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SEA WATER STRESS AFFECTS MITOCHONDRIAL PROLINE OXIDATION BUT NOT ALTERNATIVE OXIDASE ACTIVITY IN DURUM WHEAT GERMINATING SEEDLINGS

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SUMMARY - Durum wheat (*Triticum durum* Desf.) is a species well adapted to the Mediterranean environments, where it often faces salt stress due to increasing soil salinity. Recently, the central role of mitochondria in the adaptation to environmental stresses at sub cellular level has emerged. The mitochondrial defence mechanisms may contribute to depict a durum wheat plant ideotype showing higher water use efficiency under water and/or salt stress and able to be irrigated with brackish water. In particular, two important mitochondrial mechanisms involved in resistance to environmental stresses are: (i) the inhibition of proline oxidation (that parallels accumulation of proline as an osmoprotectant in the cell); and (ii) the control exerted by Alternative Oxidise (AOX) of harmful reactive oxygen species generated under stress. Durum wheat mitochondria (DWM) from early seedlings were used to study these physiological mechanisms under salt stress. Seedlings were germinated both in distilled water and in two different diluted sea water solutions, leading to either moderate or severe damage to growth. To assess the contribution of the osmotic component of stress (water stress), a parallel investigation was performed by using hyperosmotic mannitol solutions. Comparison of proline and succinate oxidation by DWM showed that early inhibition of proline oxidation should be considered a mitochondrial adaptation to stress rather than a damage to oxidative properties, while, under our experimental conditions, no increase in AOX activity under stress was observed. In conclusion, early inhibition of proline oxidation may be a useful character enhancing tolerance to salt and water stress in durum wheat.

Key words: Saline stress, water stress, durum wheat, mitochondria, proline, alternative oxidise.

RESUME *- Le blé dur (Triticum durum* Desf.*) est une espèce bien adaptée au milieu méditerranéen où il est souvent exposé au stress salin à cause d'une teneur en sel croissante dans le sol. Ces dernières années, le rôle central des mitochondries dans l'adaptation au stress environnemental au niveau subcellulaire s'est manifesté d'une manière de plus en plus évidente. Les mécanismes de défense des mitochondries pourraient contribuer à définir un idéotype de blé dur ayant une plus haute efficience d'utilisation de l'eau en conditions de stress hydrique et/ou saline et pouvant être irrigué à l'eau saumâtre. En particulier, deux principaux mécanismes contribuent à la résistance au stress environnemental: (i) l'inhibition d'oxydation de la proline (qui accompagne l'accumulation de la proline en tant qu'osmoprotecteur chez la cellule); et (ii) le contrôle, exercé par l'Oxydase Alternative (AOX), des espèces réactives délétères dérivées de l'oxygène qui sont engendrées en conditions de stress. Nous avons utilisé les mitochondries du blé dur (DWM) des jeunes plantes pour étudier ces mécanismes physiologiques en conditions de stress salin. La germination des jeunes plantes a eu lieu tant dans l'eau distillée qu'en deux différentes solutions diluées d'eau de mer, ce qui a provoqué un dégât de modéré à grave sur la croissance. Pour évaluer la contribution de la composante osmotique du stress, nous avons mené une expérience parallèle en utilisant les solutions de mannitol hyperosmotique. La comparaison de l'oxydation du succinate et de la proline par les DWM a montré que l'inhibition de l'oxydation de la proline devrait être considérée une adaptation mitochondriale au stress plutôt qu'un dégât aux propriétés oxydatives, tandis que dans nos conditions expérimentales nous n'avons observé aucune augmentation de l'activité d'AOX. En conclusion, l'inhibition d'oxydation de la proline pourrait être un caractère utile qui améliore la tolérance au stress hydrique et salin chez le blé dur.*

Mots clés*: Stress salin, stress hydrique, blé dur, mitochondrie, proline, oxydase alternative.*

INTRODUCTION

The increase in salt content due to intrusion of sea water into aquifers leads to an increase of salinity of the agricultural soils, which reduces plant growth and crop productivity in many arid and semi-arid regions of the word (McKersie and Leshem, 1994). Uptake and compartmentation of ions, as well as the production of compatible solutes, i.e. osmotic adjustment, occurring under salt stress are linked to ATP consumption. In the light of this, the effect of salt stress on ATP production is expected to be crucial (for refs. see Flagella et al., 2006). The two sub cellular organelles devoted to massive ATP synthesis are chloroplasts and mitochondria, but the effect of salt and water stress has been deeply investigated only in chloroplasts; more recently, the central role of mitochondria in stress resistance has emerged (Pastore et al., 2007).

In order to carry out a specific investigation on the effect on mitochondrial function of salt and water stress, we have chosen durum wheat early seedlings as a suitable experimental model system. In fact, mitochondrial ATP production plays an essential role in germinating seedlings and is very important for the establishment of the initial plant stand. Moreover, durum wheat is a species well adapted to the Mediterranean environments, where salt stress due to seawater intrusion into aquifers is an increasing problem (Rana and Katerji, 2000). This crop may experience salt stress when cultivated in rotation to a crop irrigated with salt water. In this case, the highest salinity level is faced during germination and seedling establishment rather than in the later developmental stages, where spring and winter rainfall prevents salt stress occurrence by leaching excess salt (Caliandro et al., 1991); therefore, salt stress occurs during the early phase of seedling growth, when mitochondrial ATP synthesis plays a key role.

Recently, we have shown that durum wheat mitochondria (DWM) from young seedlings are an early target of salt stress; in particular, the inhibition of proline oxidation was found to precede damages to mitochondrial intactness and functionality (Flagella et al., 2006). This inhibition is consistent with proline accumulation in the cell as an osmoprotectant. Anyway, whether inhibition of proline oxidation in DWM may be considered a very early damage or an adaptative response is still uncertain. A second research line we developed in the recent years deals with DWM ability to counteract oxidative damage caused by environmental stresses. DWM possess three active energy dissipating systems, namely the plant mitochondrial potassium channel, PmitoK$_{ATP}$ (Pastore et al., 1999a), the plant uncoupling protein, PUCP (Pastore et al., 2000) and the Alternative Oxidase, AOX (Pastore et al., 2001); all the three proteins are able to prevent high mitochondrial membrane potential ($\Delta\Psi$) generation and, as a consequence, they are able to dampen the production of harmful reactive oxygen species (ROS) by DWM. We have recently demonstrated that PmitoK$_{ATP}$ and PUCP may control excess ROS production when young etiolated seedlings suffer salt and water stress (Trono *et al.*, 2004), while, to date, no information about AOX activity under these conditions is available.

In the present paper we have reinvestigated the inhibition of proline oxidation by comparing proline and succinate oxidation by DWM obtained from seedlings subjected to salt stress (applied using diluted sea water solutions) and water stress (applied using hyperosmotic mannitol solutions). Interestingly, to gain novel information, we studied both washed mitochondria (i.e. a crude mitochondrial fraction obtained via differential centrifugation) and purified mitochondria (i.e. the mitochondrial fraction obtained after passage of washed organelles throughout a Percoll gradient). In fact, in our opinion, the use of both washed and purified mitochondria may be advisable to obtain a more integrated interpretation of the inhibition of proline oxidation under stress. Furthermore, we checked AOX activity in DWM from sea water-stressed and water-stressed young seedlings.

We suggest that the comprehension of the mechanisms acting at mitochondrial level to counteract salt and water stress may strongly contribute to depict an advanced durum wheat plant ideotype. The addition of novel proper metabolic and biochemical traits to classical useful morpho-physiological traits may make the new plant able to better grow and yield in semiarid areas also under moderate salt stress.

MATERIALS AND METHODS

All reagents were purchased from SIGMA Chemical Co. (St. Louis, MO). Synthetic seawater was obtained by dissolving a commercial sea salt (SPERA & Co, Margherita di Savoia, Italy) in distilled

water to obtain a solution containing 504 mM Na^+, 10 mM K^+, 54 mM Mg^{2+}, 31 mM SO_4^{2-}, 540 mM Cl^- as evaluated by DIONEX DX 600 analysis (Flagella *et al.*, 2006). Durum wheat (*Triticum durum* Desf. cv Ofanto) seeds used in this work were supplied by the Experimental Institute for Cereal Research of Foggia.

Stress was induced by using different sea water dilutions (salt stress) or hyperosmotic mannitol solutions (water stress): (i) solutions having osmotic potential equal to -0.62 MPa (22% sea water, electrical conductivity (E.C.) equal to 12 $dS \cdot m^{-1}$, or 0.25 M mannitol), which induce moderate stress; (ii) solutions having osmotic potential equal to -1.04 MPa (37% sea water, E.C. equal to 20 $dS \cdot m^{-1}$, or 0.42 M mannitol), which induce severe stress (Francois and Maas, 1994; Trono *et al.*, 2004; Flagella *et al.*, 2006). Iso-osmotic concentration of salt and mannitol solutions were checked by a vapour pressure osmometer Roebling, type 13.

Durum wheat seeds (300 g) were sown on a distilled water-saturated polyurethane foam sheet covered with a Whatman filter paper. Seeds were dark-grown for 48 h in a Heraeus HPS 1500 incubator at 25 °C and 85% relative humidity; then early seedlings (length of shoot about 0.3 cm) were used to obtain mitochondria. Stressed seedlings were germinated as described for control seedlings except that water was substituted with the sea water dilutions or mannitol solutions as above described. Stressed seedlings were harvested when they reached the same shoot length of the control (0.3 cm), after three and four days for the moderate and severe stress intensity, respectively.

Etiolated early seedlings (about 50-60 g) were removed from seeds, then mitochondria were isolated according to Pastore *et al.* (1999b) with minor modifications. The grinding buffer was 0.3 M mannitol, 4 mM cysteine, 1 mM ethylenediaminetetraacetic acid (EDTA), 30 mM 3-(N-morpholino) propanesulfonic acid (MOPS) (pH 7.50), 0.1% (w/v) defatted bovine serum albumin (BSA), 0.6% (w/v) polyvinylpyrrolidone (PVP)-360; the washing buffer was 0.3 M mannitol, 1 mM EDTA, 10 mM MOPS (pH 7.40), 0.1% (w/v) defatted BSA. The fraction of washed mitochondria was further purified by isopycnic centrifugation in a self-generating density gradient, consisting of 0.3 M sucrose, 10 mM Tris-HCl (pH 7.20) and 28% (v/v) Percoll (colloidal PVP-coated silica, Amersham Pharmacia Biotech), combined with a linear gradient of 0% (top) to 10% (bottom) PVP-40. Mitochondrial protein content was determined by the method of Lowry, using BSA as a standard.

Oxygen uptake rate was measured at 25 °C with a GILSON Oxygraph model 5/6-servo Channel pH 5, equipped with a Clark-type electrode (5331 YSI, Yellow Spring, OH) in 1.5 mL of a medium consisting of 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 0.1% (w/v) defatted BSA, and 10 mM sodium phosphate buffer (pH 7.20). As respiratory substrates, either 10 mM succinate or 20 mM proline were used. In the course of substrate oxidation, successive additions of limited amount of ADP were carried out in order to evaluate: (i) the respiratory control (RC) ratio, *i.e.* the ability of ADP to control the oxygen uptake rate expressed as a ratio between the rate measured in the presence of ADP (state 3) and the rate measured after ADP consumption (state 4); and (ii) the ADP/O ratio, *i.e.* the ratio between the nmol of phosphorylated ADP and the natom of oxygen consumed (see Flagella *et al.*, 2006).

AOX activity in succinate oxidising DWM was evaluated at 25 °C by using the above described oxygen uptake medium, as a ratio between cyanide insensitive (V_{+KCN}) and cyanide sensitive (V_{-KCN}) oxygen uptake rate, as reported in Fig. 2.

The intactness of mitochondrial membranes was evaluated oxygraphically (outer membrane), on the basis of cytochrome c oxidase latency, and photometrically (inner membrane), on the basis of malate dehydrogenase latency (see Trono *et al.*, 2004).

$\Delta\Psi$ was monitored at 25 °C by measuring safranin O fluorescence changes at λ_{ex}=520 nm and λ_{em}=570 nm, as reported in Flagella *et al.* (2006). The reaction mixture (2 mL) contained 0.3 M mannitol, 5 mM $MgCl_2$, 0.1% (w/v) defatted BSA, 10 mM MOPS (pH 7.20), 2.5 μM safranin O and 0.1 $mg \cdot mL^{-1}$ DWM protein ([safranin O]/[DWM protein] ratio value of 25).

RESULTS AND DISCUSSION

In order to gain some insight into plant bioenergetics under salt and water stress, experiments were carried out by using the moderate salt sensitive species durum wheat (*Triticum durum* Desf.). Seedlings were germinated in two different diluted sea water solutions, 22% and 37% sea water, leading to moderate and severe damage to seedling growth, respectively (Trono *et al.*, 2004; Flagella *et al.*, 2006). In order to assess the contribution of the osmotic component of the stress resulting in a water stress, a parallel investigation was performed on seedlings germinated in 0.25 and 0.42 M mannitol solutions, iso-osmotic with the two seawater solutions.

As for the DWM isolation protocol, a paper concerning hyperosmotic stress-adapted potato cells shows that the isolation protocol giving better mitochondria needs the use of media showing an osmolarity close to the one of the cells, as evaluated by plasmolysis experiments (Fratianni *et al.*, 2001). Unfortunately, the determination of tissue cell osmolarity from seedlings under our conditions is not a simple matter. Therefore, we isolated mitochondria from stressed seedlings by using the same media suitable for the control ones. In the light of this, due to the non-isotonic isolation media used, washed DWM from stressed seedlings may result in part artificially damaged by the isolation procedure; this could lead to an overestimation of the mitochondrial damages due to stress. On the other hand, the purification of these organelles leads to a selective recovery of a population of highly intact and fully functional mitochondria; therefore, purified mitochondria represent better organelles and may give underestimation of the stress-induced damages. To overcome these problems, here, we isolate both washed and purified DWM and compare the effects of the stress in both DWM populations.

Intactness and functionality of DWM from stressed seedlings

DWM were isolated from control and stressed seedlings. Since intact and coupled mitochondria are strictly required to carry out studies concerning mitochondrial metabolism, a series of experiments was performed by using both washed and purified DWM in order to ascertain structural and functional features, including the intactness of outer and inner membranes and $\Delta\Psi$ in the absence (state 1) and presence (state 4) of 5 mM succinate as oxidisable substrate.

The results relative to purified DMW were already published (Trono *et al.*, 2004), while data relative to washed DMW are reported in *Table 1*.

Table 1. Intactness of membranes and $\Delta\Psi$ in washed DWM from control and stressed seedlings

	Membrane intactness (%)								
	Control	Sea water stress				Water stress			
		Moderate	%[a]	Severe	%	Moderate	%	Severe	%
Outer membrane	83±0.7[b]	68±3.3**	82	28±3.2***	34	79±1.3*	95	67±1.5***	81
Inner membrane	94±1.0	84±1.5***	89	77±1.0***	82	86±1.0**	91	83±1.5***	88
	$\Delta\Psi$ (mV)								
	Control	Sea water stress				Water stress			
		Moderate	%	Severe	%	Moderate	%	Severe	%
State 4 $\Delta\Psi$	205±3.6	194±5.2[ns]	95	187±5.2*	91	200±5.0[ns]	97	192±6.4[ns]	94
State 1 $\Delta\Psi$	202±3.4	140±3.8***	69	100±3.0***	49	170±8.0*	84	136±5.0***	67

[a] % of the control
[b] mean value ± SE (n=4)
ns = not significant
* $P<0.05$, ** $P<0.01$, *** $P<0.001$, P represents the probability level according to the Student's t test relative to the comparison between each value with the corresponding control.

A slight reduction of the membrane intactness was observed, which was more evident in sea water stress than under water stress and under severe more than under moderate stress; on the other hand, a remarkable stripping of outer membrane under severe sea water stress was observed. No important variations were observed in the $\Delta\Psi$ values in mitochondria oxidising succinate in state 4 comparing stressed and control conditions, thus confirming substantial intactness of inner membrane. Interestingly, in this set of experiments, DWM show high state 1 $\Delta\Psi$ due to endogenous substrates (Flagella *et al.*, 2006); moreover, DWM from stressed seedlings showed lower $\Delta\Psi$ values in state 1 than control, thus suggesting progressive membrane permeability causing outflow of endogenous substrates in the course of isolation procedure as a result of increasing level of stress (Trono *et al.*, 2004; Flagella *et al.*, 2006). In the whole, in washed DWM, membrane intactness and $\Delta\Psi$ decreased with increasing strength stress and seawater caused more detrimental effects than parallel water stress. Comparison between the data from Trono *et al.* (2004) and the ones from *Table 1* shows similar trends in purified and washed DWM; moreover, as expected, washed mitochondria have a lower degree of intactness than the purified ones. In any case, washed DWM from both control and stressed seedlings were obtained with a degree of functionality sufficient to explore their oxidative properties.

Proline and succinate oxidation by DWM from stressed seedlings

Since accumulation of free proline is known to play an important role in the cell osmotic adjustment, the effect of seawater stress and parallel water stress on proline oxidation by DWM was evaluated. In particular, by using both proline and succinate as respiratory substrates, comparison was made with respect to: (i) state 3 oxygen uptake rate (*i.e.* oxidation rate); (ii) coupling between oxidation and ATP synthesis (RC ratio); and (iii) phosphorylative efficiency (ADP/O ratio). Data relative to washed DWM are summarised in *Table 2*. Data relative to purified DWM were recently published (see Trono *et al.*, 2004), but they are again reported between brackets in *Table 2* to facilitate comparison between washed and purified DWM.

Table 2. Proline- and succinate-dependent oxygen uptake rate in state 3, RC and ADP/O ratios in washed DWM under stress. The experimental conditions were as in "Materials and Methods". Values concerning purified DWM from Trono *et al.* (2004) are reported between brackets. For the explanation of data reported in bold

	Proline-dependent O_2 uptake								
	Control	Seawater stress				Water stress			
		Moderate	%[a]	Severe	%	Moderate	%	Severe	%
v[b]	47±2.7 *(180±10.6)*	34±2.0** *(91±2.4***)*	**72** ***(50)***	19±1.1*** *(31±1.8***)*	**40** ***(17)***	39±1.05[*] *(108±6.3**)*	**83** ***(60)***	26±1.9*** *(51±3.8***)*	**55** ***(28)***
RC	1.6±0.08 *(2.1±0.11)*	1.2±0.01** *(2.0±0.02[ns])*	75 *(95)*	1.0±0.03*** *(1.0±0.03***)*	nd[d] *(nd[d])*	1.3±0.02* *(2.1±0.03[ns])*	81 *(100)*	1.0±0.01*** *(1.0±0.01***)*	– *(–)*
ADP/O	2.2±0.03 *(2.5±0.04)*	1.8±0.07** *(1.9±0.08***)*	82 *(76)*	nd[d] *(nd[d])*	– *(–)*	1.9±0.03*** *(2.5±0.11[ns])*	86 *(100)*	nd *(nd)*	– *(–)*
	Succinate-dependent O_2 uptake								
	Control	Sea water stress				Water stress			
		Moderate	%	Severe	%	Moderate	%	Severe	%
v	195±11.5[c] *(510±30.0)*	155±9.2* *(450±15.0[ns])*	**79** ***(88)***	105±7.8*** *(316±16.0**)*	**54** ***(62)***	180±10.5[ns] *(540±31.6[ns])*	**92** ***(106)***	128±7.5** *(370±21.6**)*	**66** ***(72)***
RC	1.9±0.09 *(2.3±0.10)*	1.4±0.04** *(2.3±0.04[ns])*	74 *(100)*	1.0±0.01*** *(1.0±0.01***)*	nd[d] *(nd[d])*	1.6±0.05* *(2.3±0.04[ns])*	84 *(100)*	1.3±0.08** *(2.0±0.06*)*	68 *(87)*
ADP/O	1.8±0.03 *(1.8±0.03)*	1.6±0.04** *(1.7±0.08[ns])*	89 *(94)*	nd[d] *(nd[d])*	– *(–)*	1.8±0.07[ns] *(1.8±0.07[ns])*	100 *(100)*	1.5±0.04*** *(1.8±0.05[ns])*	83 *(100)*

[a] % of the control
[b] state 3 oxygen uptake rate expressed as natom $O_2 \cdot min^{-1} \cdot mg^{-1}$ of protein
[c] mean value ± SE (n=4)
[d] not determinable in mitochondria having RC ratio equal to 1, *i.e.* uncoupled (Trono *et al.*, 2004; Flagella *et al.*, 2006)
ns = not significant
*P<0.05, ** P<0.01, *** P<0.001, P represents the probability level according to the Student's t test relative to the comparison between each value with the corresponding control.

The results of *Table 2* show that both sea water stress and water stress lead to an inhibition of proline and succinate oxidation and a decrease of both coupling and phosphorylative efficiency. Consistently, we have recently reported a decrease of the rate of ATP synthesis under these conditions (Flagella *et al.*, 2006). These results are in accordance with several literature reports (Stewart *et al.*, 1977; Sells and Koeppe, 1981; Schmitt and Dizengremel, 1989). These effects were found to increase with increasing stress intensity; moreover, they were found to be more evident under sea water stress than under water stress, so confirming that under our experimental conditions the overall sea water damage is dependent on both a toxic and an osmotic component (Greenway and Munns, 1980). Interestingly, as previously found in water-stressed maize seedlings (Sells and Koeppe 1981) and other model systems (Boggess *et al.*, 1978; Schmitt and Dizengremel, 1989), we show that the decline in proline oxidation rate under stress conditions was even more dramatic than for succinate. This sharp reduction leads to the hypothesis that the imposed stress conditions affect in a specific manner mitochondrial mechanisms responsible for proline oxidation.

At this regard, by comparing washed and purified DWM with respect to the percentage values of Table 2 of both proline and succinate oxygen uptake rate under all stress conditions, an interesting finding is obtained: the inhibition of proline oxidation is more evident in purified than in washed DWM, while the inhibition of succinate oxidation is more marked in DWM washed fraction. In order to emphasise this finding, the percentage data reported in bold in the % columns of Table 2 are processed as shown in Fig. 1.

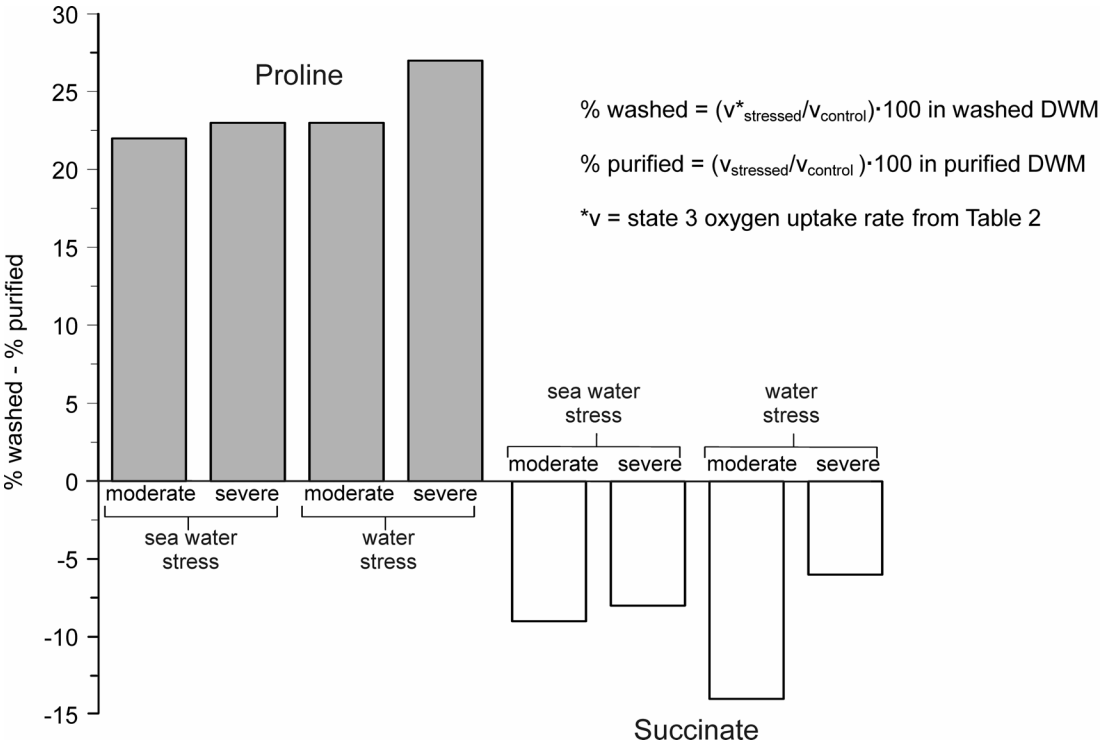

Fig. 1. Opposite behaviour of proline and succinate oxidation by washed and purified DWM from stressed seedlings. The % data are the ones reported in bold in Table 2.

The opposite effect of stresses on proline and succinate oxidation is well evident. This effect may be observed irrespective to the kind and intensity of the stress imposed.

So, a more evident inhibition of succinate oxidation was observed in the washed fraction, which contains the more damaged organelles, than in the purified one, while the inhibition of proline oxidation is much higher in purified DWM, *i.e.* the highly intact and fully functional organelles. In the light of this, it is reasonable to suppose that the alterations found in succinate oxidation represent damages due to the stress imposed. On the contrary, the decrease in the proline oxidation rate should be considered an active mitochondrial adaptation to stress rather than a generic early damage

of the oxidative properties. Consistently, we have recently showed that inhibition of proline oxidation parallels proline accumulation in durum wheat seedlings (Flagella *et al.*, 2006). Anyway, data reported in literature (Boggess *et al.*, 1976; Voetberg and Sharp, 1991) show that the increase in the proline synthesis from glutamate rather than inhibition of oxidation represents the main factor resulting in proline accumulation in stressed plant tissues; this point will merit further investigation in durum wheat.

AOX activity in DWM from stressed seedlings

The AOX branches from the respiratory chain at the level of ubiquinone and it is known to be cyanide and antimycin insensitive. Since AOX bypasses the last two sites of energy conservation associated with the cytochrome pathway, it shows a non phophorylating and, as a consequence, an energy dissipating character (Vanlerberghe and McIntosh, 1997). This appears to be useful to control both $\Delta\Psi$ and mitochondrial ROS generation, which occurs at high rate when $\Delta\Psi$ is high. As a consequence, AOX is expected to act as an antioxidant system under environmental stresses inducing oxidative stress at cellular level (Maxwell *et al.*, 1999; Pastore *et al.*, 2001 and refs. therein). Therefore, experiments were performed to evaluate its activity under our conditions of stress imposition, that were shown to enhance mitochondrial superoxide anion generation (Trono *et al.*, 2004). In this case, only the purified DWM were studied.

AOX activity in succinate oxidising DWM was evaluated by means of oxygen uptake measurements in the presence of dithiothreitol (DTT) and pyruvate, specific AOX activators (Fig. 2a) (Millar *et al.*, 1993; Vanlerberghe and McIntosh, 1997).

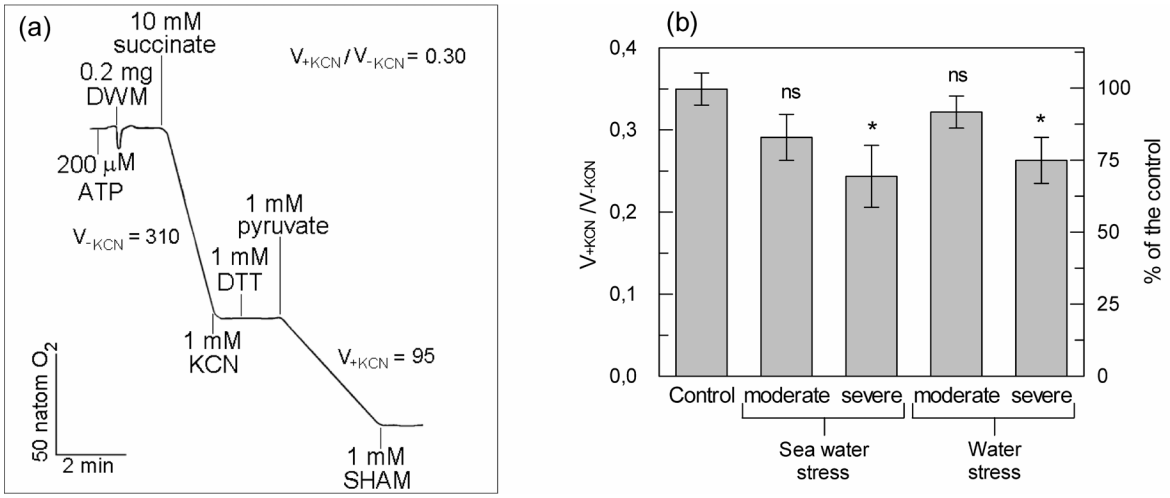

Fig. 2. AOX activity expressed as a fraction of cytochrome pathway in DWM purified from control and stressed seedlings.
(a) Measurements of oxygen uptake by DWM purified from moderately sea water-stressed seedlings were carried out in 1.5 mL of the medium reported in "Materials and Methods"; ATP (200 μM) was also present in order to activate succinate dehydrogenase. The compounds were added at the time indicated by the arrows. DTT and pyruvate are AOX activators. The numbers on the traces refer to the natom of oxygen taken up · min^{-1} · mg^{-1} of protein. The AOX activity was expressed as V_{+KCN}/V_{-KCN}, where V_{-KCN} and V_{+KCN} represent the rate before cyanide addition and the residual rate in the presence of cyanide plus AOX activators, respectively.
(b) AOX activity under control and stress conditions. Data are expressed both as mean value ± SE (n=4) and as % of the control. ns = not significant; * $P<0.05$, P represents the probability level according to the Student's t test relative to the comparison between each value and the control.

Oxygen uptake was started by adding 10 mM succinate to DWM suspended in the reaction medium; the measured oxygen uptake rate was indicated as V_{-KCN}. Then, 1 mM KCN was added which completely blocked oxygen uptake, since DWM do not spontaneously show alternative respiratory pathway (Pastore *et al.*, 2001). The addition of 1 mM DTT and 1 mM pyruvate stimulated a cyanide-insensitive oxygen uptake rate, indicated as V_{+KCN}, which was completely stopped by 1 mM salicylhydroxamic acid (SHAM), a powerful AOX inhibitor. The V_{+KCN}/V_{-KCN} ratio was used as an indicator of the AOX activity, expressed as a fraction of cytochrome pathway. This assay gives useful information about maximal activity of the AOX measured *in vitro*, which is a parameter related to the AOX amount in DWM, but is unable to test AOX contribution to overall respiration *in vivo*. To do this, further studies are required based on isotope fractionation technique (Ribas-Carbo *et al.*, 2005a).

Based on measurements shown in Fig. 2a, AOX dependent oxygen uptake in control conditions was about 35% with respect to the one dependent on cytochrome pathway. The comparison between control and stress conditions shows that the contribution of AOX functioning with respect to the cytochrome pathway was unchanged under moderate stresses and even little inhibited under severe stresses (Fig. 2b). Although this result is in apparent contrast with the widely accepted role of this enzyme as a protective system under stress conditions, it is not surprising. In fact, also Kasai *et al.* (1998) reported that, though the alternative pathway plays a critical role in germinating wheat seedlings, NaCl salinity do not affect it. Moreover, Jolivet *et al.* (1990) found that in barley the activity of the alternative pathway under NaCl stress was not modified in isolated mitochondria, while it was increased in whole leaf tissue. Finally, Lutts *et al.* (2004) found an increase in the relative importance of AOX in durum wheat callus submitted to NaCl stress, but this was lower in salt resistant cultivars than in salt sensitive ones; so, it seems that the stimulation of the alternative pathway may be not necessarily related to stress resistance. As far as durum wheat is concerned, our previous findings strongly suggest that AOX may act as an antioxidant defence system not in etiolated tissues, but in green tissues, that may contain hydroxypyruvate and glyoxylate, two powerful DWM-AOX activators (Pastore *et al.*, 2001). Consistently, Ribas-Carbo *et al.* (2005b) recently reported that in soybean leaves severe water stress caused a significant shift of electrons from the cytochrome to the alternative pathway due to a biochemical regulation other than protein synthesis. As for etiolated tissues, DWM display high activity of two different dissipating systems able to dampen mitochondrial ROS production, namely the PUCP (Pastore et *al.*, 2000) and the $PmitoK_{ATP}$ (Pastore *et al.*, 1999a). Moreover, we have recently shown that PUCP and $PmitoK_{ATP}$ are strongly activated in DWM under salt and water stress in order to act against oxidative stress (Trono *et al.*, 2004), thus replacing AOX function. Our idea of a tissue specific cross-regulation between PUCP, $PmitoK_{ATP}$ and AOX is consistent with the finding of Sluse *et al.* (1998) who reported opposite regulation by free fatty acids of PUCP and AOX, and with our observation that both $PmitoK_{ATP}$ and PUCP are directly and very rapidly activated by ROS (Pastore *et al.*, 1999a; Pastore *et al.*, 2000), while this does not occur in the case of AOX (Pastore *et al.*, 2001). For a very recent review about this topic see Pastore *et al.* (2007).

CONCLUSIONS

Durum wheat resistance to salt and water stress may be a function not only of proper morpho-physiological adaptations, but also of biochemical characteristics including mitochondria metabolism. Here, we suggest that early inhibition of proline oxidation under stress may be a useful character to improve salt and water stress tolerance; instead, the role of AOX against these stresses is questionable in durum wheat. New genotypes well fitted for Mediterranean environments showing higher water use efficiency may be hypothesised by improving mitochondria properties both via conventional and non conventional breeding.

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