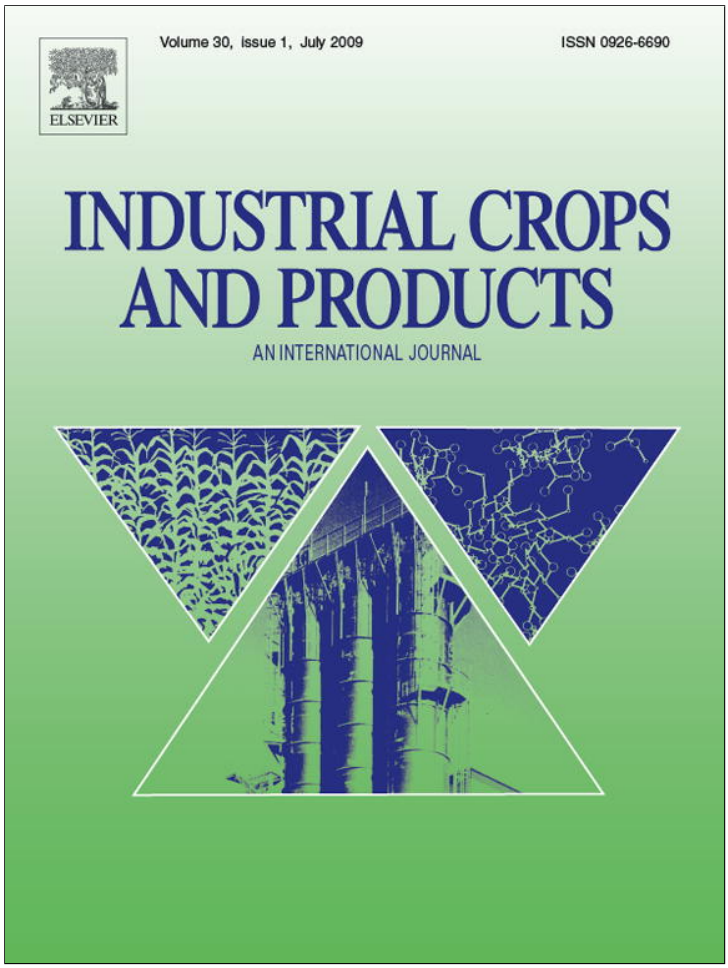

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Differential responses of two maize (*Zea mays* L.) varieties to salt stress: Changes on polyphenols composition of foliage and oxidative damages

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ABSTRACT

The leaf-structural adaptative strategies and the possible involvement of the antioxidative system in the tolerance to salt stress were investigated in relation to leaf tissue senescence of maize. Studies were carried out with seedlings of two forage maize varieties (*Aristo* and *Arper*) subjected to 0, 34, 68 and 102 mM NaCl for 6 weeks under glasshouse conditions. The leaf growth, leaf water content, H_2O_2 generation, lipid peroxidation, membranes stability index and polyphenolic compounds (total polyphenols, total flavonoids, anthocyanins and proanthocyanidins) accumulation were quantified in three leaf stages (young, mature and senescent leaves). Salt stress impacts in term of H_2O_2 generation and lipid peroxidation were more pronounced in senescent leaves. During the stress treatment, the accumulation of the major polyphenolic compounds was higher in young leaves. A significant variability in salt response was found between both varieties. The better behaviour of *Arper* salt-challenged leaves compared to those of *Aristo* may be related to their higher water content and polyphenol accumulation mainly anthocyanins which showed to participate efficiently in restriction of oxidative damages caused by the H_2O_2 generation.

1. Introduction

Salinity is an important problem to crop production in many parts of the world, especially in irrigated fields of arid and semi-arid regions (Schleiff, 2008). In several Tunisian regions, the agricultural production is affected by the rarity and the saltiness of irrigation water (Hajlaoui et al., 2007). In such areas, the high levels of NaCl affect the plant development by altering its functional state. The plant ability to acclimate to salt stress includes alterations at leaf level, associated with morphological, physiological and biochemical characteristics whereby many plants adjust to high salinity and so the low soil water availability (Çiçek and Çakirlar, 2008). Principally, salt stress induces decreases in leaf net CO_2 assimilation rate (Hajlaoui et al., 2006). This limitation of photosynthesis causes an over-reduction of photosynthetic electron chain and redirects the photon energy into processes that favour the production of reactive oxygen species (ROS), which are harmful to plant growth due to their detrimental effects on the function of the most sensitive biological macromolecules and membrane (Johnson et al., 2003). They are mainly the singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2) (Halliwell, 2006).

The hydrogen peroxide is one of the major and the most stable ROS that regulates basic acclamatory, defence and developmental processes in plants (Ślesak et al., 2007). It has an important role in cellular signaling term as second messenger (Neill et al., 2002). However, at high concentrations, it leads to oxidative stress and it will increases lipid peroxidation and electrolyte leakage (Imlay, 2003). The generation of H_2O_2 is increased in response to a wide variety of biotic and abiotic stresses, among others the salt stress (Sairam et al., 2002). The H_2O_2 can react with superoxide radicals training more active hydroxyl radicals in the presence of Fe or Cu (Van Breusegem et al., 2001). The hydroxyl radicals initiate self-propagating reactions leading to peroxidation of membrane lipids and destruction of proteins (Halliwell and Gutteridge, 1989). H_2O_2 has been also shown to promote leaf senescence through lipid peroxidation (Prochazkova et al., 2001), and induction of senescence is accompanied by an increase in endogenous H_2O_2 content (Hurng and Kao, 1994).

Plants have different adaptive mechanisms to reduce oxidative damage resulting from salt stress through a cascade of antioxidants which stopping the propagation of oxidative chain reactions. In this case, polyphenolic compounds such as phenolic acids, flavonoids, proanthocyanidins and anthocyanins play an important role in scavenging free radicals (Ksouri et al., 2007). Their synthesis is

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generally stimulated in response to biotic/abiotic stresses such as salinity (Parida et al., 2004). In a given environment, the production of polyphenols is related to the leaf carbon economy and their accumulation is enhanced when carbon production overtakes the metabolic demand for growth (Barthod et al., 2007). The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. Depending on their chemical nature and their location in the leaf, phenolic compounds also exhibit, others wide range of physiological properties. Indeed, they are implicated in responses to UV radiation, environmental pollutants, nutrients deficiency, pathogens and herbivores (Beckman, 2000).

The polyphenolic composition and antioxidant properties of corn have been the subject of many investigations and findings showed differences between cultivars (Del Pozo-Insfran et al., 2006). The gradients in concentrations of phenolic compounds within-plant, in relation to salt stress and leaf age, may reflect different requirements for dealing with abiotic stresses. Nevertheless, this relation has been researched little.

Maize is an important crop in Tunisia; it is characterized by the multiplicity of its agro-industrial uses. However, in many areas of the country, the productivity of this crop is limited because of the water or/and soil salinization. The improvement of this productivity requires understanding the mechanisms of tolerance of this plant to the saltiness.

The aims of this study was to: (i) compare the phenolic leaf content of two corn varieties subjected to different concentrations of NaCl, (ii) evaluate the degree to which the distribution of polyphenols varies with leaf age, (iii) relate the phenolic content to the free radical-scavenging and (iv) determine the most reliable leaf indicators for distinguishing salt-sensitive from salt-tolerant varieties.

2. Materials and methods

2.1. Plant material and growth conditions

Two corn (*Zea mays* L.) varieties are used: *Aristo* and *Arper*. Seeds have been provided by the Domanial Earth Office of Kairouan (Tunisia). They were germinated in Petri dishes containing two sheets of Watman no. 1 filter paper moistened with half strength Hoagland's nutrient solution. After germination, when cotyledons fully emerged, seedlings were transferred in plastic pots (45, 66, 23 cm) filled with peat/perlite mixture (2:1, v/v). Growth took place in a glazed greenhouse where the temperature for day/night was 35/24 °C, the relative humidity was 60–80% and the average of photosynthetically active radiation was 500 μmol m^{-2} s^{-1} with a photoperiod of 14 h/day. Salt treatment was started 20 days after planting. Sodium chloride was added to Hoagland nutrient solution to provide final concentrations of 0 (control), 34, 68 and 102 mM and plants were watered three times per week with approximately 0.5 l of salt solution. All measurements were made 6 weeks after final treatment concentrations were reached, when plants had achieved a steady state.

2.2. Leaf growth and leaf water content

At the end of the experiment, leaves per treatment of either variety were gathered. In those leaves, the following parameters were examined: leaf area (LA), measured with an LI-3100 leaf area meter (Li-Cor, Lincoln, NE), fresh mass (FM), dry mass (DM), measured after oven-drying at 80 °C to a constant weight. Further, the water leaf content (LWC) was calculated as (Qiujie et al., 1997):

$$\mathrm{LWC}(\mathrm{ml\,H_2O\,g^{-1}\,DM}) = \frac{\mathrm{FM} - \mathrm{DM}}{\mathrm{DM}}$$

2.3. Hydrogen peroxide (H_2O_2) estimation

The H_2O_2 concentration was determined as described by Patterson et al. (1984). Briefly, fresh leaf samples (0.5 g) were homogenized in 5 ml cold acetone, and centrifuged for 10 min at 1500 × *g*; the supernatant was used for the assay of H_2O_2. After 10 min reaction at 25 °C, 0.1 ml of 20% $TiCl_4$ and 0.2 ml of concentrated ammonia were added into supernatant (1 ml). Then the reaction mixture was centrifuged for 10 min at 1500 × *g*. The absorption at 410 nm was measured, and the H_2O_2 concentration was calculated according to the standard curve.

2.4. Determination of lipid peroxidation rate

Oxidative damage to leaf lipids, resulting from salt stress, was estimated by the content of total 2-thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA). TBARS content was estimated by the method of Cakmak and Horst (1991) with some modifications. Fresh leaf samples (0.5 g) were ground in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA), at 4 °C. Following the centrifugation at 12,000 × *g* for 5 min, an aliquot of 1 ml from the supernatant was added to 4 ml of 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) TCA. Samples were heated at 90 °C for 30 min. Thereafter, the reaction was stopped in ice bath. Centrifugation was performed at 10,000 × *g* for 5 min, and absorbance of the supernatant was read at 532 nm on a spectrophotometer (Model Camspec M330 UV/Vis) and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The malondialdehyde content was calculated using its absorption coefficient (ε) and expressed as nmol malondialdehyde g^{-1} fresh mass following the formula:

$$\mathrm{MDA}(\mathrm{nmol\,g^{-1}\,FM}) = \frac{(A_{532} - A_{600}) \times V \times 1000}{\varepsilon} \times W$$

where ε is the specific extinction coefficient (=155 mM cm^{-1}), V is the volume of crushing medium, W is the fresh weight of leaf, A_{600} is the absorbance at 600 nm wavelength and A_{532} is the absorbance at 532 nm wavelength.

2.5. Membrane stability index (MSI)

Membrane stability index (MSI %) was measured as described by Lutts et al. (1996). Leaf Samples were washed with deionized water to remove surface adhered electrolytes and cut into discs of uniform size. Leaf discs were put in closed test tubes containing 10 ml of deionized water and incubated at 25 °C for 24 h and subsequently electrical conductivity of the solution (C_1) was recorded. Samples were then autoclaved at 120 °C for 20 min and the final electrical conductivity (C_2) was obtained after equilibrium at 25 °C. The membrane stability index was defined as below:

$$\mathrm{MSI}(\%) = \left(\frac{C_1}{C_2}\right) \times 100$$

2.6. Extraction and analysis of phenolic compounds

The extraction procedure was determined using the method described by Waterman and Mole (1994) with some modifications. Briefly, lyophilised leaf samples (5 g) were extracted twice with 100 ml of 70% methanol thereafter with 100 ml of 70% acetone at the temperature of 4 °C. Then extraction was renewed with absolute methanol. The extracts were filtered, mixed and concentrated at 240 mbar pressure in a roto-evaporator (Heidolph Elektro GmbH & Co., WB 2000, Kelheim, Germany) at 40 °C. After the elimination of organic solvents, the total aqueous excerpt was centrifuged at 15,000 × *g* for 15 min then washed by the dichloromethane to

Table 1
Variation of the leaf dry matter (DM), leaf area (LA) and leaf water content (LWC) of two maize varieties (*Aristo* and *Arper*) subjected to different NaCl concentrations (0, 34, 68 and 102 mM) during 6 weeks.

NaCl (mM)	*Aristo*			*Arper*		
	DM (g)	LA (cm^2)	LWC (ml H_2O g^{-1} DM)	DM (g)	LA (cm^2)	LWC (ml H_2O g^{-1} DM)
0	5 ± 0.61 a	1301 ± 20.4 a	4.14 ± 0.66 a	5.03 ± 0.46 a′	1330 ± 19.2 a′	4.35 ± 0.49 a′
34	4.37 ± 0.42 b	1020 ± 20 b	3.94 ± 0.70 ab	5.1 ± 0.13 a′	1346 ± 11 a′	4.41 ± 0.20 a′
68	3.82 ± 0.20 c	799 ± 14.8 c	3.56 ± 0.34 ab	4 ± 0.21 b′	1019 ± 26 b′	4.36 ± 0.35 a′
102	2.72 ± 0.17 d	521 ± 16.1 d	3.20 ± 0.42 b	2.7 ± 0.33 c′	642 ± 24.3 c′	4.10 ± 0.78 a′

Values of each column followed by the same letter indicate no significant differences ($p \leq 0.05$) according to Duncan test.

remove the chlorophylls and the lipids traces. The gotten total aqueous phase was evaporated until dryness and the residual was taken in the absolute methanol, to constitute a total phase containing the set of polyphenols.

Total polyphenols were estimated according to the procedure described by Singleton and Rossi (1965). The appropriate extract dilution was oxidized with the Folin–Ciocalteu reagent (0.5 ml) and the reaction was neutralized, during 30 min at 100 °C, with 2 ml of 20% sodium carbonate (Na_2CO_3). The absorbance of the resulting blue colour was measured at 685 nm using a Camspec M330 UV/Vis spectrophotometer. Quantification was done on the basis of a standard curve of gallic acid. Results were expressed as milligrams of gallic acid equivalent per gram of dry matter.

The total flavonoid content in the samples was determined as described by Lamaison and Carnat (1991). To 1 ml of prepared extract was added 4 ml of ethanol (dilution 1/5). Then 1 ml of the analyzed solution was mixed with 1 ml of $AlCl_3$ reagent. After 10 min the absorbance was recorded at 430 nm using a Camspec M330 UV/Vis spectrophotometer. The amount of total flavonoids was calculated from a calibration curve of vitexine and results were expressed as milligrams vitexine per gram of dry matter.

The quantification of anthocyanins and proanthocyanidins was done according the method of Porter et al. (1986). In brief, 0.5 ml aliquots of prepared extracts were transferred into test tubes. After addition of 0.5 ml of methanol, 6 ml of butanol–HCl reagent (butanol:HCl, 95:5, v/v) and 0.2 ml of 2% ferric reagent (2% ferric ammonium sulfate in 2 M HCl), tubes were sealed tightly and heated, in water-bath, at a temperature of 95 °C during 40 min.

After cooling, absorbance due to proanthocyanidins was recorded at 550 nm using a Camspec M330 UV/Vis spectrophotometer. However, absorbance due to anthocyanins was determined by differences between values recorded, at 550 nm, before and after heating. The chloride cyanidin was used as a standard for both anthocyanins and proanthocyanidins and quantities were expressed arbitrarily in milligrams chloride cyanidin per gram of dry matter.

2.7. Free radical-scavenging activity of extracts

Free radical-scavenging activity of leaf polyphenolic extracts of two corn varieties, using DPPH assay, was determined as described by Akowuah et al. (2005) with some modifications. Briefly, 2 ml of a methanolic solution of DPPH (0.1 mM) were blended with 200 μl of sample polyphenolic extract, and made up with methanol to a final volume of 3 ml. After 60 min standing, the absorbance of the mixture was measured at 517 nm against methanol as blank using a Camspec M330 UV/Vis spectrophotometer. The radical-scavenging activities (%) of the tested samples were evaluated by comparison with a control which contained 2 ml of DPPH solution and 1 ml of methanol. The antioxidant activity of each sample was calculated following the formula:

$$\text{antioxidant activity (\%)} = \left[\frac{\text{Ac} - \text{As}}{\text{Ac}}\right] \times 100$$

where Ac is the absorbance of the control and As is the absorbance of the tested sample after 60 min.

2.8. Statistical analysis

Results were examined statistically by using the one-way analysis of variance (ANOVA) with the varieties, salinity treatment and leaf stages as factors, and their interactions was performed for the whole data set using SPSS for Windows: Version 13.0 (standard version). In all experiments, each analysis is the mean of minimum five independent measurements ($n = 5$). Duncan's multiple-range test was used for comparison of means among different levels within a factor. In figures, the spread of values is shown as error bars representing standard errors of the means.

3. Results

3.1. Leaf growth and leaf water content

Growth and structural characteristics of leaves are presented in Table 1. The increasing of salt stress influenced significantly the leaf dry mass (DM) of both varieties. At moderate salinity (34 and 68 mM), the dry matter of *Arper* leaves was faintly higher than those of *Aristo*. However, leaves treated with 102 mM have practically the same value of DM in both varieties; it was around 2.7 g. The leaf area (LA) was also significantly reduced with increasing salt stress. Moreover, intraspecific differences linked to this parameter were observed between the two maize varieties. Dealing with NaCl treatments, the averages of leaf area showed to be greatly higher in *Arper* than in *Aristo*. In the last variety the reduction of DM and LA was associated with a gradually and significantly decrease of leaf water content. The lowest value of this parameter was observed at 102 mM NaCl (3.2 ml H_2O g^{-1} DM). Contrarily in *Arper*, the leaf water content remained statistically constant through all NaCl treatments; it was approximately 4.3 ± 0.13 ml H_2O g^{-1} DM.

3.2. Hydrogen peroxide generation

Salt stress often leads to the production of reactive oxygen spices (ROS) such as $O_2^{\bullet -}$ and H_2O_2 in plant tissues. The last is one of the main and the most stable ROS. Results on H_2O_2 content recorded in salt treated leaves of maize showed significant variations at various stages and between the two varieties. Indeed, the H_2O_2 accumulation was raised significantly with the increase of saltiness (Table 2). In that issue, it has been noted that, in both varieties, this accumulation becomes important particularly in senescent leaves then, with one slightly weaker degree, at mature leaves. The raise of H_2O_2 concentration, according to salt stress and leaf tissue senescence, is not of the same extent in both varieties. Indeed, at all levels of salt stress and all leaf stages, the generation trend of H_2O_2 concentration in *Aristo* was greater than in *Arper*. For example, in leaves treated with 102 mM NaCl, where the generation of ROS/H_2O_2 was important, the difference of H_2O_2 content between the two varieties, at any leaf stage, was fairly around 2.5 μmol H_2O_2 g^{-1} FM.

Table 2
Hydrogen peroxide (H_2O_2) content (μmol g^{-1} FM) of three leaf stages of two maize varieties (*Aristo* and *Arper*) treated with different concentrations of NaCl (0, 34, 68 and 102 mM).

Leaf-stage	Variety	NaCl (mM)			
		0	34	68	102
Young leaves	*Aristo*	1.62 ± 0.37 a	3.23 ± 0.24 b	5.47 ± 0.59 c	6.67 ± 0.61 d
	Arper	1.20 ± 0.27 a′	2.52 ± 0.54 b′	2.85 ± 0.41 b′	3.89 ± 0.25 c′
Mature leaves	*Aristo*	2.03 ± 0.50 a	4.21 ± 0.29 b	7.01 ± 0.35 c	8.42 ± 0.23 d
	Arper	1.43 ± 0.54 a′	3.32 ± 0.29 b′	4.40 ± 0.54 c′	6.21 ± 0.29 d′
Senescent leaves	*Aristo*	2.50 ± 0.50 a	5.20 ± 0.44 b	8.20 ± 0.43 c	9.20 ± 0.27 d
	Arper	1.90 ± 0.22 a′	4.30 ± 0.44 b′	5.86 ± 0.31 c′	7.11 ± 0.23 d′

Values in each line followed by the same letter indicate no significant differences ($p \leq 0.05$) according to Duncan test.

Table 3
Variation of the malondialdehyde (nmol MDA g^{-1} FM) amounts in three leaf stages of two maize varieties (*Aristo* and *Arper*) submitted to different NaCl concentrations (0, 34, 68 and 102 mM).

Leaf-stage	Variety	NaCl (mM)			
		0	34	68	102
Young leaves	*Aristo*	13.1 ± 0.9 a	19.2 ± 2.8 b	24.0 ± 3.0 c	26.0 ± 1.7 c
	Arper	12.3 ± 2.5 a′	16.2 ± 2.9 b′	16.4 ± 2.6 b′	18.2 ± 1.3 b′
Mature leaves	*Aristo*	17.2 ± 2.1 a	22.8 ± 2.6 b	35.0 ± 2.8 c	45.2 ± 1.9 d
	Arper	17.0 ± 1.3 a′	22.2 ± 2.0 b′	25.4 ± 1.8 b′	36.2 ± 4.4 c′
Senescent leaves	*Aristo*	20.4 ± 2.4 a	27.0 ± 2.3 b	41.8 ± 1.5 c	54.8 ± 2.1 d
	Arper	18.4 ± 1.3 a′	25.3 ± 1.8 b′	38.0 ± 3.3 c′	45.0 ± 2.7 d′

Values in each line followed by the same letter indicate no significant differences ($p \leq 0.05$) according to Duncan test.

3.3. Lipid peroxidation

The oxidative damage was observed as malondialdehyde (MDA) content, which is a product of lipid peroxidation. Results reported in Table 3 showed that, in both varieties, lipid peroxidation was influenced by salt stress. As compared with control, NaCl increased the leaf MDA content. However, there was a significant NaCl × variety × tissue senescence interaction. Indeed, the MDA accumulation was more pronounced in senescent leaves than in mature and/or young leaves. Moreover this accumulation was greater in *Aristo* than in *Arper*. In the young leaves of this last variety, contrarily to those of *Aristo*, the amount of MDA remained statistically unchanged with increasing salinity. In mature and senescent leaves, the amount of MDA increased significantly and progressively with increasing salt stress. In both varieties, the highest values of MDA content were registered in senescent leaves of seedlings treated with 102 NaCl mM. They were approximately 45 and 55 nmol MDA g^{-1} FM for *Arper* and *Aristo*, respectively.

In order to determine the contribution of H_2O_2 generation to lipid peroxidation we put in relation the MDA amount with the H_2O_2 accumulation (Fig. 1). In both varieties, the linear correlation between lipid peroxidation and H_2O_2 generation indicated that these parameters may not be independent or are co-regulated. In fact, the H_2O_2 generation, in leaves of both varieties could elucidate approximately 80% of lipid peroxidation changes of leaf membranes.

3.4. Membrane stability index

The membrane stability index (MSI), estimated as electrolyte leakage, decreased significantly under salinity stress in both varieties at three stages of leaves. This decrease was much more apparent in senescent leaves than in young leaves. In addition, MSI values were higher in *Arper* as compared with *Aristo* at all leaf stages and treatments (Table 4).

The relationship between MSI and H_2O_2 generation reported in Fig. 2 showed a high negative correlation. The two varieties followed similar linear patterns. However, the slope of the linear correlation was the highest in *Aristo* and the lowest in *Arper*: where H_2O_2 content did overlap, the response of *Aristo* was more variable than that of *Arper*. These results suggest that MSI susceptibility to H_2O_2 accumulation was higher in *Aristo* ($r^2 = 94$) than in *Arper* ($r^2 = 84$).

3.5. Leaf phenolic compounds

3.5.1. Total polyphenols accumulation

The pattern and degree of within-plant variation of total polyphenols were different across varieties and NaCl treatments. In both varieties, the amount of total polyphenols increased with salinity but decreased with leaf tissue senescence (Table 5). Dealing with all treatments of salinity, the greater accumulation of total polyphenols was showed, principally, in young and mature leaves. In these stages, the first detectable increase of polyphenols content, in two varieties, was localised at lower level of salinity (34 mM

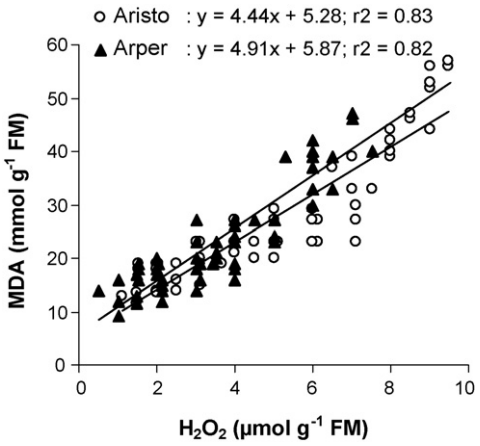

Fig. 1. Correlation between malondialdehyde (MDA) amounts and hydrogen peroxide (H_2O_2) content in leaves of two maize varieties (*Aristo* and *Arper*). The correlation has been established while considering the individual values (5 by treatment) of the two parameters alongside the three leaf stages.

Table 4
Changes of membrane stability index (%) with NaCl treatments (0, 34, 68 and 102 mM) at three leaf stages of two maize varieties (*Aristo* and *Arper*).

Leaf-stage	Variety	NaCl (mM)			
		0	34	68	102
Young leaves	*Aristo*	91.6 ± 2.07 a	87.6 ± 0.89 b	72.2 ± 2.38 c	62.6 ± 3.57 d
	Arper	92.2 ± 1.48 a′	91.2 ± 1.64 a′	79.6 ± 2.60 b′	65.4 ± 2.88 c′
Mature leaves	*Aristo*	89.6 ± 1.81 a	80.6 ± 2.34 b	60.6 ± 1.34 c	52.0 ± 2.00 d
	Arper	90.2 ± 0.48 a′	85.3 ± 2.49 b′	66.1 ± 2.30 c′	59.3 ± 2.04 d′
Senescent leaves	*Aristo*	87.6 ± 1.34 a	70.0 ± 2.03 b	52.4 ± 2.07 c	39.8 ± 2.28 d
	Arper	87.3 ± 0.48 a′	74.2 ± 3.70 b′	52.0 ± 4.00 c′	46.6 ± 2.30 d′

Values in each line followed by the same letter indicate no significant differences ($p \leq 0.05$) according to Duncan test.

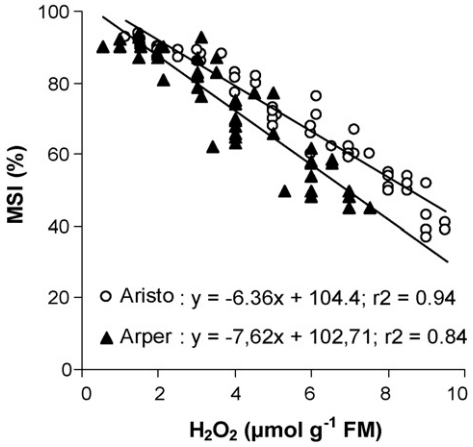

Fig. 2. Relationship between membrane stability index (MSI) and hydrogen peroxide (H_2O_2) content in leaves of two maize varieties (*Aristo* and *Arper*). The correlation has been established while considering the individual values (5 by treatment) of the two parameters alongside the three leaf stages.

NaCl) and it continuous to increase significantly at treatments of 68 and 102 mM NaCl. However, in senescent leaves, the behaviour of two varieties was different. Indeed, *Aristo* maintained a fairly steady level of polyphenols whereas *Arper* indicated a significant raise over salt stress. At all circumstances, the more pronounced increase of polyphenols concentration, particularly at high salinity, was observed in the variety of *Arper* compared to *Aristo* (Table 5). For example, in leaves treated with 102 mM NaCl, the concentration of total polyphenols of *Aristo* was around 13.3, 11.6 and 5.3 mg g^{-1} DM, respectively, in young, mature and senescent leaves. For this treatment and in the same leaf stages, means of *Arper* were, respectively, in the range of 18.4, 15.1 and 7.5 mg g^{-1} DM.

3.5.2. Total flavonoids content

In salt treated plants of the two varieties, the variation of total flavonoids concentration, between leaves of different ages was similar to polyphenols content. This variation led to significant ($p \leq 0.05$) differences among treatments, leaf stages and varieties. Results reported in Table 5 showed that the flavonoids content decreased with leaf tissue senescence and increased with salt stress. This accretion was much more apparent in the young and mature leaves of the two varieties. For *Aristo*, the increase rates of flavonoids accumulation in young and mature leaves treated with 102 mM NaCl, compared to control ones, were, respectively, almost 78% and 75%. For *Arper* and at the same leaf stages, these rates were higher; they were, respectively, around 86% and 88%. In the oldest leaf stage, although the two varieties showed the same average of flavonoids concentration at 102 mM NaCl, but the increase rate of flavonoids accumulation, compared to control senescent leaves, was higher in *Aristo* (61%) than in *Arper* (53%). In addition, at all leaf stages, the first significant accumulation of flavonoids was commenced at 34 mM NaCl for *Arper* variety and at 68 mM NaCl for *Aristo* variety.

3.5.3. Proanthocyanidins content

Changes observed in proanthocyanidins content in the leaf stages were related both to the relative age of each stage and to the concentration of NaCl in the nutrient medium. As seen in Table 5, the proanthocyanidins content improved substantially with increased salt stress. The proanthocyanidins accumulation reached its highest values in the young and mature leaves, decreasing in the oldest leaves. Considerable differences were observed between the average values of varieties among NaCl concentration and leaf stages. This appeared that *Arper* has more susceptibility to NaCl for proanthocyanidins synthesis and accumulation than *Aristo* especially in young leaves. Indeed, for 102 mM NaCl, the proantho-

Table 5
Effects of NaCl treatments (0, 34, 68 and 102 mM) on the concentration of total phenols, total flavonoids, proanthocyanidins and anthocyanins (mg g^{-1} DM) at three leaf stages of two corn varieties (*Aristo* and *Arper*).

Leaf-stage	NaCl (mM)	Total phenols		Total flavonoids		Proanthocyanidins		Anthocyanins	
		Aristo	*Arper*	*Aristo*	*Arper*	*Aristo*	*Arper*	*Aristo*	*Arper*
Young leaves	0	3.24 ± 0.40 a	3.59 ± 0.54 a′	1.21 ± 0.25 a	1.21 ± 0.26 a′	0.70 ± 0.39 a	1.32 ± 0.45 a′	0.43 ± 0.22 a	0.29 ± 0.12 a′
	34	4.16 ± 0.34 b	6.21 ± 0.79 b′	1.31 ± 0.43 a	2.06 ± 0.59 b′	0.93 ± 0.14 a	2.46 ± 0.45 b′	0.91 ± 0.09 b	1.67 ± 0.16 b′
	68	9.40 ± 0.90 c	12.76 ± 1.04 c′	4.00 ± 0.73 b	6.00 ± 0.70 c′	2.33 ± 0.32 b	4.36 ± 0.53 c′	1.07 ± 0.18 b	2.40 ± 0.36 c′
	102	13.31 ± 0.86 d	18.45 ± 0.62 d′	5.60 ± 0.54 c	9.20 ± 0.44 d′	3.87 ± 0.44 c	5.72 ± 0.54 d′	1.84 ± 0.07 c	3.53 ± 0.29 d′
Mature leaves	0	2.96 ± 0.34 a	3.24 ± 0.48 a′	1.11 ± 0.12 a	1.08 ± 0.23 a′	0.68 ± 0.42 a	1.12 ± 0.39 a′	0.38 ± 0.25 a	0.27 ± 0.10 a′
	34	4.66 ± 0.38 b	5.87 ± 0.87 b′	1.13 ± 0.22 a	2.60 ± 0.54 b′	0.86 ± 0.18 a	2.06 ± 0.55 b′	0.87 ± 0.10 b	1.21 ± 0.31 b′
	68	8.60 ± 0.29 c	11.44 ± 0.82 c′	3.83 ± 0.20 b	5.20 ± 0.44 c′	2.53 ± 0.29 b	3.94 ± 0.24 c′	1.58 ± 0.17 c	1.91 ± 0.12 c′
	102	11.67 ± 0.82 d	15.13 ± 0.18 d′	4.10 ± 0.41 b	8.60 ± 0.22 d′	4.12 ± 0.73 c	4.27 ± 0.54 c′	1.67 ± 0.16 d	3.26 ± 0.36 d′
Senescent leaves	0	2.92 ± 0.35 a	2.03 ± 0.53 a′	1.07 ± 0.11 a	1.28 ± 0.41 a′	0.52 ± 0.43 a	0.52 ± 0.41 a′	0.32 ± 0.27 a	0.23 ± 0.11 a′
	34	2.46 ± 0.24 a	4.10 ± 0.66 b′	1.07 ± 0.13 a	2.04 ± 0.63 b′	0.93 ± 0.14 b	0.98 ± 0.12 b′	0.45 ± 0.31 a	1.08 ± 0.17 b′
	68	3.19 ± 0.71 a	5.18 ± 0.50 c′	1.64 ± 0.49 b	2.40 ± 0.54 b′	1.08 ± 0.17 b	1.47 ± 0.16 c′	0.47 ± 0.17 a	1.31 ± 0.42 b′
	102	5.38 ± 0.72 b	7.51 ± 0.57 d′	2.60 ± 0.54 c	2.60 ± 0.54 b′	2.13 ± 0.29 c	2.53 ± 0.29 d′	0.64 ± 0.16 a	2.05 ± 0.10 c′

For all leaf stages, values of each variety followed by the same letter indicate no significant differences ($p \leq 0.05$) according to Duncan test.

Table 6
Effect of different levels of salt stress (0, 34, 68 and 102 mM) on the antioxidant activity percentage of polyphenolic compounds extracted from leaves of two maize varieties (*Aristo* and *Arper*) seedlings.

NaCl (mM)	Antioxidant activity (%)	
	Aristo	*Arper*
0	5.4 ± 2.4 a	6.0 ± 2.3 a′
34	8.2 ± 2.0 a	10.0 ± 2.1 a′
68	23.6 ± 4.2 b	35.6 ± 3.9 b′
102	32.4 ± 3.9 c	48.6 ± 3.2 c′

Values of each column followed by the same letter indicate no significant differences ($p \leq 0.05$) according to Duncan test.

cyanidins content in young leaves was almost 5.7 mg g^{-1} DM for *Arper* and solely 3.8 mg g^{-1} DM for *Aristo*.

3.5.4. Anthocyanins content

In both tested varieties, the anthocyanins concentrations showed net variation among leaf position having different tissue ages. For both varieties and along with all salinity treatments, the anthocyanins content reached in the young leaves, decreased very slightly in mature leaves and considerably in senescent leaves. However, the stimulatory effect of NaCl on anthocyanins accumulation was more pronounced in *Arper* than in *Aristo* (Table 5). This difference of behaviour between the two varieties becomes obvious especially at the youngest and the oldest leaves treated with 102 mM NaCl. Indeed, for this treatment, the anthocyanins content of *Aristo* was approximately 1.8 and 0.6 mg g^{-1} DM in young and senescent leaves, respectively. However, for *Arper* variety, values were considerably higher; they were, respectively, around 3.5 and 2 mg g^{-1} DM in young and senescent leaves.

3.6. Free radical-scavenging activity

The free radical-scavenging activity of leaf extracts was used to explain the response of the two corn varieties under salinity treatments. It was determined using DPPH assay and it was expressed in percentage of antioxidant activity. Data presented in Table 6 indicated that, in both varieties, the percentage of antioxidant activity of leaf extracts remained statistically constant during the treatment of 34 mM NaCl. While it increased notably once the level of salinity concentration was reached to 68 mM and it continuous to increasing significantly at 102 mM NaCl. The trend of rise of the antioxidant activity was more pronounced for the *Arper* variety compared to *Aristo*, especially at higher salt stress treatments (68 and 102 mM NaCl). For example, the free radical-scavenging activity of polyphenolic compounds extracted from the leaves of seedlings treated with 102 mM NaCl was around 49% for *Arper* and only 32% for *Aristo*.

4. Discussion

Under salt stress the leaf area in both maize varieties decreased earlier than leaf dry matter and leaf water content (Table 1). This behaviour confirms that, in general, the first symptom of salt stress in the plants is a restriction in leaf expansion (Alarcón et al., 1993). The reduction in leaf area under saline conditions can be considered as an avoidance mechanism, which minimise water losses when the stomata are closed, which happens to many species under osmotic stress (Ribeiro et al., 2006). This seems to be efficiently achieved by *Arper* variety which succeeded to maintain steady the leaf water content through all NaCl treatments. Under saline conditions, the reduction in total leaf area can be explained by a decrease in leaf turgor, changes in cell wall properties or a decreased photosynthesis rate (Franco et al., 1997). As expected, the decreased in leaf dry mass of treated plants (Table 1) could be considered a direct effect of the observed leaf area reduction. In this setting, the dry matter of *Arper* leaves compared to those of *Aristo* was faintly higher especially at moderate salinity.

Salinity involves an oxidative stress that manifesting by a large generation of reactive oxygen species. We thought that this phenomenon depends on cultivars, and on aging process of leaf tissue. In order to elucidate the mechanism of the oxidative stress tolerance of salt treated corn leaves during the course of senescence, in this work we studied the differences in the levels of polyphenolic compounds, which considered as antioxidants. Comparative response studies can clarify the salt tolerance mechanism in maize.

In both varieties, leaves grown under salt stress conditions revealed signs of oxidative stress. One of the signs was the generation of hydrogen peroxide. Our data indicated a substantial accumulation of H_2O_2 with increasing salt stress and leaf tissue senescence. This accumulation was greater in *Aristo* than in *Arper* (Table 2) and it was accompanied, in both varieties, by a decrease in net CO_2 assimilation and electron transfer rate (data not shown). The H_2O_2 generation, with salt stress conditions and/or leaf tissue senescence, confirms of already reported results (Prochazkova et al., 2001). The accurate mechanisms entailing ROS accumulation, in particularly H_2O_2, during environmental stress are not entirely clear. However, some authors thought that the disruption or inhibition of the mitochondrial electron transfer chain (Johnson et al., 2003) or photosynthetic apparatus (Ślesak et al., 2007) are major factors. Others researchers suggested that H_2O_2 is also produced via several enzyme-mediated reactions, such as glycolate oxidase, oxalate oxidase and NADPH oxidase (Svedruzic et al., 2005).

In both varieties, the leaf H_2O_2 accumulation resulted in a marked increase in MDA content and a decrese in MSI, especially, in senescent leaves of seedlings treated with 68 and 102 mM NaCl. These findings indicate that H_2O_2 brings about lipid peroxidation leading to membrane damages. The high positive correlation between H_2O_2 generation and MDA amount (Fig. 1), which was negatively associated with the increase of MSI (Fig. 2), confirmed this hypothesis. Various works have examined the generation of ROS/H_2O_2 and lipid peroxidation in plants in relation to salinity stress demonstrated increased lipid peroxidation and decreased membrane stability index during senescence and salinity increasing (Hurng and Kao, 1994; Sairam et al., 2002). Moreover, others studies (Shalata and Tal, 1998; Juan et al., 2005) with different plant species have concluded that lipid peroxidation in salt-sensitive lines increased more than in those of salt-tolerant. In our experiment, the *Aristo* variety showed greater lipid peroxidation, and so, the more oxidative stress.

The accretion of ROS/H_2O_2, under salt stress, is generally coupled with changes in net carbon gain (Barthod et al., 2007). Since it may strongly affect the biosynthesis of carbon-based secondary compounds, particularly leaf polyphenols (Tattini et al., 2006), which serve key functions in protection of plants against oxidative damage. The foliar concentrations of these secondary compounds (total polyphenols, total flavonoids; anthocyanins and proanthocyanidins), in the present study, increased notably with salt stress. The enhanced level of polyphenolic compunds in different tissues under salt stress conditions has been reported in a number of plants (Navarro et al., 2006; Tattini et al., 2006). Tattini et al. (2006) mentioned a close relationship between oxidative stress tolerance and flavonoids accumulation. In the same meaning, Hendrich (2006) suggested that the incorporation of flavonoids into the lipid bilayer is sometimes the first step in the sequence of events induced by polyphenolic compounds. The accumulation of diverse phenolic monomers, in response to environmental stress has been shown to be a consequence of activity of specific isoforms of phenyl ammonia lyase (Rohde et al., 2004).

Earlier studies in many higher plants revealed that the accumulation of polyphenols was found to be higher in salt tolerant cultivar

than in the salt sensitive one (Wahid and Ghazanfar, 2006; Ksouri et al., 2007). In the present study, the *Arper* variety reserved always the higher accumulation of polyphenols, flavonoids, anthocyanins and proanthocyanidins, suggesting the salt tolerant nature of this variety.

The order of variation of polyphenolic compounds according leaf ontogeny showed that, in both varieties, young leaves of control or treated seedlings have the greater amounts. This confirms the hypothesis that the ability to induce polyphenols accumulation may sometimes be limited to the juvenile leaf stage (Murray et al., 1994), or is lost with increasing leaf age and reduced sensitivity to environmental stress, as showed in sugarcane (Nozzolillo et al., 1990).

Polyphenolic structure has the ability to eliminate radical species thus preventing the propagation of oxidative chain reactions (Sgherri and Navari-Izzo, 1995). In this setting, the leaf polyphenolic extracts of two varieties were evaluated for their capacity to scavenge hydroxyl radical using the DPPH assay which has been used extensively as free radical to estimate reducing substances. As shown in Table 6, all the extracts found to have the ability to scavenge hydroxyl radicals. However, their antioxidant activities have been sharply increased especially at 68 and 102 mM NaCl. Similar results have been reported by Ksouri et al. (2007) for the antioxidant activity of polyphenolic extracts of salt treated *Cakile maritima* leaves. In our study, it is difficult to explain faithfully the increase of antioxidant activity with salt stress, but we thought that NaCl stimulate the synthesis of new polyphenolic derivates that are potent antioxidants.

Our results revealed also that the *Arper* variety for which the phenolic compounds is high expressed showed the strong antioxidant activities. Such result can explain why this variety, compared to *Aristo*, has the lowest values of H_2O_2 content and consequently less lipid peroxidation. The effectiveness of the antioxidant activity of *Arper* extract is probably related to the high proportions of flavonoids, including anthocyanins. Indeed, at all circumstances, the *Arper* leaf extracts are more enriched in flavonoids and anthocyanins than those of *Aristo* (Table 5). Some authors suggested that, among polyphenols, the anthocyanins are considered very good antioxidant agents (Skerget et al., 2005). Their high activity being attributed to their peculiar structure, namely the oxonium ion in the C ring (Wang et al., 1997). Furthermore, in some small fruits (Moyer et al., 2002) and caneberries (Wada and Ou, 2002), the antioxidant capacity has been correlated to a significant degree with anthocyanins content, indicating that anthocyanins may govern to a certain extent the antioxidant capacity of several plant tissues.

5. Conclusion

In conclusion, this study strongly supports the idea that polyphenols play significant physiological role in maize salinity tolerance particularly against oxidative damage. In salt treated maize, the accumulation and remobilisation of the major phenolics compounds were shown to be affected by leaf tissue senescence. The youngest leaves have the most part of this accumulation and seem to be more protected against the reactive oxygen species. A notable distinction between the two tested varieties was observed in the present study. The *Arper* variety showed to be more tolerant to salt stress than *Aristo* as indicated by the response of leaf growth, leaf water content, membrane stability index and polyphenols accumulation and their related antioxidant activity in the presence of NaCl. The occurrence of flavonoids in phenolics compounds such as anthocyanins is of key importance in the protection against the oxidative damages.

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