well as controlled environment (Farrukh, 2002; El Yacoubi *et al*., 2004). In callus cultures selected for in vitro NaCl tolerance, Sabbah and Tal (1990) have found that no electrolytes were the main contributors to the decrease in osmotic potential. Furthermore, Basu *et al*. (2002) reported that K^+ was the first candidate to counteract the negative water potential of outside medium when exposed to salt stress. In our research, we selected salt-tolerant calli of sugarcane in order to study the physiological and biochemical adaptative mechanisms implied in their salt tolerance.

The present report describes *in vitro* technique as an efficient method to study the effect of NaCl stress and ionic solutes accumulation in callus tissue of height wheat genotypes differing in salt tolerance. The aims of this study were to compare Na^+, Cl^- and K^+ accumulation in non-selected and salt-tolerant calli to identify the discriminate ions in tolerance to salinity.

Materials and methods

Plant material

Eight wheat *Triticum durum* Desf.) genotypes "Sebou" (septoriose tolerant), "Ourgh", "Anouar", "Tomouh" and "Tarek" (wide adaptation)", "Amjad" and "Massa" (semi-arid and arid zones) and "Marzak" (major fungal diseases tolerant) were used for the experiments. The seeds were obtained from NIAR (National Institute of Agronomic Research), Rabat, Morocco.

Callus establishment

Seedlings were raised of the eight wheat genotypes under greenhouse. After of anthesis, the immature embryos which the size is ranging between 0.8 and 1.5 mm were taken and used as explants. Callus cultures for the eight genotypes were induced from mature embryos following procedures outlined by Koutoua *et al*. (2007). Callus was initiated from the immature

embryos by culturing on Murashige and Skoog (1962) medium (MS) with vitamin B5 (Gamborg *e*t al., 1968), containing 30 g/l glucose, 8.0 g/l agar and 3.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D). This callogenesis medium (MC) was adjusted to pH 5.8 with NaOH (0.1 N), autoclaved at 120 °C and 1 bar for 30 min and dispensed in Petri dish (30 ml in each Petri dish). The cultures were placed in a growth chamber at 28 ± 2 °C under photoperiod of light/dark (16/8 h). Illumination was supplied by cool white fluorescent tubes at approximately 2,000 lux light intensity. After 4 weeks culture, calli were obtained to start growing on the stress media.

In vitro selection procedure

Calli were separated from the explants and approximately 200 mg were subcultivated to MC medium with increase in NaCl for further proliferation and to initiate a shock selection. Calli were incubated during 16 weeks while passing successively on MC medium enriched gradually in NaCl (saline stress): M1: MC medium + 4 g/l NaCl; M2: MC medium + 8 g/l NaCl; M3: MC medium + 12 g/l NaCl; M4: MC medium + 16 g/l NaCl. The control callus was subcultured on NaCl-free medium and was designated as non-selected callus line. After 16 weeks (4 x 4 weeks), the evaluation of calli to salt stress was carried according to El Yacoubi et al. (2004) who mentioned the following characteristics:

- Control calli: calli became yellowish and normal growth;
- Tolerant calli to salinity: calli became yellowish and normal growth as observed to control;
- Sensitive calli to salinity: calli became brown except for small groups of cells that remained light in color and the growth was inhibited compared to control.

The tolerant and sensitive calli were transferred to the non-saline MC medium for 8 weeks for further proliferation (independence test). After that, calli were transferred again to the selection medium (16 g/l NaCl) for 8 weeks for stabilization (stability test). Control (non-selected) calli were continuously cultivated in MC medium without NaCl. Briefly, the assay was initiated according to the following scheme: (1) calli from the control medium were transferred to non-saline medium during 16 weeks (8 x 2 weeks); (2) calli from the selected line (tolerant and sensitive) were transferred to non-saline medium for 8 weeks and to medium with 16 g/l NaCl added for again 8 weeks.

Callus growth determination

The fresh weight of control, salt tolerant and salt sensitive calli was determined at 0 time (w_o) and 8 weeks after the beginning of the experiment (w_f) on media containing 0 or 16 mg/l NaCl to estimate the extend of adaptation achieved by selected tissues. Calli were harvested and relative fresh weight growth rate (r) of calli was calculated as $(w_f - w_o)/w_o$.

Evaluation of the mechanisms of tolerance

To understand the mechanisms involved in salt tolerance, water content, sodium (Na^+), potassium (K^+) and chloride (Cl^-) accumulation as well as ratio K^+/Na^+ were determined in calli (control, tolerant and sensitive) at the end of experiment.

Water content

The water content (wc) of calli was calculated by the following formula: wc (%) = [(fw - dw)/fw] x 100.

Ions measurement

For ions determination, calli were rinsed for 5 min in cold distilled water in order to the maximum solute present in the apoplast, while avoiding a substantial elimination of cytoplasmic solutes (Sacchi *et al.*, 1995). Calli were oven-dried at 80 °C for 48 h, 200 mg of dry calli were finely ground and the powder was recovered in crucibles. Sodium (Na^+) and potassium (K^+) were determined by flame spectrophotometric method at 590 and 680 nm, respectively (Novozamsky *et al.*, 1993). Hundred (100) mg oven dried samples were digested in 5 ml of nitric acid (100%). Chloride was measured by titration with silver nitrate (2%) in the

presence of some drops of chromate potassium (1%) according Cotlove (1965). Ions concentrations are expressed in mg/g dw (dried weight) basis with NaCl (Na^+ and Cl^-) and KCl (K^+) as standard.

Statistical analysis

For each experiment, 30 calli were used (5 per Petri dish). A two-way analysis of variance of data for all the parameters was computed SAS statistical software. Differences in mean values were tested by analysis of variance, and significance levels were obtained with LSD test at 5%. Data are the means of three replicates.

Results

Callus growth

In the absence of stress, relative fresh weight growth (*r*) was similar for both control and salt tolerant calli. Salt effect results in a significant reduction ($P<0.05$) in salt sensitive calli *r* (Table 1) while no significant reduction was observed in NaCl-tolerant ones (Figure 1). In the case of NaCl-sensitive calli, *r* decreased from 1.59 in the absence of stress to 0.85 at 16 g/l NaCl after 16 weeks; this decrease corresponded to 46.54%. *r* of salt tolerant calli was about 1.65 in the absence of stress and about 1.60 after 16 weeks at 16 g/l NaCl. The significant difference observed between the types of calli in their growth indicated that salt-tolerant calli grew faster than salt-sensitive calli when they were cultivated in the presence of NaCl.

Water content

The water content varies according to genotype and type of calli (Table 2). However, no significant difference was observed between wheat genotype for the same type of callus. Water content of calli of the eight genotypes decreased significantly under salt stress. The salt sensitive calli were the lowest than salt-tolerant calli. These results are confirmed by the analysis of variance which reveals a significant effect at 5% of water content with salt stress and the interaction genotype x types of callus and stress x types of callus (Table 1). Water content passes from 89.97% of fresh weight in control calli to 78.0 and 41.56% of fresh weight, respectively in tolerant and sensitive calli (Figure 2). Salinity decreases water content of calli; however, wc is significantly high ($P<0.05$) in selected sensitive calli than selected tolerant ones. The genotype of wheat keeps water differently in presence of salt: Control > Tolerant > Sensitive.

Table 1. Results of two-ways variance analysis for r, wc and ions content accumulation in salt sensitive and salt tolerant calli of wheat.

Parameter	Stress	Genotype	Type of callus	Interaction (genotype x type of callus)	Interaction (stress x type of callus)
r	22.6**	2.46 *	113.2*	0.69 *	18.5*
WC	31.2*	2.25 ns	47.0 **	18.9 *	20.6*
K^+	2.87*	15.00 **	130.4 **	3.5 *	3.3*
Na^+	10.4*	11.37 *	127.3*	4.8 *	4.1*
Cl^-	13.0*	11.56*	199.8*	4.1*	8.6*
K^+/Na^+	3.6*	1.24*	1.93*	1.30*	1.4*

r: relative fresh weight growth; wc: water content; F-ratios are given for the main effects of the following levels of classification: stress (i.e. presence of NaCl in the media), type of calli and interaction between these levels of classification (ns, not-significant; *significant at $P = 0.05$).

Table 2. Comparison between callus water content of eight wheat genotypes after treatment with 16 g/l NaCl.

Genotype	Water content (%)		
	Control	Tolerant	Sensitive
Sebou	87.61 ± 1.7 a	79.45 ± 1.5 b	43.09 ± 1.2 c
Anouar	91.82 ± 2.6 a	81.99 ± 2.6 b	42.55 ± 1.7 c
Marzak	93.36 ± 1.3 a	77.37± 1.6 b	44.34 ± 1.8 c
Ourgh	88.71 ± 1.9 a	78.14 ± 1.8 b	40.52 ± 1.4 c
Tarek	89.54 ± 1.4 a	76.84 ± 1.3 b	38.62 ± 1.9 c
Tomouh	90.71 ± 2.4 a	80.00 ± 2.3 b	45.91 ± 1.3 c
Massa	89.43 ± 1.5 a	73.05 ± 1.8 b	39.82 ± 1.7 c
Amjad	88.55 ± 2.2 a	77.83 ± 1.4 b	37.60 ± 1.4 c

In row and column, values followed by a different letter are significantly different (LSD test at 5%). Each value represents the mean of three replicates; ± SD (standard deviation).

K+ content

Table 3, shows the concentrations of K^+ in wheat calli with eight genotypes. Selected (tolerant and sensitive) calli line accumulated significantly less K^+ ($P<0.05$) as compared with non-selected (control) callus line.

However, weaker concentrations of accumulated K^+ were obtained in salt-sensitive calli. K^+ concentration decreased under salt stress, it varies according to genotype and type of calli. From 14.90 mg/g dw in control, K^+ content reached 12.43 mg/g dw for salt tolerant calli and it passes from 11.52 mg/g dw in salt sensitive calli (Figure 3). The reduction corresponded, respectively, to 16.58 and 22.68% of K^+ content as compared to control. K+ content of salt-tolerant and salt-sensitive calli of Ourgh and Tarek genotypes are statistically identical. On the other hand, K^+ content of Tomouh genotype seems to be independent of the salinity because no significant difference was observed in the three type of calli.

Table 3. Callus K^+ content of eight wheat genotypes after treatment with 16 g/l NaCl.

Genotype	Concentration of K^+ (mg/g dw)		
	Control	Tolerant	Sensitive
Sebou	17.31 ± 0.8 a	13.96 ± 0.7 d	12.22 ± 0.6 c
Anouar	15.81 ± 0.5 b	13.34 ± 0.5 d	11.69 ± 0.3 c
Marzak	12.93 ± 0.69c	12.00 ± 0.3 c	10.71± 0.3 a
Ourgh	13.69 ± 0.9 d	10.20 ± 0.3 a	10.54 ± 0.7 a
Tarek	15.80 ± 0.5 b	12.02 ± 0.3 c	11.93 ± 0.8 c
Tomouh	13.27 ± 0.8 cd	12.65 ± 0.5 cd	12.44 ± 0.5 c
Massa	15.24 ± 0.7 b	11.83 ± 0.2 c	10.90 ± 0.0 a
Amjad	15.13 ± 0.78 b	13.43 ± 0.38 d	11.72 ± 0.74 c

In row and column, values followed by a different letter are significantly different (LSD test at 5%). Each value represents the mean of three replicates; ± SD (standard deviation).

Furthermore, analysis of variance (Table 1) showed the significant effect of K^+ content, genotype and type of callus. Otherwise, significant interaction at 5% was observed between genotype x types of callus stress x types of callus. The comparison of K^+ content revealed that the genotypes were regrouped in four groups with control: Sebou (group 1) > Anouar = Tarek = Massa = Amjad (group 2) > Ourgh (group 3) ≥ Tomouth = Marzak (group 4); three groups with salt tolerant calli: Sebou = Anouar = Amjad (group 1) ≥ Tomouh = Tarek = Massa = Marzak (group 2) > Ourgh (group 3); and two groups with salt sensitive calli: Sebou = Anouar = Tarek = Tomouth = Amjad (group 1) > Marzak = Ourgh = Massa (group 2).

Table 4. Callus Na^+ content of eight wheat genotypes after treatment with 16 g/l NaCl.

Genotype	Concentration of Na^+ (mg/g dw)		
	Control	Tolerant	Sensitive
Sebou	7.88 ± 0.8 a	32.65 ± 0.4 e	37.67 ± 0.8 f
Anouar	9.34 ± 0.1 b	32.63 ± 1.2 e	36.23 ± 0.8 f
Marzak	10.82 ± 1.0 c	36.18 ± 0.4 f	49.20 ± 0.9 a
Ourgh	10.12 ± 0.6 bc	28.24 ± 0.7 a	43.76 ± 1.0 g
Tarek	11.70 ± 0.5 cd	33.10 ± 0.8 e	51.66 ± 0.9 a
Tomouh	12.60 ± 0.3 d	36.96 ± 0.9 f	46.46 ± 1.8 g
Massa	9.73 ± 0.3 b	37.84 ± 0.6 f	44.65 ± 0.6 g
Amjad	9.41 ± 0.5 b	36.38 ± 1.1 f	47.62 ± 1.5 g

In row and column, values followed by a different letter are significantly different (LSD test at 5%). Each value represents the mean of three replicates; ± SD (standard deviation).

Na^+ content

Presence of NaCl salt in the culture medium significantly ($P<0.05$) increased the Na^+ content in selected calli line of eight genotypes. Non selected calli were the lowest and salt-sensitive calli the highest follow-up of salt-tolerant calli (Table 4). Salt-tolerant and salt-sensitive calli were respectively three and four times higher in this attribute as compared to control. Na^+ concentration increased under salt stress, it varies according to genotype and type of calli. From 10.20 mg/g dw in control, Na^+ content increase to reach 34.25 mg/g dw for salt-tolerant calli and then 44.66 mg/g dw in salt-sensitive calli (Fig. 4). Besides, analysis of variance (Table 1) showed a significant effect between Na^+ content, genotype and type of callus and their interaction. The comparison of Na^+ content revealed that the genotypes were classified in four groups with control: Tomouth (group 1) ≥ Tarek = Marzak = Ourgh (group 2) ≥ Massa = Amjad = Anouar (group 3) > Sebou (group 4); three groups with salt-tolerant calli: Massa = Tomouh = Amjad = Marzak (group 1) > Tarek = Sebou = Anouar (group 2) > Ourgh (group 3); and three groups with salt-sensitive calli: Tarek = Marzak (group 1) > Amjad =

Tomouth = Massa = Ourgh (group 2) > Sebou = Anouar (group 3).

Table 5. Callus K^+/Na^+ ratio of eight wheat genotypes after treatment with 16 g/l NaCl.

Genotype	Concentration of K^+/Na^+		
	Control	Tolerant	Sensitive
Sebou	2.20 ± 0.10 a	0.42 ± 0.03 d	0.32 ± 0.01 c
Anouar	1.69 ± 0.08 b	0.41 ± 0.01 d	0.31 ± 0.04 c
Marzak	1.20 ± 0.09 c	0.33 ± 0.01 a	0.21 ± 0.01 e
Ourgh	1.35 ± 0.03 c	0.36 ± 0.02 ad	0.24 ± 0.03 e
Tarek	1.35 ± 0.08 c	0.38 ± 0.01 d	0.23 ± 0.02 e
Tomouh	1.05 ± 0.04 d	0.34 ± 0.03 a	0.26 ± 0.02 ce
Massa	1.59 ± 0.06 b	0.31 ± 0.01 a	0.24 ± 0.01 e
Amjad	1.67 ± 0.08 b	0.36 ± 0.03 ad	0.24 ± 0.02 e

In row and column, values followed by a different letter are significantly different (LSD test at 5%). Each value represents the mean of three replicates; ± SD (standard deviation).

K^+/Na^+ ratio

Accumulation of K^+/Na^+ ratio in selected calli line decreased significantly ($P < 0.05$) under salt stress as compared with control. The reduction was lower in salt-sensitive calli than that of salt-tolerant calli (Table 5). From 1.51 in control, K^+/Na^+ ratio reached 0.36 for salt-tolerant calli and then decreases again until 0.26 in salt-sensitive calli (Figure 5). Moreover, analysis of variance (Table 1) showed the significant effect of K^+/Na^+ ratio, genotype and type of callus. In addition, significant interaction at 5% was observed between genotype x types of callus stress x types of callus. Furthermore, comparison of K^+/Na^+ ratio indicated four genotype groups with control: Sebou (group 1) > Anouar = Massa = Amjad (group 2) > Ourgh = Tarek = Marzak (group 3) > Tomouh (group 4); two genotype groups with salt tolerant calli: Sebou = Anouar = Tarek (group 1) ≥ Ourgh = Amjad = Marzak = Tomouh = Massa (group 2); and two genotype groups with salt-sensitive calli: Sebou = Anouar = Tomouh = Tarek (group 1) ≥ Marzak = Ourgh = Massa = Amjad (group 2).

Table 6. Callus Cl^- content of eight wheat genotypes after treatment with 16 g/l NaCl.

Genotype	Concentration of Cl^- (mg/g dw)		
	Control	Tolerant	Sensitive
Sebou	39.88 ± 1.9 a	50.97 ± 2.0 d	58.72 ± 2.0 c
Anouar	36.56 ± 1.1 b	56.14 ± 1.3 e	60.20 ± 1.6 c
Marzak	37.30 ± 1.7 b	57.98 ± 1.1 e	75.34 ± 2.9 a
Ourgh	41.73 ± 1.0 a	63.52 ± 2.3 f	80.14 ± 2.3 e
Tarek	35.08 ± 1.6 b	55.03 ± 1.7 e	73.86 ± 2.1 a
Tomouh	37.30 ± 1.1 b	64.26 ± 1.9 f	56.13 ± 1.3 c
Massa	47.27 ± 1.0 d	74.97 ± 2.2 a	68.89 ± 1.8 b
Amjad	45.79 ± 1.3 cd	69.80 ± 2.0 b	78.66 ± 2.1 e

In row and column, values followed by a different letter are significantly different (LSD test at 5%). Each value represents the mean of three replicates; ± SD (standard deviation).

Cl^- content

Both types of calli (tolerant and sensitive) accumulated different quantities of Cl^- when exposed to NaCl (Table 6). In response to NaCl, Cl^- content increased significantly ($P < 0.05$) in the both types of calli as compared to control. However, this accumulation was lower in salt-tolerant calli than salt-sensitive ones. Cl^- content increased under salt stress, it varies according to genotype and type of calli. From 40.11 mg/g dw in control, Cl^- content increase until to 61.58 mg/g dw for salt-tolerant calli while it increased to reach 68.99 mg/g dw in salt-sensitive calli (Fig. 6). Moreover, Cl^- content was higher than that of any individual cation (K^+; Na^+) content in the eight wheat genotypes calli under NaCl stress. The analysis of variance showed that the accumulation of Cl^- in callus presents a significant effect on genotype, type of callus and their interaction (Table 1). The comparison of Cl^- content revealed that the genotypes were regrouped in three groups with control: Massa = Amjad (group 1) > Ourgh = Sebou (group 2) > Anouar = Marzak = Tarek = Tomouh (group 3); five groups with salt-tolerant calli: Massa (group 1) > Amjad (group 2) > Tomouh = Ourgh (group 3) > Marzak = Anouar = Tarek (group 4) > Sebou (group 5); and three groups with salt-sensitive calli: Ourgh = Amjad (group 1) > Marzak = Tarek (group 2) > Massa (group 3) > Anouar = Tomouh = Sebou (group 4).

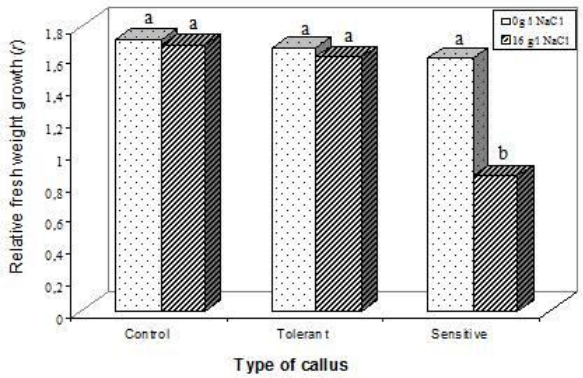

Fig. 1. Relative fresh weight growth (*r*) of control, NaCl-tolerant and NaCl-sensitive wheat on non-saline or saline media after 16 weeks of culture. Values followed by a different letter are significantly different (LSD test at 5%). Each value represents the mean of three replicates.

Discussion

The maximum potential of wheat crops is seldom attained because of limitations on morphological and physiological processes imposed by stress (Krizek, 1981). Salt is becoming increasingly limiting factor in many areas of agricultural production and is among the most important environmental factors that limit crop productivity. Salinity is an environmental stress which is a major barrier to productivity of agricultural crops throughout the world. Crops exposed to this stressful environment are observed initially to have reduced growth rates. If salt stress is more severe the response is manifested visually in a number of specific and recognizable symptoms (Rains, 1989). In the last years, *in vitro* selection has seemed to be the methodological solution to cope with this problem, since assessment of salt tolerance by this method requires relatively little space and time, as well as controlled environment. Nowadays, many studies have tried to develop salt tolerant plants through the use of tissue and cell culture. The first step of these methods was the selection of cell lines exhibiting enhanced tolerance to salinity. Salt-tolerant cell lines have been developed in several plants such as tomato (Kripkyy *et al*., 2001), rice (Basu *et al*., 2002) sunflower (Alvarez et al., 2003) and Catharanthus roseus (Elkahoui *et al*., 2005). This suggests that tissue culture selection is an adequate model to select tolerant clone from overall non-tolerant populations and to research the adaptative mechanisms of plants living in saline environment.

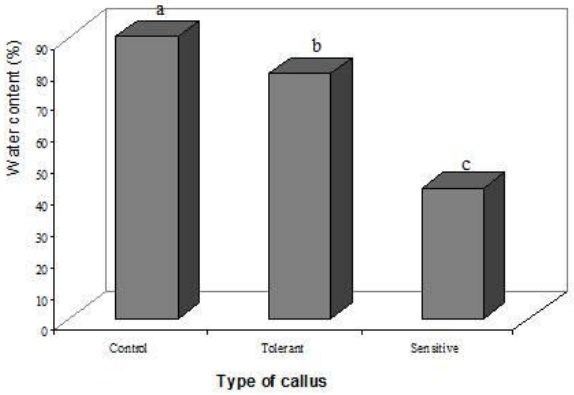

Fig. 2. Mean of water content of eight wheat genotypes according to treatment with 16 g/l NaCl. Values followed by a different letter are significantly different (LSD test at 5%). Each value represents the mean of three replicates.

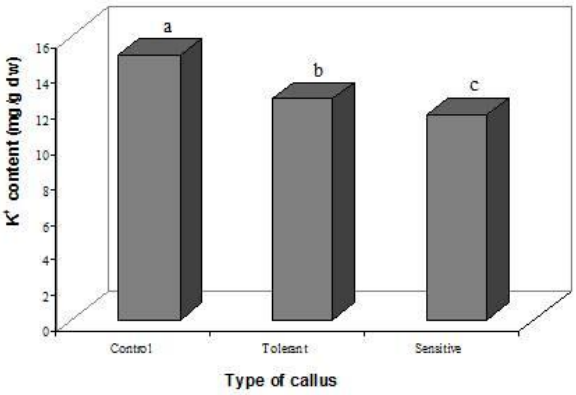

Fig. 3. Mean of K^+ content of eight wheat genotypes according to treatment with 16 g/l NaCl. Values followed by a different letter are significantly different (LSD test at 5%). Each value represents the mean of three replicates; dw: dry weight.

We developed salt-tolerant wheat calli using *in vitro* selection techniques. Sustained growth of selected calli in NaCl medium indicated that the tissues were tolerant as reported in rice (Basu *et al*., 2002). These authors reported that rice calli selected for salt

tolerance maintained high viability and regrowth capacity in the presence of NaCl in comparison with the non-selected calli. El-Sayed and Kirkwood (1992) reported that salinity reduced callus fresh weight and increased dry matter. In the present study, the relative fresh weight growth of callus was higher with salt sensitive calli and showed almost the same rate at control. On the other hand, sensitive calli were sharply decreased in this attribute at salt treatment as compared to control. This result indicated that salt-tolerant calli grew faster than salt-sensitive calli when were cultivated in the presence of NaCl. Similar results were reported in several plants Fallon and Philips, 1989; Purushotham *et al.*, 1998, Naureen and Naqvi, 2010). Salinity stress caused a significant decrease in water content of selected (salt-tolerant and salt-sensitive) calli. This reduction was also significantly marked by the genotype. Similar results were reported by Errabii *et al.* (2006) in sugarcane. However, this reduction more affects salt-sensitive calli than salt-tolerant ones as mentioned by Nguyen *et al.* (2005) at rice. In other hand, Almansouri *et al.* (1999) reported that in some varieties of wheat, water content is not affected by the saline stress.

A culture in NaCl medium enabled the salt-sensitive calli to accumulate more inorganic solutes (Na^+ and Cl) in comparison with salt-tolerant calli while salt-sensitive calli accumulated less quantity of K^+ in NaCl free medium. Cl^- content was higher than that of any individual cation (K^+; Na^+) content in the eight wheat genotypes calli under NaCl stress. These results are in accordance with the findings of Farruth (2002), in which that Cl^- content was the highest ion in calli. Selected (tolerant and sensitive) calli accumulate more Na^+ and Cl^- in comparison with non-selected (control) calli. This suggests that gradual adaptation of calli in response to culture in saline medium is directly related to uptake of Na^+ and Cl^-. Unlike Na^+, Cl^- was more accumulated in salt-sensitive calli than in salt-tolerant calli indicating that Na^+ and Cl^- toxicity may be the main part of salt effect on sugarcane calli; thus the salt-tolerance of selected calli may be due to their ability to limit tissue accumulation of Na^+ and Cl^-. The excessive accumulation of Na^+ and Cl^- known for their toxicity on cells is likely responsible for the reduced growth and even sensitive callus necrosis (Rochdi *et al.*, 2003). Na^+ and Cl^- ions seem to play a key role in vitro salt tolerance. Moreover, Gandonou *et al.* (2005) and Badu *et al.* (2007) have reported that calli of the salt-tolerant accumulated more Na^+ and Cl^- than that of the salt-sensitive in sugarcane and rice, respectively under *in vivo* conditions.

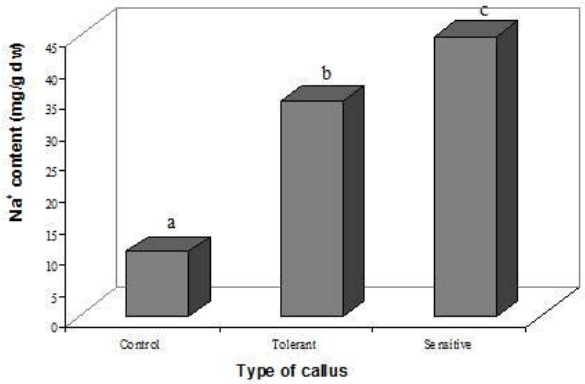

Fig. 4. Mean of Na^+ content of eight wheat genotypes according to treatment with 16 g/l NaCl. Values followed by a different letter are significantly different (LSD test at 5%). Each value represents the mean of three replicates; dw: dry weight.

The fact that the salt-tolerant calli accumulated less Na+ and Cl- than salt sensitive ones when both were cultivated in the same NaCl medium in this study suggests that *in vitro* selection techniques could allow salt-tolerant mechanisms different to those occurred naturally in tissue. It was found that salt-tolerant and salt-sensitive calli of sugarcane accumulated same quantity of Na^+ and Cl^- when both were cultivated on same NaCl medium (Gandonou *et al.*, 2006). The accumulation of in great quantity of Na^+ has been reported in other species such as rice (Ndayiragije and Lutts, 2006), sugarcane (Gandonou *et al.*, 2005), citrus (El Yacoubi *et al.*, 2004), ryegrass (Haouala *et al.*, 2007). The accumulation of this ion has been extensively discussed in several works. Piri *et al.* (1994)

and Lutts *et al.* (1996) have found a large quantity of Na^+ in salt sensitive calli compared to tolerant calli in wheat and rice, respectively. In addition, in rice (Basu *et al.* 2002) and corn (Mansour *et al.* 2005), opposite results were obtained. These authors have found more Na^+ in cell clusters of salt-resistant and less in salt-sensitive ones. Gandonou *et al.* (2005) reported the same content of Na^+ in both salt-tolerant and salt-sensitive calli. The authors suggest that the harmful effect of Na^+ depends on the accumulation place. Indeed, this accumulation is toxic in salt-sensitive calli when it is carried out in cytoplasm and nontoxic for salt-tolerant calli when it takes place in vacuole. In this case, the vacuolar sequestration would play an important role in water balance maintenance of calli by increasing the osmotic pressure in the cells.

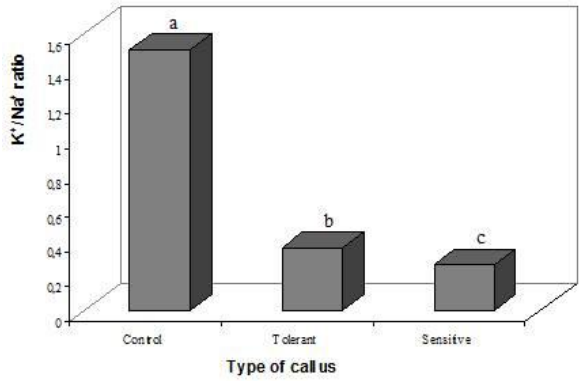

Fig. 5. Mean of $K+/Na^+$ ratio of eight wheat genotypes according to treatment with 16 g/l NaCl. Values followed by a different letter are significantly different (LSD test at 5%). Each value represents the mean of three replicates; dw: dry weight.

We noted a significant difference between types of callus and between wheat genotypes as for their capacity to accumulate Na^+ under NaCl stress. However, although Na^+ and Cl^- content were high in the selected tolerant calli than control, but they remains nevertheless lower than that of the selected sensitive ones. A similar trend was reported by Errabii *et al.* (2006) and Haouala *et al.* (2007) in sugarcane and ryegrass, respectively. They showed that the cell capacity accumulation of Na^+ and Cl^- in presence of NaCl differs according to genotypes. Callus tissue of salt-tolerant accumulated more Na^+ and Cl- than that of salt sensitive genotype. The data indicated that Na^+ exclusion operative in wheat plants was expressed in callus tissue. These results are not in accordance with the earlier findings in sorghum (Yang *et al.*, 1990) and tomato (Cano *et al.*, 1998), in which Na^+ exclusion as a salt-tolerant mechanism operated at plant level, was not expressed in the cell cultures.

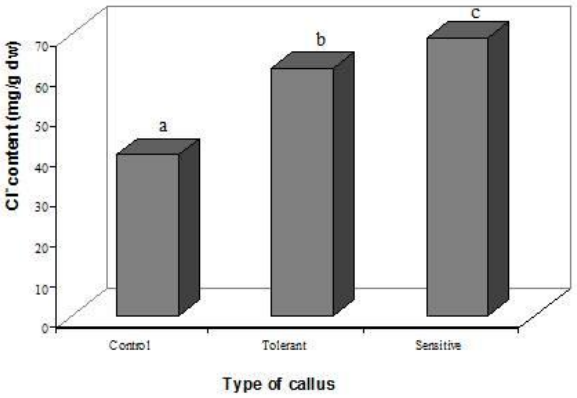

Fig. 6. Mean of Cl^- content of eight wheat genotypes according to treatment with 16 g/l NaCl. Values followed by a different letter are significantly different (LSD test at 5%). Each value represents the mean of three replicates; dw: dry weight.

Moreover, the fact that the Na^+ and Cl^- content were appreciably high in salt tolerant calli compared to control, suggests a possible accumulation of Na^+ and Cl^- on cytosol or vacuolar. The vacuolar sequestration of these ions would be thus responsible for the weak toxic effect of Na^+ and Cl^- in cytoplasm while ensuring the maintenance of hydrous balance of selected calli by increasing osmotic pressure in salt-tolerant cells (Brini *et al.*, 2007). Piri *et al.* (1994) implied the ionic partitioning as means of tolerance of salinity. In our study, the behavior of Na^+ allowed to distinguish three different groups of wheat genotype which discriminate the salt tolerant calli o those of the sensitive ones to salinity. Thus with salt-tolerant calli, Massa = Tomouh = Amjad = Marzak (group 1) > Tarek = Sebou = Anouar (group 2) > Ourgh (group 3); and with salt-sensitive calli: Tarek = Marzak (group 1) > Amjad =

Tomouth = Massa = Ourgh (group 2) > Sebou = Anouar (group 3). On the other hand with Cl^-, five groups were identify in salt-tolerant calli: Massa (group 1) > Amjad (group 2) > Tomouh = Ourgh (group 3) > Marzak = Anouar = Tarek (group 4) > Sebou (group 5); while wheat genotypes were classified in three groups with salt-sensitive calli: Ourgh = Amjad (group 1) > Marzak = Tarek (group 2) > Massa (group 3) > Anouar = Tomouh = Sebou (group 4). So Na^+ and Cl^- appeared to be responsible, at least partially, for imparting salt-tolerance to the calli of wheat NaCl tolerant calli.

Potassium (K^+) is known to play a main role in osmotic adjustment during stress (Santos-Diaz and Ochoa-Alejo, 1994; Wu *et al.* 1996). Salt-tolerant calli maintained higher K^+ concentration than salt-sensitive ones when both were cultivated in NaCl medium indicating that the salt-tolerance of the first was related to their capacity to maintain high K^+ content in the presence of the excess of Na^+. These results were in agreement with those reported by several authors (Mezni *et al.*, 2002; El Yacoubi *et al.*, 2004; Gandonou *et al.*, 2005), in which that calli of the in *vitro* salt-tolerant sugarcane cultivar maintained high level of K^+ in comparison with that of the salt-sensitive when both were cultivated in the presence of NaCl. Besides, with Ourgh and Tarek genotypes, there is no significant difference between the salt-tolerant calli and the sensitive ones in the capacity to accumulate K^+. On the other hand, K^+ content of Tomouh genotype seems to be independent of salt stress because no significant difference was observed between control, salt-tolerant and salt-sensitive calli. So, K^+ content of calli can be regarded as a criterion of tolerance/sensitivity to saline stress only for some wheat genotypes. Babourina *et al.* (2000) reported that high content of Na^+ involve a Na^+/ATPase of plasmalemma. This causes a disturbance of the membrane permeability involving an efflux K^+ starting from the cytosol. Thus, El Yacoubi et al. (2004) showed that salt-tolerance of citrange calli is associated to K^+ accumulation. The efficiency with which K^+ is absorbed and used for the metabolic needs would be thus a component of salt-tolerance as mentioned by Piri *et al.* (1994). Behavior of K^+ permitted to classify the eight genotypes of wheat in three different groups with salt-tolerant calli: Sebou = Anouar = Amjad (group 1) ≥ Tomouh = Tarek = Massa = Marzak (group 2) > Ourgh (group 3); and two groups with salt sensitive calli: Sebou = Anouar = Tarek = Tomouth = Amjad (group 1) > Marzak = Ourgh = Massa (group 2) according to their response to salinity. For better apprehending these concepts salt-tolerance and salt-sensitivity, some researchers prefer to use the ionic selectivity which is often expressed by K^+/Na^+ ratio as a criterion of selected cell line and classification of genotype with respect to salinity (Babourina *et al.*, 2000). Maathuis and Amtmann (1999) also suggested that the important element in the salt tolerance is the ability to maintain on a high level the K^+/Na^+ ratio in cytosol. The depressive action of salt entails a decrease of K^+/Na^+ ratio. Therefore, most tolerant genotypes are those which generally maintain the highest K^+/Na^+ ratio (Sairam *et al.*, 2002). The results obtained with the eight wheat genotypes showed that salt-sensitive calli exhibit a low K^+/Na^+ ratio compared to salt tolerant ones. These results are in agreement with those of Lutts *et al.* (1996) in rice, and Ashraf and Ahmad (2000) in cotton. They showed that the most salt-tolerant cells are those which present the highest K^+/Na^+ ratio. So in our case, the high K^+/Na^+ ratio found in Sebou, Anouar and Tarek genotypes seem to indicate that these three wheat genotypes are most tolerant with salinity. To the contrary, Marzak, Ourgh, Massa and Amjad which have the lowest K^+/Na^+ ratio could be the most sensitive wheat genotype to salt stress. K^+/Na^+ ratio was used by Wahid (2004) and Gandonou *et al.* (2005) in sugarcane, and Ndayiragije and Lutts (2006) in rice as a criterion of genotypes classification. On the other hand, Wilson *et al.* (2000) and Qian *et al.* (2001) were mentioned that K^+/Na^+ ratio was identical with some sugarcane genotypes. In this case, K^+/Na^+ ratio cannot be to use as a criterion of salt-tolerance selection.

Conclusion

The response of eight wheat genotypes examined in this study was different to salt stress. *In vitro* selection techniques can be used to generate salt-tolerant cell lines in wheat and to study physiological and biochemical indicators of salinity tolerance in this plant. The effect of salt stress decreased the water content and K^+ but it increased Na^+ and Cl^- content. The selected tolerant calli accumulate less Na^+ and Cl^- compared to selected sensitive calli. The decrease in water content, relative fresh weight growth and K^+ content is lower in salt tolerant calli than salt sensitive ones. Thus salt tolerance seemed to be related to the efficiency of a tissue to modulate the level of inorganic solutes in response to salt stress. These results indicate that Na^+ and Cl^- exclusion combined to less K^+ accumulation are the main option to counteract the negative effects of salt stress in wheat tolerant calli. The mechanism is found to be associated with the better compartmentation of high amount of Na^+ and Cl^-, since these ions exclusion mechanism which is marker of salt tolerance of whole plant was expressed in the callus tissue. *In vitro* selection techniques can be used to generate salt-tolerant callus lines in wheat.

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