RESPONSES OF LEAF ELONGATION, TURGOR, OSMOTIC ADJUSTMENT AND ABA IN 3 DURUM WHEAT (*TRITICUM DURUM* L.) VARIETIES UNDER WATER STRESS IMPOSED BY PEG

M. MAHDID[1,2], A. KAMELI[2], C. EHLERT[3] and T. SIMONNEAU[3]
1 - Faculté des sciences Biologiques, Université des Sciences et de la Technologie Houari Boumediene, BP 32 El Alia, Bab Ezzouar, Alger, ALGÉRIE.
2 - Département de Biologie, Ecole Normale Supérieure, Vieux Kouba, Alger, ALGÉRIE.
3 - Laboratoire d'Ecophysiologie des Plantes sous Stress Environnementaux (LEPSE), SupAgro-INRA, 2 place viala Montpellier, FRANCE.

SUMMARY

In order to study the changes in water status, osmotic adjustment and turgor during a water stress period in wheat plants ; 3 varieties of durum wheat *Triticum durum* were subjected to water stress of 5 bars using PEG_{6000} in hydroponics solution for one week. The growth of leaf 3 was monitored in relation to water status (RWC, osmotic potential and turgor pressure) in addition to the level of acid abscisic (ABA). Results showed that stressed plants maintained turgor due to osmotic adjustment despite the decrease in growth rates. Results suggest that turgor is not the only factor regulating growth during the stress period and other factors such as cell wall extensibility are involved. Measurements of ABA concentration showed an increase in the elongation zone in Inrat 69 and MBB with maintenance of RWC in both varieties, suggesting a role of ABA in the increase of hydraulic conductivity in the cells of Elongation zone indicating a positive role of ABA in leaf elongation during water stress.

Key Words **:** Water stress, PEG, Turgor, Osmotic Adjustment, ABA, Durum wheat.

ملخص

لغرض دراسة تغيرات الحالة المائية للنبات، التعديل الأسموزي و ضغط الانتفاخ أثناء مرحلة الإجهاد المائي لنباتات القمح الصلب المزروعة في وسط مائي، أخضعت 3 أصناف من القمح الصلب *Triticum durum* إلى إجهاد مائي بواسطة مادة PEG_{6000} بجهد قدره 5 بار لفترة أسبوع، وتم تتبع نمو الورقة 3 مع الحالات المائية من محتوى نسبي مائي، جهد أسموزي و ضغط الإنتفاخ وكذلك مقدار هرمون حمض الأبسيسيك ABA. بينت النتائج المتحصل عليها محافظة النباتات المجهدة على ضغط الانتفاخ بفضل عملية التعديل الأسموزي، بالرغم من انخفاض معدلات النمو. يظهر من ذلك أن ضغط الإنتفاخ لم يحدد أو لم ينظم وحده نمو النبات أثناء فترة الإجهاد، وقد يعود تأثر النمو إلى عوامل أخرى كتصلب الجدران الخلوية. كما بينت قياسات هرمون الأبسيسيك في منطقة الاستطالة زيادة في كمياته في صنفي إنرات 69 ومحمد بن بشير وبمقارنة ذلك مع المحافظة على المحتوى النسبي المائي في الصنفين، يوحي ذلك بوجود دور للـــ ABA في زيادة الناقلية المائية لخلايا منطقة الإستطالة مما يتبين الدور الموجب للهرمون في النمو الورقي أثناء الإجهاد المائي.

الكلمات المفتاحية : الإجهاد المائي، PEG، ضغط الإنتفاخ، التعديل الأسموزي، حمض الأبسيسيك، القمح الصلب.

INTRODUCTION

Durum wheat is subjected to the effects of variable stress in arid and semi arid regions. In these regions, water stress is the limiting factor in plant growth and production. Plants resort to many adaptive strategies in response to this abiotic stress (Turner, 1979).

The control of leaf elongation in plants growing under water and salt stress remains a subject of extensive debate. Some authors have emphasized the role of cell turgor or leaf water status in determining leaf elongation rates (Frensch 1997; Hsiao *et al*., 1998; Tang and Boyer 2002), while others have emphasized the cell wall extensibility (Nonami and Boyer 1990; Cramer and Bowman 1991 ; Neumann 1993; Neumann *et al*., 1994), or signals from roots such a ABA (Passioura 1988; Saab and Sharp 1989; Gowing *et al*. 1990; Davies and Zhang 1991).

Turner (1979) described some mechanisms of water stress tolerance in plants such as drought escape, avoidance and tolerance to low water potential. However, in fact, all these plant strategies depend on certain specific plant adaptations to water deficit conditions (Turner, 1979 ; Chaves *et al*., 2003). According to Serraj & Sinclair (2002), osmotic adjustment is one of the major physiological phenomena vital for sustaining growth of plants under osmotic stress, which allows the maintenance of turgor and thus the maintenance of growth.

When plants are kept fully turgid throughout such sudden environmental changes, by placing their roots in a pressure chamber and raising the pressure so that the leaf xylem sap is maintained at atmospheric pressure, both the transient and persistent changes in leaf elongation rate disappear, All these responses show that water relations are responsible for the sudden changes in leaf elongation rate resulting from sudden changes in water stress and putative root signals play not part. However, at a time scale of days, pressurization fails to maintain high rates of leaf elongation of plants in either saline or drying soil, indicating that root signals such an abscisic acid (ABA) are overriding water relations effects. ABA has been proposed to influence growth in response to drought or salinity through changes in cell wall extensibility (Cramer and Bowman 1991, Neumann 1993, Neumann *et al*., 1994), and has been shown to influence both tissue hydraulic and stomatal conductivity (Collins and Kerrigan, 1974; Davies and Zhang, 1991; Freundl *et al*., 2000; Hose *et al*., 2000). Much attention has been paid to the growth-inhibiting role of ABA in leaves (Cramer *et al*., 1998; Dodd and Davies, 1996; He and Cramer, 1996; Thompson *et al*., 1997). ABA can also enhance turgor and growth by increasing solute transport (Roberts and Snowman 2000). Little attention has been paid to a potential role of ABA in facilitating growth resumption following the application of stress (Thompson *et al*., 1997). A recent report indicated a 6-fold increase in ABA in the distal portion of the leaf elongation zone following the addition of salt (Fricke *et al*., 2004).

The aim of the present study was to test the role of turgor in LER (Leaf Elongation Rate) recovery and the role of osmotic adjustment in turgor recovery under water

deficit. This work also attempts to clarify a possible role of ABA in resumption of LER after stress. An attempt was made to test whether differences in drought tolerance between the varieties correlated with differences in ABA accumulation and water status.

MATERIALS AND METHODS

Plant material and growth conditions :

The experiments were conducted using 3 durum wheat (*Triticum durum* L.) varieties: Inrat 69, Mohamed-Ben-Bachir (MBB), and Oued-Zenati (OZ), obtained from ITGC institute Algiers and selected on the basis of growth analysis and differential responses to water stress. The seeds were surface sterilized with 0.5% NaCl for 15 min, washed 3 times with distilled water then germinated on soaked filter paper in Petri dishes. After 6 days, seedlings of similar sizes were transferred to aerated nutrient solutions in 10 litres plastic containers under controlled environmental conditions. With a photoperiod of 14 h at 400 µmol PAR, 24/20c° day/night temperature, a RH (Relative Humidity) air : 65%, and VPD (Vapour Pressure Deficit) : between 0.8-1. The hydroponics' solution was continuously aerated using air pump. Osmotic stress was induced using PEG 6000 at 5 bars (195.4g/l).

The diluted nutrient solution contained : [$CaSO_4.2H_2O$: 0.5; KNO_3: 0.8; KH_2PO_4: 0.3; $MgSO_4.7H_2O$: 0.2; NH_4NO_3: 0.4; Fe-EDTA : 0.02; H_3BO_3: 0.008; $MnSO_4.H_2O$: 1.10^{-3}; $Na_2MoO_4.2H_2O$: $0,1.10^{-3}$; $ZnSO_4.7H_2O$: $0,2.10^{-3}$; $CuSO_4.5H_2O$: $0,2.10^{-3}$] mM. The pH maintained between 5.5 - 5.8. The solution was renewed every 4 days.

LER (Leaf Elongation Rate) was continuously measured on leaf 3 when it reached 6-8 cm length, using manual slid-rule by cm units (1 cm=10 mm). All measurements and Poly Ethylene Glycol (PEG) addition were made in the morning approximately 4 hours into the day, since growth patterns were found to change during the day.

Extraction and physiological measurements

The growing leaf three was disclosed, the location of the elongation zone (EZ) of the growing leaf and the exact distance of growth zone was found to be 3 cm long from leaf base (Hu *et al*., 2000) it was verified by measuring displacement rates along the leaf axis by the pricking method (Schnyder *et al*., 1987). Remaining leaf above the EZ is the mature zone (MZ).

Leaf tissue was quickly cut into small segments put into microtubes containing a small plastic sieve, sealed and quickly plunged into liquid nitrogen. Samples were thawed and then spun for 10 min. at 10.000 t/min. in the refrigerated centrifuge. Samples (about 20 µL) were collected and stored in freezer (-20°c) until their analysis.

The osmotic potential was measured on the expressed sap using vapor pressure osmometer VAPRO 5520, after calibration with standards solution of NaCl of known osmotic potential, reading are expressed in m mol/kg. To convert to pressure units it was assumed that 40 m.mol/kg water =1 bar.

Water potential was measured using a pressure chamber in different levels of leaf. To avoid the damage of EZ, the enclosed third leaf was left covered into the second leaf.

The Relative Water Content (RWC) was determined from as follows :

$$RWC\ (\%) = \frac{(FW - DW)}{(TW) - (DW)} 100$$

Where: FW= fresh weight; DW= dry weight; TW = turgid weight.

Osmotic pressure at full turgor $\pi 100$ was measured as described below :

$$\pi 100 = \frac{\pi\ (RWC - 10)}{90}$$

Turgor was measured using direct method by Cell-pressure probe technique was measured in epidermal cells of the elongating zone of the third leaf. The plant (was kept) in nutrient solution was placed vertically and attached by a plastic ribbon on plate plastic bar. To gain access to the growth zone of the third leaf, the coleoptiles, first and second leaves were separated. The newly exposed (uncovered) zone of third leaf was covered with thin film of Vaseline.

Turgor was measured by cell impalement using a cell pressure probe made of borosilicate microcapillaris (Harvard Apparatus Ltd., Edenbridge, UK) which were sharpened and bevelled to obtain a tip diameter of 6 µm and filled with silicon oil (type AS4, Wacker, Munich, Germany). The plant was placed in front of the pressure probe and a video microscope system (Leica, Buffalo, NY, USA) was used to adjust the microcapillary in front of the growth zone of leaf 3. Cell impalement resulted in the immediate appearance of a oil/cell sap meniscus in the capillary. Adjusting the pressure in the microcapillary, the meniscus was brought back to its initial position and the required pressure value was read on a digital pressure indicator (DPI 260, Druck, Leicester, UK). Readings were repeated several times for one cell by moving the meniscus forwards and backwards.

Biochemical measurements

Total carbohydrates were measured using the phenol sulfuric acid according to the method of Dubois *et al.,* (1956). Glucose, fructose and sucrose were estimated after enzymatic transformation to NADH, the latter is estimated at 340 nm on microplates. The transformation of glucose was obtained using Hexokinase (HK) to yield G-6-P which is then transformed using another enzyme glucose-6-phosphate dehydrogenase (G6PDH) to yield NADH. Fructose transformation requires a third enzyme Phospho Gluco Isomerase (PGI) which transform Fructose-6-phosphate (F6P) to Glucose-6-phosphate (G6P). Sucrose is first hydrolysis using β-fructosidase to yield Glucose and fructose, which are then estimated using the previously described enzymes.

The potassium concentration in leaf sap was measured by a flame photometer (SPECTRAA 220fs Varian) after a calibration with the standard solutions of KCl.

All biochemical measurements were made using the expressed sap extracted using liquid nitrogen.

ABA determination

Acid abscisic (ABA) was determined on leaf extracts using a radioimmunoassay. Leaf samples were collected from the growing zone of leaf three and the mature lamina of leaf two. Samples were cut into 5-10 mm segments, placed into Eppendorf tubes and stored at -80°C pending analysis. The segments were lyophilized and cut into thin pieces. Pure water was added (20 times the dry weight of the tissue). ABA was extracted by agitation at 4°C overnight. The tubes were then centrifuged to discard the tissue fragments and 50µL of supernatant was used for ABA assay. Concentration of (+) cis-trans-ABA was estimated using the method described by Quarrie *et al.,* (1988). The extracts were incubated for 45 min in the presence of a known amounts of 3[H]- ABA and specific monoclonal antibodies for (+)-ABA (Barrieu and Simonneau 2000). Ammonium sulphate was then added for precipitation of ABA-antibody complexes that were separated by centrifugation while the supernatant was discarded. The precipitate was re-dissolved in water; the radioactivity was fixed and measured using liquid scintillation (PACKARD 1600 TR). Estimation of ABA concentration was made using standard solutions containing known amounts of ABA and treated in the same way as the samples.

The essays were conducted to determine the reliability of measurement of ABA from sap comparing with the classical method using extracts by agitation (Quarrie *et al.,* 1988). The measurements of ABA from sap were very close to those of the classic method (Fig. 1).

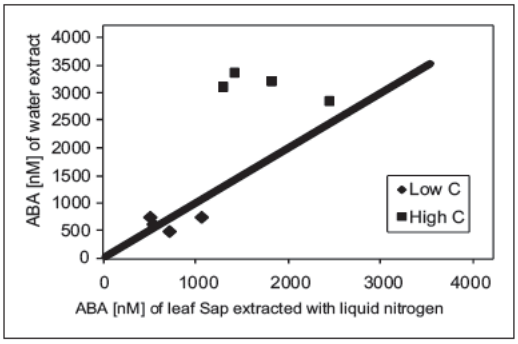

Figure 1 : The reliability and the conformity of the measurement method of ABA from the sap extracted by liquid nitrogen and that which extracted by water agitation. A comparison between two methods in low and high concentrations.

Statistical analysis

T tests was performed using R software (allowing for differences in variance) in order to test for differences between any two groups (Benjamini and Hochberg 1995).

RESULTS

The growth of leaf three exposed to PEG showed a decrease at the first day of stress particularly in OZ. The comparison of LER before and after stress indicated better recovery in MBB and Inrat 69 at 2 and 3 days following the addition of PEG. The % recovery of LER in OZ exhibited a late recovery at the end of stress (Fig.2).

Osmotic potential at full turgor (π100) in EZ showed a decrease in the 3 varieties (Inrat 69, MBB and OZ) during the stress period; however the time and degree of decrease was almost similar in these 3 varieties during the period of stress except of MBB at 4 days of stress (Fig. 3).

RWC of EZ decreased in all varieties during the first day of stress and increased during day 2 in MBB and day 6 in Inrat 69 reaching the water content close to prestress values. In OZ however, RWC remained low after stress (Fig.4)

Direct method measurements of turgor using the pressure probe on single cells in EZ indicated maintenance of turgor even so high values in MBB at first day with Inrat 69 at end of period of stress except in OZ at 3 days of stress, (Fig. 5).

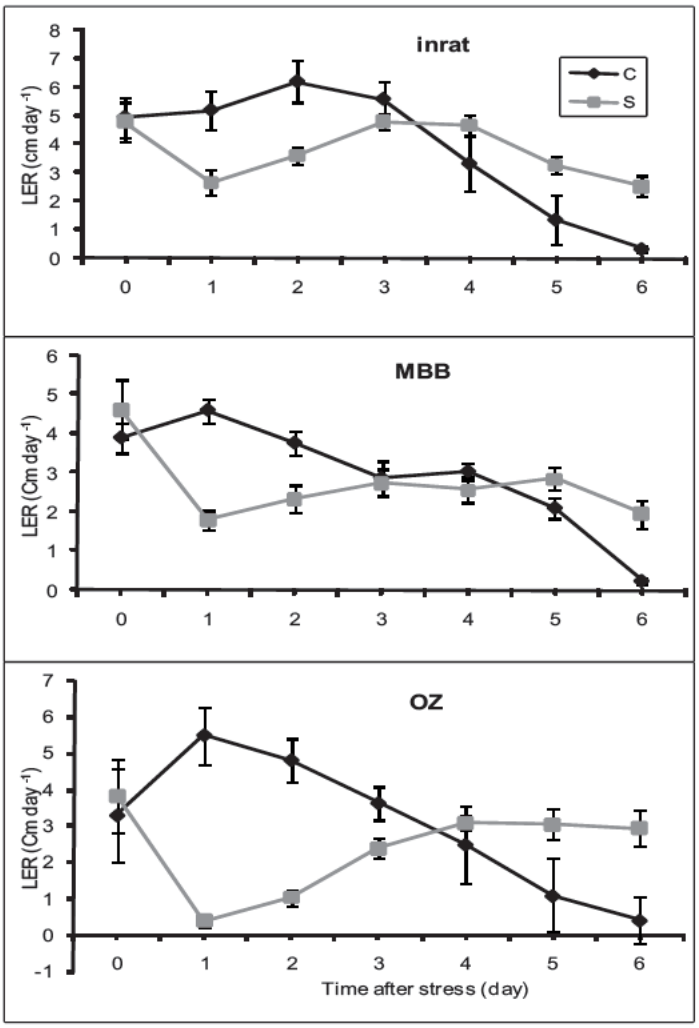

Figure 2 : The effect of PEG on Leaf Elongation Rate (LER) of third growing leaf of 3 varieties durum wheat during 6 days of stress by addition of PEG. Values are the means of 10 replicates ± SE.

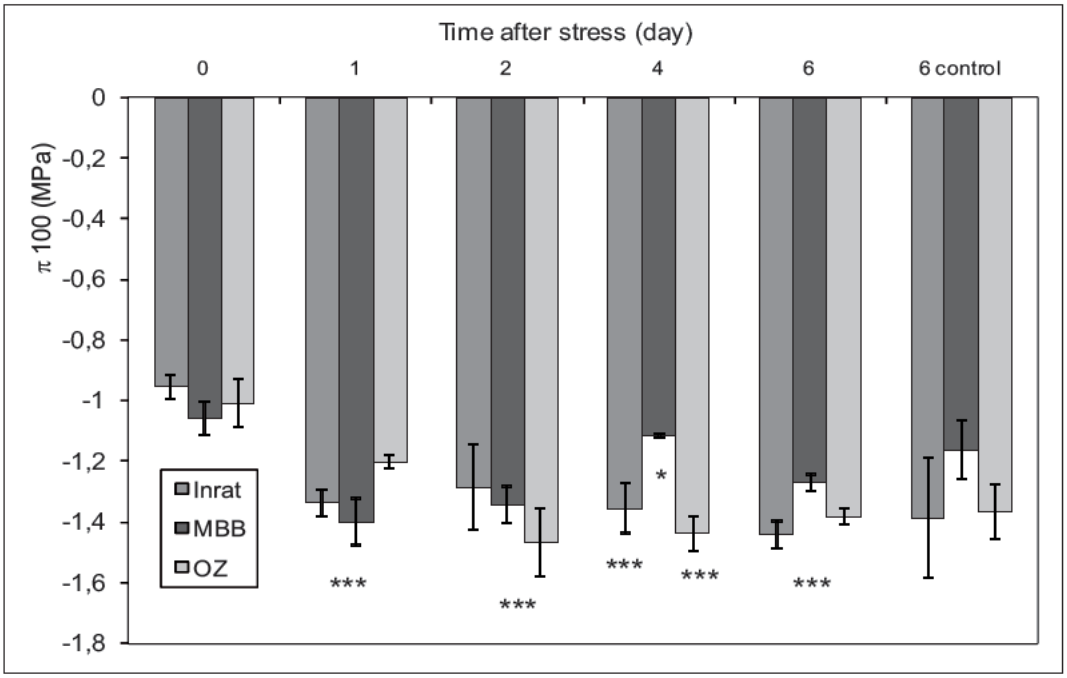

Figure 3 : Changes in osmotic potential at full turgor of bulk-tissue in EZ of growing leaf three of 3 varieties durum wheat before and following the addition of PEG to the root medium. Values are the means of 4 replicates ± SE (ns, non-significant; *, P≤0.05; **, P≤0.01; ***, P≤0.001).

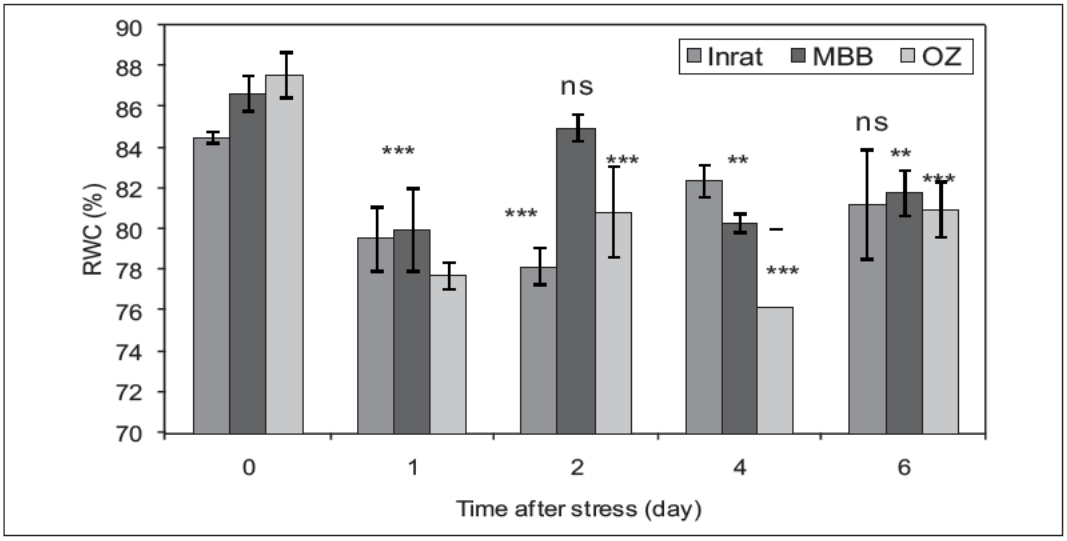

Figure 4 : Relative water content (RWC) of growing leaf three of 3 varieties durum wheat before and following the addition of PEG to the root medium. Values are the means of 4 replicates ± SE. (ns, non-significant ; *, P≤0.05; **, P≤0.01; ***, P≤0.001).

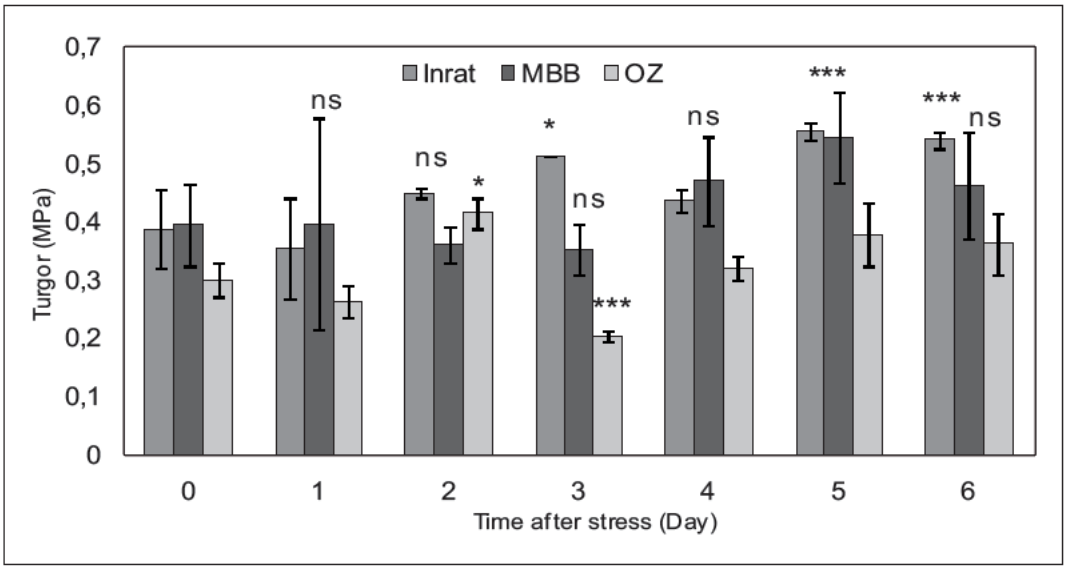

Figure 5 : Changes in turgor pressure measured by pressure probe in elongation zone of leaf three of wheat before and following the addition of PEG to the root medium. Values are the means of 6 replicates ± SE (ns, non-significant ; *,P≤0.05; **, P≤0.01; ***, P≤0.001).

An accumulation of total soluble sugars was recorded in EZ in all varieties during the stress period particularly in OZ (at the end of stress) (Fig. 6).

The estimation of individual sugar concentrations revealed an increase in glucose, fructose and sucrose in the EZ of all varieties particularly in Inrat 69 and OZ after the imposition of stress (Table 1).

Inrat 69 and OZ showed a transient increase in K+ at 1 day of stress in the EZ (MBB was not measured). Whereas in mature zone, Inrat 69 and MBB showed a considerable accumulation at day 2 and day 6, with moderate accumulation of K+ in MBB after the second day of stress (Fig. 7). ABA content rapidly rose in the expanded leaf two in response to PEG imposition, the increase was higher in Inrat 69 and MBB at first day in both elongation and mature zone after stress (Fig. 8).

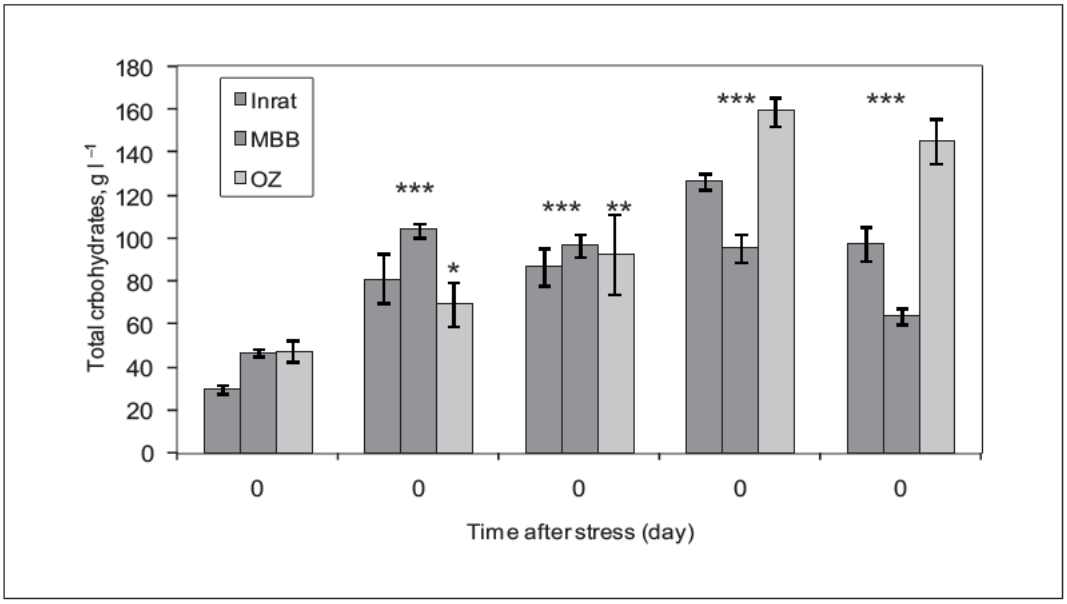

Figure 6 : Changes in total carbohydrates measured from the sap of bulk-tissue in elongation zone of growing leaf three of wheat in 3 varieties before and following the addition of PEG to the root medium. Values are the means of 4 replicates ± SE (ns, non-significant ; *, P≤0.05; **, P≤0.01; ***, P≤0.001).

Tableau 1 : Concentrations of glucose, fructose and sucrose (m mol kg-1) in elongation zone measured from the sap of leaf tissue between 0 day (before stress) and 7 day (aftar stress), followed by the difference in concentrations between the two times. Values are the means of 4 replicates ± SE (ns, non-significant ; *, P≤0.05; **, P≤0.01; ***, P≤0.001).

Varieties	sugars	0	1	2	4	6	Δ (0- 6)day	
Inrat	Glu	50,9±2,99	210,58±11,82	168,14±15,25	150,76±8,08	170±22,94	119,1	***
	Fruc	22,56±2,36	69,2±7,32	55,2±5,01	47,82±6,00	50,64±4,93	28,08	***
	Suc	4,33±0,85	22,2±6,7	38,24±3,7	57,16±1,85	62,67±7,93	58,34	***
MBB	Glu	76,01±10,46	108,23±11,57	106,11±15,25	131,24±9,60	92,39±7,12	16,38	*
	Fruc	26,36±3,20	36,96±2,99	41,13±5,01	51,94±6,08	42±5,82	15,64	*
	Suc	5,62±0,80	33,16±1,56	24,97±3,70	16,6±4,80	6,7±1,45	1,08	ns
OZ	Glu	52,28±5,92	116,55±10,47	134,8±10,47	214,73±3,19	189,37±5,48	137,09	***
	Fruc	19,16±0,25	41,71±2,18	44,8±2,18	57,68±2,41	77,15±8,29	57,99	***
	Suc	3,34±1,19	6,12±1,61	17,92±1,61	61,1±,04	33,71±0,81	30,37	***

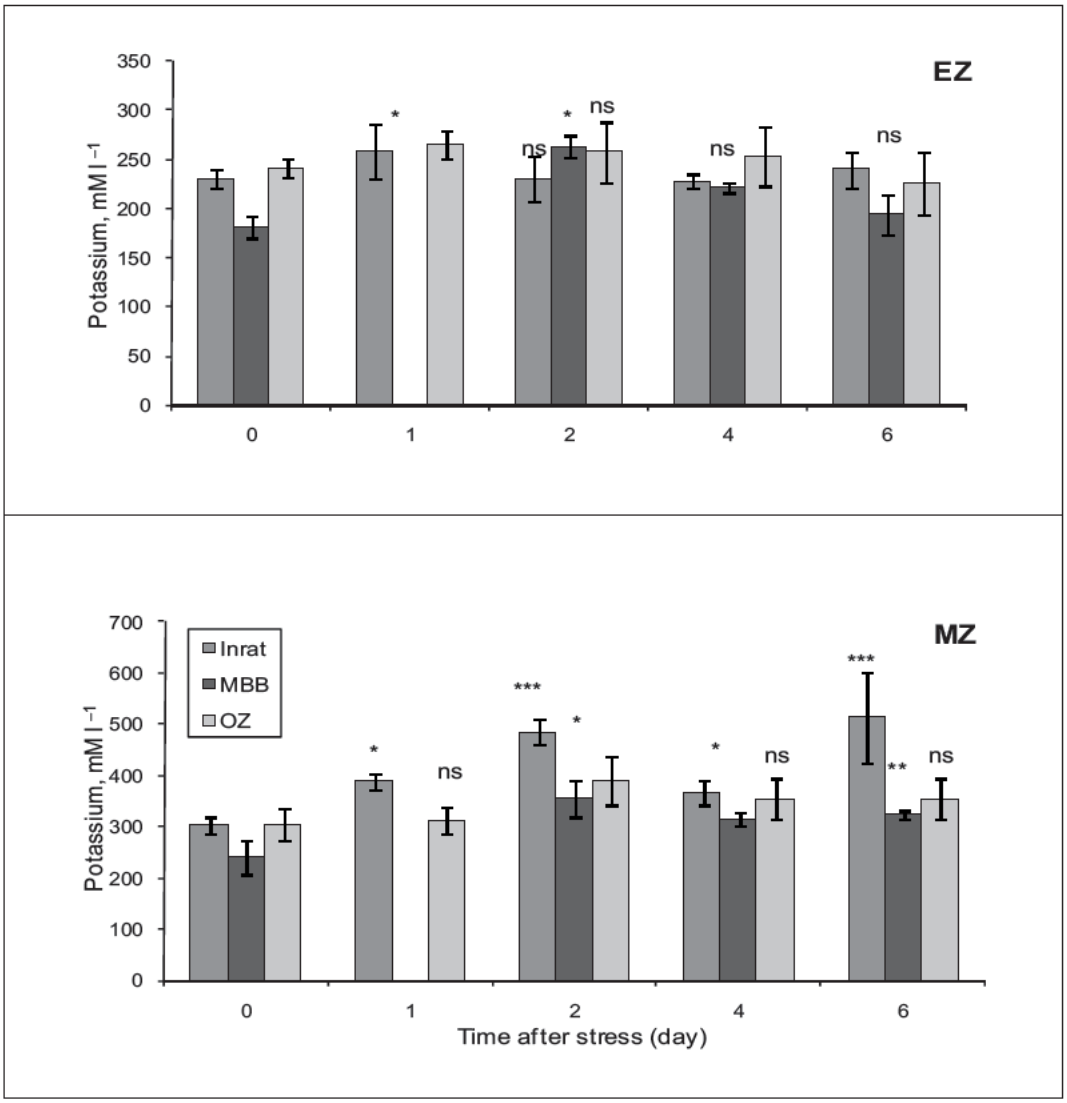

Figure 7 : Changes of potassium measured from the sap of bulk-tissue in elongation (EZ) and mature zone (MZ) of growing leaf three of wheat in 3 varieties, before and following the addition of PEG to the root medium. (MBB at 1 day was not measured). Values are the means of 4 replicates ± SE (ns, non-significant ; *, P≤0.05; **, P≤0.01; ***, P≤0.001).

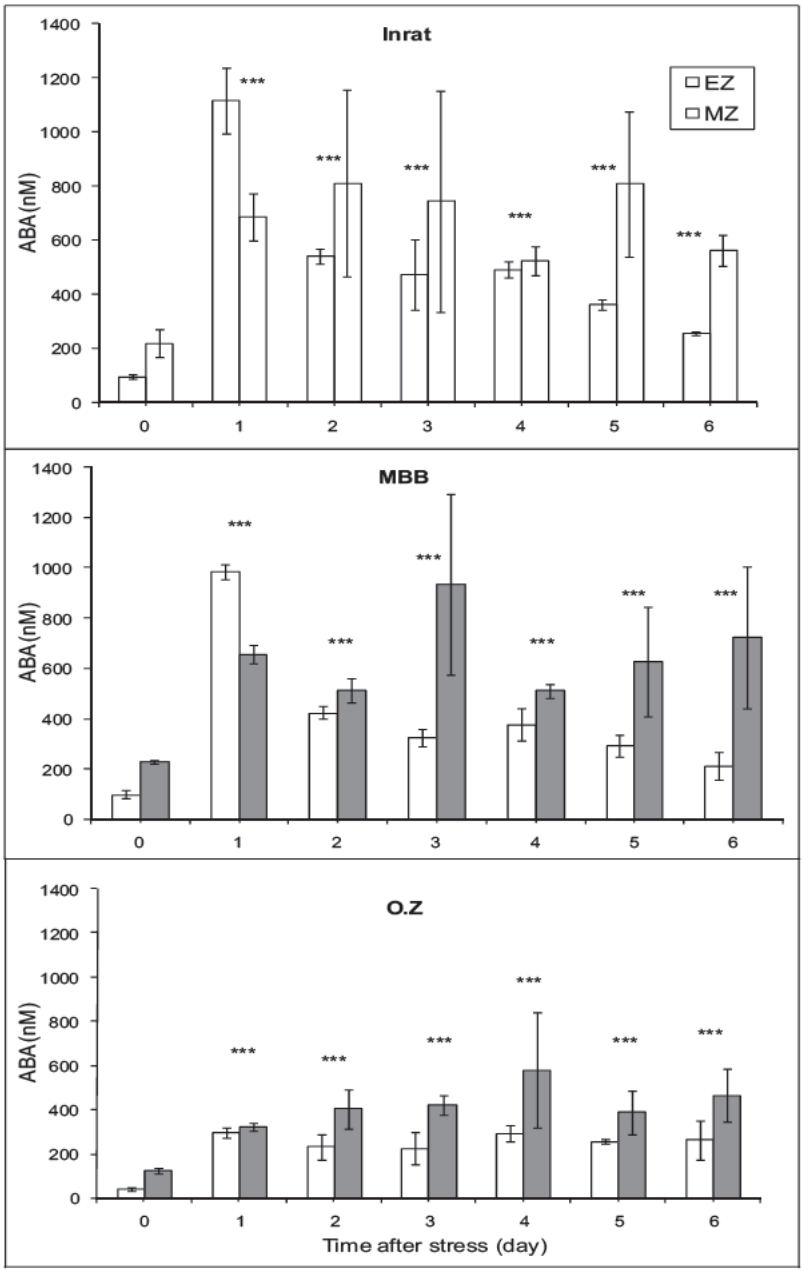

Figure 8 : ABA concentration in elongation and mature zone leaf three (EZ; MZ) of wheat before and following the addition of PEG to the root medium. Results represent means ± SE of 4-6 samples. (ns, non-significant ; *, P≤0.05; **, P≤0.01; ***, P≤0.001).

DISCUSSION

Osmotic Adjustment and turgor

The significant differences found in the % recovery of LER between the varieties may indicate differences in water stress tolerance between the varieties within a period of few days. These differences in tolerance are probably due to the genetic variability between the studied varieties. The question of whether long term responses are different from few hour responses is an important point to be raised in this discussion. On the long time scale other and more complex responses such as morphological, anatomical and physiological responses may be involved.

The cells adjusted osmotically around the elongation zone, which indicates the need of elongating cells to generate and maintain turgor and RWC.

The continued decrease of osmotic potential after 1-2 day resulted in higher magnitude of osmotic adjustment (OA) which could be the cause of growth resumption.

Osmotic components and properties associated with genotypic differences in OA, was reported in many studies for some time such as those of Morgan (1991), Morgan and Tan (1996) on wheat. Recently, complex genetic control for OA has also been supported by QTL analysis in a population of sunflower plants in field conditions, by the identification of genomic regions associated with water status traits and OA under water-stressed conditions (Poormohammad Kiani *et al.,* 2007). We suggest that a better understanding of this control at physiological level may help in breeding plants for arid and semi-arid conditions.

The same magnitude of turgor in growth recovery was noticed in all varieties. The recovery of turgor is probably due to OA of tissues described previously.

In another study (Bouchabké 2003), LER was found to differ in the growing tissue of 5 lines of maize and turgor was not maintained under long term water deficit. These different turgor responses in these lines were mainly due to genetic variability. It is well known that cell turgor, hence favourable water status of plants is important to expansion growth. However, the role of turgor in the recovery phase is also an important question to be raised. Turgor was shown to recover in the growing leaf tissue at a long time after applying stress due to OA. It is not clear from the results why growth failed to return to pre-stress values despite full turgor recovery. One explanation is the involvement of other factors such as cell wall extensibility.

Pressurization technique was used to cause step increases in (presumably) the turgor of growing cells, in order to prevent the cessation and the decrease of LER several hours after the addition of PEG or NaCl to the root medium, indicating that water relations are responsible for the sudden changes in LER, but failed to prevent an inhibition of leaf growth under salt stress over a time scales of 24 hours or scale time of days (Passioura and Munns 2000), there is much evidence to suggest that hormonal signals and/or wall extensibility rather than water relations are controlling.

In many studies, the extensibility of the walls strongly decreased in conditions of water or saline stress. This cell-wall harde-

ning (rigidity) leads to a significant decline in growth rate (Nonami & Boyer 1990; Neumann *et al.,* 1994; Lu& Neumann 1998).

Van Volkenburgh and Boyer (1985) have shown that shoot turgor does not change during growth inhibition under water deficits. Matsuda and Riazi (1981) observed that bulk-leaf turgor was largely unaffected in the basal leaf zone of osmotically stressed barley seedlings. Michelena and Boyer (1982) reached the same conclusion in maize. Termaat *et al.,* (1985) assumed that turgor and therefore, the processes generating turgor, are not limiting shoot growth. Turgor, although it presumably is necessary for growth, is not regulating shoot growth but is overridden by some other factor. Fricke and Peters (2002), concluded that leaf and cell elongation rates in barley exposed to NaCl are not limited by the magnitude of cell turgor.

The results of this study indicated the role of turgor recovery in leaf elongation recovery; however, full turgor recovery alone does not necessarily result in full growth recovery which suggested that other factors such as cell wall extensibility changes may play a major role in expansion growth recovery under long term stress.

Solute were found to accumulate in first day after applying stress which indicates the importance of these solute (especially sugars) in OA in the growing leaf tissue. Most of the studies on solute accumulation under stress conditions in wheat Carbohydrates: (Munns and Weir 1981; Kameli and Lösel 1993; Kerepesi and Galiba 2000), Potassium: (Munns *et al.,* 1979), were made under long term stress conditions.

The extent of total carbohydrates accumulation showed similar pattern in EZ of all varieties i.e MBB showed the highest accumulation followed by Waha then Inrat 69 and less in OZ which showed the least level of accumulation. This pattern was in agreement with the changes in OA.

Substantial accumulation of potassium (K+) was found in MZ compared to EZ which may be due to the presence of small vacuoles occupying a limited volume of the cells in the growing tissue. The increase in K+ concentration in MZ occurred in Inrat 69 and MBB during the stress period, for the osmotic balance with EZ.

K+ is an important cation in most biological systems. It is the most abundant cation in plant cells, constituting up to 10% of the total plant dry weight. It plays an important role in basic functions at the cellular and whole plant level, including OA, osmotic balance, and maintenance of membrane potential and electrical neutralization of anionic groups.

ABA

Recently many reports focused on the role of acid abscisic (ABA) in the LER recovery. ABA may facilitate growth resumption following the application of stress (Fricke *et al.,* 2004). For example, ABA has been proposed to stimulate leaf growth in water-stressed maize by preventing excess production of the growth-inhibitory hormone ethylene (Sharp and LeNoble 2002).

Another positive role of ABA on leaf growth has been evidenced as the effect of the hormone on stomatal closure favouring the maintenance of turgor in the growing

organs (Thompson *et al.,* 2007). Sansberro *et al.,* (2004) concluded that ABA might have promoted higher turgor thus inducing increased growth in terms of volume (as noticed by higher leaf expansion) as well as shoot elongation. ABA can also enhance turgor and growth by increasing solute transport (Roberts and Snowman 2000) and photosynthetic import (Jones *et al.,* 1987) towards growing cells or by stimulating the hydraulic conductivity on the pathway that provides the growing tissues with water (Parent *et al.,* 2009; Sade *et al.,* 2010). Most of these conclusions on the positive effects of ABA on water status and LER are in agreement with this study.

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